Studies on Isolation, Distribution, Biotyping, Characterization, Production of Antibiotic, Bioemulsifier and Plasmid pUPI126 encoded Indole Acetic Acid (IAA) production and its role in plant growth promotion by *Acinetobacter* species from Rhizosphere of Wheat.

A Thesis Submitted To
The University of Pune
For the Degree of
Doctor of Philosophy
In Microbiology

BY
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Dedicated to Ti. Sow Aai, Anna & Sunil.....

Contents :

Declaration

Certified that the work incorporated in the thesis "Studies on Isolation, Distribution, Biotyping, Characterization, Production of Antibiotic, Bioemulsifier and Plasmid pUPI126 encoded Indole-3-Acetic Acid (IAA) production and its role in plant growth promotion by *Acinetobacter* species from Rhizosphere of Wheat" submitted by Mrs. Shilpa Sunil Mujumdar (Reg. No.: PGS/Ph. D /Micro. 17270-72; dated 9.9.1997), was carried out by the candidate under my supervision at Department of Microbiology, University of Pune, Pune. Such material as has been obtained from other sources has been duly acknowledged in thesis.

B.A. Chopade

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Abbreviations :

Antibiotics			Metals			Others		
Ap	:	Ampicillin	Au		Gold	M	:	Molar (Moles/ liter)
Am	:	Amoxicillin	Ag	:	Silver	mM	:	Mill molar
Cx	:	Cloxacillin	Al	:	Aluminium	µl	:	Micro liter
Ce	:	Cephaloridine	As^{2+}	:	Arsenite	g	:	Gram
Ct	:	Cephotaxime	As^{4+}	:	Arsenate	Mg	:	Milligram
Cz	:	Cephazolin	Bi	:	Bismuth	L	:	Liter
Sm	:	Streptomycin	Cd	:	Cadmium	ml	:	Milliliter
Km	:	Kanamycin	Cr	:	Chromium	^0C	:	Degree Celsius
Gm	:	Gentamicin	Cu	:	Copper	h	:	Hour (s)
Ak	:	Amikacin	Hg	:	Mercury	min.	:	Minute (s)
Tc	:	Tetracycline	Li	:	Lithium	$EUml^{-1}$	:	Emulsion unit per milliliter
Tp	:	Trimethoprim	Mo	:	Molybdate	v/v	:	Volume by volume
Na	:	Nalidixic acid	Ni	:	Nickel	w/v	:	Weight by volume
Cf	:	Cefuroxime	Pb	:	Lead	cm	:	Centimeter.
Rf	:	Rifampicin	Sb	:	Antimony	mm	:	Millimeter
Cm	:	Chloramphenicol	Se	:	Selenium	%	:	Percentage
Pb	:	Polymyxin B	Sn	:	Stansium	CFU	:	Colony forming units
Pn	:	Penicillin	W	:	Tungstate	TVC	:	Total viable count
			Te	:	Tellurium	TBC	:	Total bacterial count
			Pt	:	Platinum	MIC	:	Minimal inhibitory concentration
			V	:	Vanadium	SIC	:	Sub-inhibitory concentration
			St	:	Strontium	IAA	:	Indole acetic acid
			Zn	:	Zinc	Prn/Pyr	:	Pyrrolnitrin
						SD	:	Standard deviation
						df	:	Degree of freedom

Fig.3B

Fig.3B. Effect of *A.* genospecies 3A 28 bioinoculum on different crop plants.
G : Black bean (*Phaseolus vulgaris*); H : Rajama (*Phaseolus vulgaris*);
I : Groundnut (*Arachis hypogea*); J : Coriander (*Coriander Cilantro*);
K : Spinach (*Spinacia olerecea*); L : Tomato (*Lycopersicon esculentum*).
C : Control; S : Sample.

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Mrs. Shilpa S. Mujumdar

ABSTRACT

Four Gram-negative bacterial genera consisting *Pseudomonas, Acinetobacter, Moraxella* and *Serratia* were isolated from the rhizosphere of wheat. Members of genus *Acinetobacter* were found to be one of the dominant bacteria (31.3 %) next to *Pseudomonas* (35.7 %) in the rhizosphere of wheat. *Moraxella* (28 %) and *Serratia* (4.5 %) were also present in rhizosphere of wheat. *Acinetobacter* spp. were selected for further studies. *Acinetobacter* minimal medium (AMM) and Holton's medium without cefsulodin were found to be most suitable media for isolation of *Acinetobacter* spp. from the rhizosphere of wheat. Maximum number of *Acinetobacter* strains (23) were found in rhizosphere of wheat (RS) than compared to rhizoplane (RP) and nonrhizosphere soils (NRS).

37 *Acinetobacter* strains were isolated from rhizosphere of wheat and biotyped into five genospecies by employing Bouvet & Grimont system, these were *A. calcoaceticus* (4), *A.* genospecies 3 (3), *A. baumannii* (8), *A. haemolyticus* (11) and *A. junii* (10). For all *Acinetobacter* strains temperature 28^{0}C, pH 7 and aeration of 150 rpm was found to be optimal conditions for growth of *Acinetobacter* spp. Fifteen carbon and 13 nitrogen sources were utilized by almost all *Acinetobacter* spp. 13 organic acids, except lactic acid was utilized by almost all *Acinetobacter* spp. Enzymes such as chitinase (4), pectinase (4), xylanase (3), protease(8), urease (27), lipase (17), amylase (6), phosphatase (35), L- asparginase (36), β-glycosidase (33) and nitrate reductase (16) were produced by *Acinetobacter* genospecies. *Acinetobacter* showed resistance to β-lactam antibiotics such as Pn, Cf, Ct, Cx, Ax and Ap, moderate resistance to aminoglycoside antibiotics like as Km, Sm, Am, Gm, Tb, and Am and was sensitive to other antibiotics such as Rif, Nal and Tc except chloramphenicol (1024 μg/ml). *Acinetobacter* genospecies exhibited resistance to toxic metal salts such as tellurium, selenium, lead, arsenate (1024 μg/ml) and arsenite (512 μg/ml).

A. haemolyticus A19 was found to produce antibiotic which was inducible by co cultivation method with (2:1) optimum proportion. Antibiotic showed antimicrobial activity against plant as well as human pathogenic bacteria and fungi. Purification of antibiotic was carried out by extraction with ethylacetate followed by silica gel column chromatography using chloroform : methanol (1:10) system and further purification was done by preparative thin layer chromatography. Yield of antibiotic was found to be 15 mg/liter and was active at very low concentration such as 50μg /ml. It is stable at room temperature up to 6 months and sparingly water soluble. ^{1}H-NMR analysis revealed this antibiotic as a pyrrolnitrin. It was found that pyrrolnitrin production in *A. haemolyticus* A19 was encoded by plasmid pUPI126 of molecular weight of 40 Kb. Plasmid pUPI126 was transformed to *E. coli* HB101 at frequency of 5 x 10^5 per μg of DNA. It was also conjugally self transferred to *E. coli* HB101 rif^r mutant at a frequency of 5.9 x 10^8 per recipient cells. Plasmid pUPI126 was 100 % stable in *Acinetobacter* and 95 % in *E. coli* HB101. Both transformants and transconjugants showed antibiotic production. This is a first report of plasmid pUPI126 mediated pyrrolnitrin antibiotic production in genus *Acinetobacter*.

Two genospecies of *Acinetobacter* consisting of *A. baumannii* A25, A13, A16 and *A. junii* A6 were found to produce bioemulsifier. Different triglycerides such as groundnut oil, mustard oil, sunflower oil and sesame oil were utilized as substrate by these *Acinetobacter* spp. Different hydrocarbons like petrol, kerosene and toluene were also utilized by these *Acinetobacter* strains. *A baumannii* A25 showed maximum bioemulsifier activity of 1200 EU ml^1 at 28, 37 and 40^{0}C and at pH 6,7, and 8. Yield of bioemulsifier was found to be 6.6 gm/liter.

Partially purified bioemulsifier contain protein (85%), polysaccharide (14 %) and reducing sugars (1%). Reconstitution (99%) of this bioemulsifier was achieved by mixing protein and polysaccharide parts of this bioemulsifier. It reduced viscosity of oil by 60 % and increased viscosity of distilled water by 10 %. Esterase produced by *A. baumannii* A25 in cell free supernatant was 190 Uml^{-1} at 48 h where as cell associated esterase was found to be 245 Umg^{-1} at 36 h. Cell surface hydrophobicity of *A. baumannii* A25 towards groundnut oil was maximum for hexadecane lower than groundnut oil and lowest for xylene. Poor congered binding to the cells of *A. baumannii* A25 showed its capsulated nature. This partially purified bioemulsifier was stable at room temperature (28-30^{0}C) for 6 months and 10 and 50^{0}C for 5 and 3 days respectively. It reduced surface tension of water to 63 dynes/cm. CMC of bioemulsifier was found to be 40mg/ml. This bioemulsifier was named as " Chopadesan". This is the first report of novel bioemulsifier production by *A. baumannii* A25 isolated from the rhizosphere of wheat.

All strains of *Acinetobacter* were screened for production of plant growth promoting hormone like indole acetic acid (IAA). Only eight *Acinetobacter* strains exhibited indole acetic acid production, and were found to belong to *A. junii* A6, *A. baumannii* A13, A16, A18 and A30, *A.* genospecies 3 A15 and A28 and *A. haemolyticus* A19. IAA produced by each eight *Acinetobacter* genospecies was extracted by ethyl acetate (1:1) and purified by preparative thin layer chromatography (TLC). Purified IAA was confirmed by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and infrared spectrum (IR) analysis. The ^{1}H-NMR of purified eight IAA samples was found to be same as that of ^{1}H-NMR of standard IAA. The IR spectrum of purified eight IAA samples was found to be same as that of standard IR spectrum. The melting point of purified IAA was found to be 168^{0}C which, is similar to standard IAA. Pot experiments showed significant increase in plant growth inoculated with *A. junii* A6, *A. baumannii* A13, A16, A18 and A30, *A.* genospecies 3 A15 and A28 and *A. haemolyticus* A19 as compared to control plants. IAA production was encoded by plasmid pUPI126 in *A. haemolyticus* A19. All eight strains of *Acinetobacter* contain a plasmid pUPI126 of 40kb. Plasmid pUPI126 was transformed in to *E. coli* HB101 at a frequency of 5 x10^{-5} per μg of DNA and *E. coli* HB101 (pUPI126) transformants also showed IAA activity. This is the first report of plasmid pUPI126 encoded IAA production in the genus *Acinetobacter*.

Rifampicin resistant mutants of eight indole acetic acid (IAA) producing *Acinetobacter* strains were isolated. Rif^r mutants showed presence of plasmid pUPI126 with same molecular weight of 40 Kb. Almost all rif^r mutants showed 95 % stability. *A. haemolyticus* A19.1, *A. baumannii* A18.1, A16.1, A13.1 and *A.* genospecies 3 A15.1, showed maximum IAA production in mid-stationary phase (60 h). *A. junii* A6.1 in early stationary phase (36 h) and *A.* genospecies 3 A28.1 and *A. baumannii* A30.1 in late stationary phase (60 and 72 h) same as parent. Wild as well as rif^r mutants of all *Acinetobacter* spp. survived up to 95 % in lignite carrier for up to 4 months. There was 80 % decrease in the survival of *Acinetobacter* after 4 to 6 months.

Acinetobacter bioinoculum was prepared using lignite as a carrier. Field trials of *Acinetobacter* bioinoculum were carried out. *Acinetobacter* bioinoculum increased grain yield of wheat by 60 % and *Acinetobacter* bioinoculum and half dose of fertilizer (Urea: 0.63Kg/100m^2 and Single Super Phosphate (SSP): 1.3 KG/100m^2) increased grain yield of wheat by 75 % as compared to controls and standards. Standard bioinoculums (*Azotobacter* and *Azospirillum)* increased the yield of wheat grains with only 10 to 12 %. *Acinetobacter* was found to colonize wheat roots successfully at each life stage of wheat plant. In treated wheat plants, increase in number of tillers (12 to 13) and height (87.84cm) of the plant was observed in comparison with controls and standard bioinoculums. Dry weight of single wheat plant treated with *Acinetobacter* bioinoculum was 25g.

1000grain weight of was found to be maximum for wheat seeds treated with *Acinetobacter* bioinoculum (62.66 g) as compared to controls and standards. Chlorophyll content of *Acinetobacter* bioinoculum treated plants was significantly more in quantity (7.74 mg/g) than standard bioinoculum (3.30 mg/g) and controls (0.8 mg/g). IAA oxidase activity was also recorded more (0.16 μg/min/mg of protein) in *Acinetobacter* bioinoculum treated wheat plants. *Acinetobacter* bioinoculum was treated with 12 different crop plants and vegetables were found to be suitable for each of them. These plants include: i) *Phaseolus vulgaris* ii) *Sorghum bicolor*, iii) *Pennisetum americanum* iv) *Zea maize* v) *Arachis hypogea* vi) *Vigna radiata* vii.) *Cicer arietinum* viii) *Coriander cilantro* ix) *Spinacia olerecea* x) *Lycopersicon esculentum* xi) *Glycine max* and xii) *Phaseolus vulgaris*. Thus *Acinetobacter* bioinoculum was found to be successful as a biofertilizer for these crop plants as well as vegetables.

Plasmid profiles were studied for all 37 *Acinetobacter* strains. All *Acinetobacter* strains harbor at least one plasmid of molecular weight of 40Kb. Plasmid pUPI126 also encoded with Km^r and Te^r genes. Plasmid pUPI126 was conjugally selftransferred to *E. coli* HB101 at frequency of 8.7×10^{-6} per recipient cells. Plasmid pUPI126 was transformed to *E. coli* HB101 at frequency of 5×10^5 per μg of DNA. Plasmid pUPI126 was found to be 90 % stable in *E. coli* K12 HB101 and 98 % stable in *A. haemolyticus* A19. Transformants (PUPI126) also showed pyrrolnitrin as well as IAA production as wild *A. haemolyticus* A19. Transformation between rif^r mutant of *E. coli* HB101 and with *A. baumannii* (A13, A16, A18, A30), *A.* genospecies 3 (A15, A28) and *A. junii* A6 showed frequencies in the range of 1×10^7 to 3×10^5 per μg of DNA. Along with IAA production, tellurium and kanamycin markers were also transformed.

Overall, this research work has revealed the role of *Acinetobacter* in rhizosphere of wheat. It promotes plant growth by producing plant growth promoting hormones such as IAA. It is able to produce antibiotic, pyrrolnitrin, which has broad antimicrobial spectrum. It was also observed that these *Acinetobacter* spp. are able to solubilize phosphate, and also can produce chitinase and other enzymes such as L-asparginase, pectinase, xylanase and protease. They are also able to produce bioemulsifier. IAA production and antibiotic production is encoded by plasmid pUPI126. Thus *Acinetobacter* can act as plant growth promoting rhizobacteria and in biocontrol. This thesis describes the role of *Acinetobacter* in the rhizosphere of wheat. This work has opened a new field of research in biology of *Acinetobacter* and *Acinetobacter* has been established as a novel rhizobacteria. Findings of this work would act as a strong foundation on which further investigations can be done in future.

CHAPTER 1

General Introduction and Reviews

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General Introduction and Reviews

A. General Introduction

1.1 Definition and current taxonomic status of Genus *Acinetobacter*
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A. General Introduction :

1.1. Definition and current taxonomic status of Genus *Acinetobacter* :

Acinetobacter is an aerobic, Gram-negative, non-motile, non-spore forming, oxidase negative, catalase positive, capsulated coccobacilli (Dhakephalkar & Chopade, 1994). It is ubiquitous in nature and mostly present in soil, water, food, sewage and in hospital environment. It is the only Gram-negative bacteria present on the human skin (Patil & Chopade, 2001). The G+C content of *Acinetobacter* is 39-47 moles %. The genus *Acinetobacter* was first delineated by Brisou & Prevot in 1954. Previously some oxidase positive strains were also considered as a genus *Acinetobacter* but after several investigations only oxidase negative *Acinetobacter* strains were considered as genus *Acinetobacter* (Lessel, 1971, Juni & Heym, 1980). *Acinetobacter* is relatively recently studied group of microorganisms. This is because, previously this bacterium was known by 40 different names such as *Acinetobacter anitratus*, *Bacterium anitratum*, *Herella vaginicola*, *Lingelsheimia anitrata*, *Mima polymorpha*, *Alcaligenes haemolysans* etc. and hence there has been a lot of confusion about the systematics of *Acinetobacter* (Henriksen, 1973 ; Henriksen & Bovre, 1976).

With the development of chromosomal DNA transformation assay by Juni (1972 & 1978), a genuine method of confirmation of *Acinetobacter* was developed. This assay is specific only for *Acinetobacter* species and based on this assay, the authenticity of genus *Acinetobacter* was established (Juni, 1972 & 1978). In numerical analysis of 291 *Acinetobacter* strains, Pagel & Seyfirend (1976) made use of 89 characteristics based on morphological, physiological, nutritional and biochemical properties to divide strains into two major groups corresponding to the two major nutritional groups established by Baumann et al (1968). These are dependent on ability of *Acinetobacter* to produce or lack of the ability to produce acid from sugars. Acid producing subspecies have been designated as *anitratus* and the non-acid producing subspecies have been designated as *lwoffii*. API 20 NE system was also used for classification of *Acinetobacter* (Towner & Chopade 1987), but all *Acinetobacter* species can not be biotyped by this system. *A. calcoaceticus* was included in the Bergeys Manual of Systematic Bacteriology (Juni,1984). At present the systematics of *Acinetobacter* is well defined and it consists of 21 genospecies (John, et al 1994; Bouvet & Grimont 1986 & 1987).

Previously, in 1971 *Acinetobacter* was classified under the family *Neisseriaceae*. However, recently *Acinetobacter* is included into the family *Moraxellaceae* by Rossau, et al (1991). It was observed by Rossau et al, that neither transformation studies nor phenotypic, chemotaxonomic, and nucleic acid hybridization (Baumann & Stanier, 1968; Bovre, 1970) studies could provide substantial evidence about the more remote relationships between *Moraxella*, *Acinetobacter* and

allied bacteria. Roussau's classification is mainly by DNA-rRNA hybridization studies, that the genera *Moraxella, Psychrobacter* and *Acinetobacter* and related organisms form a distinct, separate cluster of taxa.

1.2. Biotyping of *Acinetobacter* :

The present declaration of species within the genus *Acinetobacter* is totally unsatisfactory. Only two genospecies, *A. calcoaceticus* and *A. lwoffii* are on the approved list of bacterial names (Shkerman et al, 1980). It should be noted that genus *Acinetobacter* is biochemically and genetically heterogenus. The detailed and more successful biotyping of the genus *Acinetobacter* was carried out by Bouvet & Grimont (1986 & 1987). These studies mainly carried out on the basis of DNA hybridization and biochemical performance of the strains isolated mainly from clinical sources and some from soil, and water environment. 12 phenotypically distinct hybridization group and four additional species were identified and named as *A. baumannii, A. haemolyticus, A. junii,* and *A. johnsonii.* (Bouvet & Grimont, 1986 &1987 ; Bouvet & Jeanjean, 1989). The latest position of *Acinetobacter* has resulted in delineation of the genus into 17 genospecies and 19 biotypes (Bouvet & Grimont, 1986 & 1987).

The most common genospecies of *Acinetobacter* are including *A. baumannii, A. calcoaceticus, A. lwoffii, A. junii, A. johnsonii,* and *A.* genospecies 3, 6 and 11. DNA retardness studies carried out in strains of *Acinetobacter* showed five DNA retardness groups. (Nishimura, et al, 1988; Kairiyama, et al, 1980). The group 5 contains radiation resistance *Acinetobacter* strain named as *A. radioresistens,* isolated from soil (Ino & Nishimura, 1990). Esterase and polymorphism electrophoretic studies differentiate strains of *A. baumannii, A. haemolyticus* and *A. johnsonii. Acinetobacter* genospecies were also classified on the basis of cellular fatty acid patterns and composition. (Kaempfer,1993). On the basis of presence of 3-OH dodecanoic acid two main groups were identified. All currently recognized *Acinetobacter* genospecies could be characterized by major amount of hexadecanic acid (16:0). i.) Cis –9 –hexadecanoic acid (cis-9-16:1), ii.) octadecanoic acid (18:1) and iii) 3-OH dodecanoic acid (3-OH 12:1) (Kaempfer,1993).

1.3. Importance of *Acinetobacter* :

It is very important to study this genus as it is one of the clinically and industrially important bacteria.

Importance of *Acinetobacter* is as follows :

- *Acinetobacter* is one of the plant growth promoting rhizobacteria which promote plant growth by producing Indole –3-acetic acid (IAA) (Huddedar et al, 2002).
- *Acinetobacter* produces antimicrobial substances such as antibiotic and siderophores. (Huddedar, et al 2001).
- *Acinetobacter* solubilize phosphate and promote plant growth (Leinhos, 1994).

- *Acinetobacter* produces anti-tumor and antileukamic agents like asparginase, glutaminase (Ammon et al, 1988).
- *Acinetobacter* species are resistant to commonly used antibiotics and act as a reservoir of plasmids particularly in hospital environment (Chopade et al, 1985).
- *Acinetobacter* is one of the opportunistic human pathogen and causative agent of number of diseases (Patwardhan, 1991; Patil and Chopade 2001; Bergogene-Berezin, 1987).
- *Acinetobacter* is most common organism in food spoilage (Gibbs, et al 1982; Gennari, 1985; 1992; 1993; Saha & Chopade, 2001).
- Protein rich biomass can be produced by *Acinetobacter* spp. (Koh, et al, 1987).
- *Acinetobacter* is able to degrade variety of hazardous aromatic pollutants, complex hydrocarbons, fatty acids, alicyclic compounds and alkanes (Fewson, 1991).
- *Acinetobacter* spp. is used in phosphate removal from waste (Pauli & Kaitala, 1995).
- *Acinetobacter baumannii* is used in removal of silver from photographic waste water (Shakibaie et al, 1999).
- Lipases, glucose dehydrogenases, aspartylglycosylamidase, aminohydrolases, are also produced by *Acinetobacter* spp. (Singer et al, 1985).
- Enzymes like Acc-I, Acc-II, Acc-III and Acc-IV restriction endonucleases are produced by *Acinetobacter* spp. (Singer & Finnerty, 1986)
- *Acinetobacter* produces polymeric bioemulsifers (Gutnick & Rosenberg,1977; Gutnick, et al, 1991; Patil & Chopade, 2001).
- *Acinetobacter* spp. is an important model bacterium in understanding of genetics, structure, organization and regulation in microbial system (Towner & Vivian, 1977).

1.4. Gene transfer in *Acinetobacter* :

Acinetobacter contains plasmid/s, an extrachromosomal, circular DNA, and co-transfer of resistance to number of antibiotics and metals (Dhakephalkar & Chopade, 1994; Deshpande & Chopade, 1994). Antibiotic resistance of *Acinetobacter* plasmids is serious concern to medical community. Spread of these resistant plasmids to the other organisms makes clear the highest epidemiological significance of *Acinetobacter.* Metal resistance of *Acinetobacter* is very important in environmental safety point of view as it affects ecosystem, especially on food chain. Heavy metal pollutions such as cadmium polluted water and food can cause Itai-Itai disease and mercury-polluted water and food can cause Mina-Mata syndrome (Krishnamurti, 1991). The transfer of these plasmids and their effect was mainly depends on ability to survive and multiply in the host. The plasmid transfer in *Acinetobacter* mainly take place by 3 ways, which are briefly discussed below.

a). Conjugation :

Towner & Vivian (1976a) had first reported conjugation in genus *Acinetobacter* using strain EBF65/65 and a broad host range plasmid RP4 as a mobilizing vector. Vakeria et al, (1984), subsequently added several genes concern with mandelate metabolism to this map using conjugation mediated by RP4. However, transferable silver resistance was a rare phenomenon, Deshpande & Chopade (1994), was found that plasmid encoded silver resistance was transferable by conjugation in *Acinetobacter*. The chromosomal organization of *Acinetobacter* differs from enteric bacteria, but is similar to *Pseudomonas*. A variety of plasmids belonging to different incompatibility groups have now been shown to be capable of transfer by conjugation to *Acinetobacter* from enteric bacteria, although not all are stably maintained (Towner & Vivian, 1977; Chopade et al, 1985; Deshpande & Chopade 1994, Shakibaie, et al, 1999). Conjugal mobilization of cloning vectors from *E. coli* HB101(pRK2013) in to *A. calcoaceticus* BD413 was carried out by Singer & Finnerty (1986). However, many questions remained unanswered about the nature of both RP4 and PAV1 mediated conjugation in *A. calcoaceticus*.

B) Transformation :

The transfer of genetic material by transformation in *Acinetobacter calcoaceticus* was demonstrated by Juni & Janik (1969). The transformation was carried out on the surface of agar plates using capsulated mutants of strain BD4 as recipients. The microencapsulated mutant BD413 was shown to transform at higher frequencies than other capsule mutants tested. The plate transformation assay proved useful for taxonomic studies. Several similarities were noted between chromosomal transformation (Cruze, et al, 1979) and plasmid transformation in *Acinetobacter* spp. : i) Plasmid transformation was not dependent upon the addition of calcium, magnesium, rubidium salts nor upon pre-incubation of recipient cell cultures in specific buffers at reduced temperatures. ii) Plasmid transformation was dependent upon culture and transformation conditions that favor rapid recipient cell growth, iii) Perturbation that retarded recipient cell growth resulted in depressed transformation frequencies, iv.) The inability to transform *Acinetobacter* spp. strain HO1-N with plasmid DNA paralleled its poor performance as a chromosomal DNA recipient in earlier studies (Cruze et al, 1979 ; Singer et al, 1986).

At least two *Acinetobacter* strains and their derivatives are naturally transformable. One of these strain formerly designated *Acinetobacter calcoaceticus* BD4 has recently been renamed *Acinetobacter* spp. strain BD4. The high competence for natural transformation of strain BD4 and its mini encapsulated mutant strain BD413, facilitated molecular studies of catabolic pathways. Physiology of natural transformation in strain BD413 has been intensively studied. High competence is induced immediately after the transition from the lag phase to the exponential growth phase and gradually declines thereafter. DNA uptake is strongly dependent on divalent cations, such as Ca^{+2}, Mn^{+2}or Mg^{+2} and has been shown to require energy. Furthermore

Acinetobacter spp strain BD413 dose not discriminate between heterologus and homologues DNA and takes up DNA in single stranded form before it is integrated in to the chromosome via a RecA – dependent recombination mechanism (Porstendorfer, et al, 1997). Recently, *Acinetobacter* spp. strain BD413 was examined to capture and integrate transgenic sugerbeet DNA based on homologus recombination (Gebhard & Smalla, 1998).

C) Transduction :

The presence of phages in *A. calcoaceticus* has been demonstrated by various author (Hartman, et al, 1960; Twarog & Blouse, 1968; Ackermann & Grochu, 1973; Vieu et al, 1979). Twarog & Blouse (1968) described a small short tailed transducing phage (BP1) for *Acinetobacter*, which was morphologically similar to coliphages T3 and T7, which was thought to transduce arginine and histidine genes in strain ATCC 15150.

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B. Rhizosphere of Wheat : A Review.

1.6 Introduction :

The root soil interface is a complex system referred to as the rhizosphere. The rhizosphere encompasses the soil immediately adjacent to a root. (Hiltner, 1904; Kleeberger, et al, 1983). There were many microorganisms in rhizosphere region, and the rhizosphere microorganisms play important roles in plant development. The rhizosphere has indefinite dimensions, depending on the soil and plant. The greatest effect of the plant appear on the root surface and in the soil in contact with the root, but effects may extend for several millimeters beyond the root where fungus mycelium penetrates the soil from the rhizosphere which is the food base. Clark (1949), proposed the term "rhizoplane" for the external root surface and closely adhering particles of soil and debris. However, the term rhizosphere is widely accepted than rhizoplane.

Most rhizosphere microorganisms are saprophytes, but not all, and their relations to plants are incidental. Some microorganisms live on the root surface, where as, others penetrate the root. Some are restricted to the cortical cells, but others go deeper, passing between the cells and invading them. Some are innocuous and others are destructive or have favorable effects on development of the host. Interrelations between plants and microorganisms, Hoffmann (1914) reported that bacteria were generally more numerous adjacent to plant roots than in soil. Microbial cells were much more abundant in the rhizosphere than in the soil (Starkey, 1929; Thom & Humfeld, 1932). According to the plate method, increase in number of aerobic bacteria was much higher than that of actinomycetes and filamentous fungi. Certain bacterial types were more affected by rhizosphere conditions than others. For example, *Agrobacterium radiobacter* was affected proportionally more than the general bacterial population (Starket, 1958). The fact that microbial population is dense on plant roots at all stages of plant growth because it has 2-fold importance: the plant roots affect microbial development, and plant intern was affected by the increased activity of microorganisms in the rhizosphere. It was also observed by Linford (1942), that large colonies of bacteria were developed about the young growing roots. Rovira (1956), observed that bacteria were present on the dry seeds and that they developed rapidly on the roots as the seeds germinated. Rovira (1956), clearly indicated that there was extensive bacterial development on seeds of oats and tomatoes almost from the start of germination.

The non sporulating, gram negative rods are the most prominent group of bacteria in the rhizosphere. Fungi and actinomycetes are also present but their population was lesser than bacteria (Rovira, 1962a, 1965a & 1965). Greater rhizosphere effect was seen with bacteria than with actinomycetes or fungi (Subba Rao, 1986). Root exudates, which directly or indirectly influence the quality and quantity of microorganisms in the root region, include amino acids, sugars, organic acids, vitamins, nucleotides and many other unidentified substances (Subba Rao, 1986). In number

of plant microbe association, symbiotic and pathogenic interactions takes place in the rhizosphere, which affects on the population of microorganisms present in the rhizosphere (Newman, 1978; Subba Rao, 1986; Campbell, 1982).

1.6.1 Importance of wheat :

Among the major world crops wheat is pre-dominant both in regard to its antiquity and its importance as a food of mankind. Although rice is the principal food of a large portion of the human race, a greater amount of wheat is grown and this in the form of bread, constitute the chief food of the most highly civilized races. On account of particularly physical and chemical quantities of the gluten of its grain, wheat makes more potable and better bread than any other cereals. Thus wheat is the most important staple food of the world, covering 200 million hectors. In India, it is grown in about 23 million hectors, which is about 20 % of the area under cereals. The annual production is about 36 million tonnes of grains (Rangaswami, 1996; Rangarajan et al, 2002). Byproducts of wheat obtained while manufacturing are most valuable foods for all kinds of stocks. In addition to its agricultural value, wheat straw is utilized in the manufacture of mattresses, straw hats and paper. Major wheat producing countries are USA, China, India, Australia, Russia, and Mexico. Thus it is important to study rhizosphere of wheat, which is the base of wheat plant, and on which yield of wheat plant relies.

1.7. Distribution of bacteria in the rhizosphere of wheat :

Wheat rhizosphere includes large number of gram-negative bacteria, some fungi and actinomycetes. The percent distribution of bacteria in the wheat rhizosphere was not done in detail. Kleeberger et al (1983), first time, showed distribution of rhizosphere microflora of wheat with special reference to Gram negative bacteria (Table 1). Kleeberger et al (1983), classified these microflora in three major groups, viz. *Pseudomona, Enterobacter* and spore formers (genus *Bacillus*). Additional gram-negative bacteria were representatives of genera *Alcaligenes, Acinetobacter, Chromobacterium* and *Flavobacterium*, were grouped together. Similarly the socalled Coryneforme bacteria, which included *Corynebacteria, Arthrobacteria, Mycobacteria* and representative of genus *Cellulomonas*, were treated as one group. Kleeberger et al (1983), observed that, Coryneforme bacteria were dominant, followed by *Pseudomonas*, few *Enterobacter* group and spore formers were found. The most dominant gram negative bacteria found was *Pseudomonas* spp. after that *Enterobacter* spp and lesser, *Citrobacter freundii* and *Serratia* spp. Different *Pseudomonas* spp. observed were *P. fluorescens, P. putida, P. stutzeri* and *Pseudomonas* spp., *Enterobacter* spp. found were, *E. agglomerans* I, *E. agglomerans* II, and *E. cloacae*. *Azospirilla* in spite of thorough screening were not found. Kleeberger et al (1983) stated that, *Azospirilla* are not a substantial part of rhizosphere of cereals in temperate regions. A very homogenous spp. found in rhizosphere of wheat is *P. fluorescens*. Thus Kleeberger et al (1983), found that 80 % of gram-negative bacteria were occupied in total bacterial flora of wheat rhizosphere. In Gram positive

Table. 1. Bacteria present in rhizosphere of wheat.

Bacteria	References
Gram negative bacteria	
Pseudomonas spp.	Kleeberger et al, 1983, Linhos & Vocek, 1994
P. fluorescens	Kleeberger et al, 1983
P. putida	Kleeberger et al, 1983
P. stutzeri	Kleeberger et al, 1983
Enterobacter spp.	Kleeberger et al, 1983
E. agglomerans I	Kleeberger et al, 1983
E. agglomerans II	Kleeberger et al, 1983
E. cloacae	Kleeberger et al, 1983
Citrobacter freundii	Kleeberger et al, 1983
Serratia spp.	Kleeberger et al, 1983
S. marcescens	Kleeberger et al, 1983
Azospirillum spp.	Kleeberger et al, 1983
Azotobacter spp.	Kleeberger et al, 1983, Linhos & Vocek, 1994
Alcaligenes spp.	Kleeberger et al, 1983
Acinetobacter spp.	Kleeberger et al, 1983
Chromobacterium spp.	Kleeberger et al, 1983
Flavobacterium spp.	Kleeberger et al, 1983
Arthrobacter spp.	Kleeberger et al, 1983
Mycobacterium spp.	Kleeberger et al, 1983
Cellulomonas spp.	Kleeberger et al, 1983
Gram positive bacteria	
Bacillus spp.	Mavingui, 1992; Kleeberger et al, 1983
Bacillus polymyxa	Mavingui, 1992.

bacteria, Mavingui (1992), found out more number of *Bacillus polymyxa* (70%) were present in rhizoplane than rhizosphere and non rhizosphere soil.

1.8. Different interactions in rhizosphere of wheat :

Two major interactions take place in wheat rhizosphere. One is symbiotic type and the other is pathogenic.

Symbiotic interactions between wheat roots and rhizosphere bacteria :

Wheat is mainly infected by fungal pathogens than bacterial. Table 2, indicates the list of wheat diseases and causative organisms. The major symbiotic interaction between wheat plant and *P. fluorescens* was studied in detail. It is known that take-all is the major disease caused by a wheat pathogenic fungi *Gaeumannomyces graminis* var *tritici*. It was found that, *P. fluorescens*, a plant growth promoting rhizobacteria, naturally present in wheat rhizosphere inhibits the growth of many fungal pathogens including *Gaeumannomyces graminis* var *tritici* by producing an antibiotic phenazine-1-carboxilic acid (PCA) (Brisbane et al, 1987; Gurusiddaiah et al, 1986; Weller & Cook, 1984) and acetylphloroglucinols 2,4-diacetyl-phloroglucinol (Keel et al, 1992; Vincent et al, 1991; Harrison et al, 1993; Dutrecq et al, 1991). Phenazine mediated antibiosis is the primary mechanisms by which strain 2-79 suppress take- all (Bauer et al, 1991; Thomashow & Weller, 1988; Thomashow et al, 1990) and is well known to play an active role in take-all suppression by other *Pseudomonas* as well. Another species of *P. aureofaciens* also produces Phenazine (PCA), and able to suppress the take-all. *P. fluorescens* also produces a siderophore known as CHAO, and other antifungal and antimicrobial compounds such as pyrrolnitrin , pyoluteorin , cyanide etc and able to suppress tan spot caused by *Pyrenophora tritici-repentis* (Pfender , et al, 1993;). It was also found that *P. fluorescens* has not only a role in plant protection but also played an important role in plant growth stimulation by producing, auxins such as IAA, cytokinine and hormones and also having ability to solubilize phosphate (Leinhos & Vocek, 1994; Leinhos, 1994; Georgakopoulos, et al, 1994). Other *Pseudomonas* spp., also showed symbiotic relation with wheat plant. *P. aurantiaca* can inhibit the growth of wheat pathogen such as *F. oxysporium* (Garagulya, et al, 1974; Pidoplichko, et al, 1974), *P. aeruginosa* inhibit a wheat plant pathogen *Septoria tritici* (Flaishman et al, 1990). There are other bacteria such as *Enterobacter agglomerans, Azotobacter* spp., *Azospirillum* spp. and *Bacillus polymyxa* also showed symbiotic actions by employing different modes of plant growth promotion. But for wheat plant a strong interaction was exhibited by beneficial *Pseudomonas* spp. Thus *P. fluorescens* significantly increase plant growth under gnotobiotic conditions and in the field (Kloepper & Schroth, 1981).

Table. 2. Diseases of wheat prevalent in India.

Name of the pathogenic microorganism	Name of the Disease	Reference
Fungi	**Fungal diseases**	Rangaswami, 1996
Puccinia graminis,	Black rust	
Puccinia recondite	Brown or orange rust	Rangaswami, 1996
P. striiformis	Yellow or stripe rust	Rangaswami, 1996
Ustilago nuda triticischar	Loose smut	Rangaswami, 1996
Urocystis agropyri (preuss) Schroet	Flag smut	Rangaswami, 1996
Tilleria caries	Rough- spored bunt	Rangaswami, 1994
T. foetida (Wallr) Liro	Smooth- spored bunt	Rangaswami, 1994
Neovossia indica (Mitra) Mundk	Karnal bunt	Rangaswami, 1994
Pythium graminicolum subr.	Foot rot	Rangaswami, 1994
P. arrhchnomanes dresch		
Erysiphe graminis vr. Tritici (D.C.)	Powdery mildew	Rangaswami, 1998
E.P. Marchal		
Alternaria triticina	Leaf blight	Rangaswami, 1998
Septoria tritici	Spickled leaf blotch	Rangaswami, 1998
Helimenthosporium sativum pammel	Leaf spot or black point	Rangaswami, 1998
Gaeumannomyces graminis var tritici	Take-all	Rangaswami, 1998
Rhizoctonia solani or Fusarium spp.	Seedling blight	Rangaswami, 1998
Sclerotinia sclerotiorum	Sclerotina rot	Rangaswami, 1996
Cladosporium herbarum	Leaf spot	Rangaswami, 1996
Bacteria	**Bacterial diseases**	
Angunia tritici (nematode) + *Ornybacterium tritici* (bacteria)	Yellow ear rot	Rangaswami, 1996
Xanthomonas translumcerns var. undulosum	Black-chaff	Rangaswami, 1998
Dilophora alopecuri (Fr.) Fr.	Dilophora leaf spot	Rangaswami, 1994
Virus	**Viral diseases**	
Chire , Mosaic (viruses)	Molya disease	Rangaswami, 1996

Pathogenic interactions by bacteria from rhizosphere of wheat :

The main pathogens for wheat are mainly fungi. But it was also observed by Elliott & Lynch (1984) that winter wheat (*Triticum aestivum* L.) roots can be colonized by large number of *Pseudomonas* capable of inhibiting winter wheat root growth. The organisms are aggressive root colonizers and appeared to be associated with heavy crop residues (Elliott & Lynch, 1985b; Fredrickcson et, al 1987). The inhibitory effects on wheat root growth was greater at an incubation temperature of 15^{0}C than at 20^{0}C (Elliott & Lyunch, 1984; Papendick & Miller, 1977). But their inhibitory action is not found. The inhibition of wheat roots was taken place due to the production of toxin by deleterious *Pseudomonas* spp. (Fedrickson & Elliot, 1985a). The similar results were also observed by Stroo, et al (1988). Another wheat plant pathogenic bacteria found in rhizosphere is *Xanthomonas translumcerns* var *undulosum*, a causative agent of black-chaff (Rangaswami, 1996).

1.9 Root exudates of wheat plant :

Root exudates are the compounds excreted by the plants, which act as nutrients for rhizosphere microflora (Subba Rao, 1986). Most of the studies on root exudates have been done in plants grown under aseptic conditions. The great varieties of organic substances available at the root region by the way of root exudates from roots which directly or indirectly influence the quality and quantity of the microorganisms in the rhizosphere. Root exudates are specific for each plant by nature and amount of substances, thus exudates are dependent on the species of plant, age and environmental conditions under which they grow (Bhuvaneshwari & Subba Rao, 1957; Dey et al. 1968; McDougall & Rovira, 1970; Rovira, 1965; Vancura, 1964). The substances exudated from wheat plant root include amino acids, sugars, organic acids, vitamins, nucleotides and many other unidentified substances. Table 3. shows the list of root exudates of wheat plant. It was observed that root exudates influence the proliferation and survival of root infecting pathogens in soil either through soil fungistatus or inhibition of pathogen in the rhizosphere (Subba Rao, 1986). It was also reported that root exudates can serve as chemo-attractants for rhizosphere bacteria at very low concentrations, such as compounds like benzonate can attract *Azospirillum* spp. (Loake et al, 1993), luteolin attracts *Agrobacterium tumefaciens* and *Rhizobium meliloti* (Bauer & Gaetano-Aolles, 1991; Caetano-Anolles et al, 1988; Dharmatilake & Bauer, 1992), phenolic compound acetosyringone and sugars such as sucrose, glucose and fructose all attract *Agrobacterium tumefaciens* (Ashby et al, 1987; Loake et al, 1988) and at higher concentrations certain amino acids can attract *Pseudomonas lachrymans* (Chet et al, 1973) and *Pseudomonas aeruginosa* (Nikata, et al, 1992). Recently, Overbeek & van Elsas (1995), reported the induction of promoter activity in *Pseudomonas fluorescens* mutants in the rhizosphere of wheat.

Table. 3. Root exudates of wheat plant.

Sugars	Organic acids	Amino acids	Others	References
D- manitol	malonic acid, quinic acid, m-	Leucine, valine, aminobutyric	Flavonone, adenine,	Subba Rao, N.S. (1986)
Sucrose	tartaric acid, oxalic acid, malic	acid, glutamine, α-alanine,	guanine, uredine, cytidine,	Subba Rao, N.S. (1986)
glucose	acid, acetic acid, citric acid,	aspargine, serine, glutamic	invertase, amylase,	Overbeek & van Elsas (1995)
rhamnose	succinic acid , fumaric acid,	acid, aspartic acid,	proteases, adenosine, A-	Overbeek & van Elsas (1995)
arabinose	propionic acid, butyric acid,	cystine/cysreine, glycine,	acetyl	Vancura, (1964)
xylose	valeric acid, γ-aminobutyric	tyrosine, lysine, methionine,	D-glucosamine, glycerol,	Bhuvaneshvary & Rao, (1957)
raffinose	acid, hydroxybutyric acid, D-	phenylanaline, threonine,	inosine, putrescine,	Rovira, A.D. 1965 &1969
D-mannose	galactonic acid lactone, pyruvic	proline, alanine, L-gluco-	D,L- carnitine, adinosine,	Overbeek & van Elsas (1995)
D-trehalose	acid,	thymine, proline	2-aminoethanol	Overbeek & van Elsas (1995)
D-arabitol	α-ketoglutaric acid, bromo			Overbeek & van Elsas (1995)
fructose,	succinic acid, urocanic acid.			Overbeek & van Elsas (1995)
D-galactose				

2.0. Wheat root colonization by rhizobacteria :

Soil microbiologists and plant pathologists have recently succeeded in altering the native microflora of plant roots to achieve either biological control of soil borne diseases (Klopper et al, 1980; Scher & Baker, 1980; 1982; Sivasithamparam & Parker, 1978; Smiley, 1978;1979; Weller & Cook, 1983) or increased plant growth (Kloepper & Schroth, 1981; 1981b; Suslow & Schroth, 1982). When the proper bacterial strain was used plant roots were extensively colonized by the introduced strain, which suggest a close bacteria-plant association that allows beneficial plant growth or disease protection. The use of microorganisms to promote plant growth has become more important during the past decades and colonization is the first step for successful biocontrol (Brown, 1974; Burr & Caesar, 1984; Gaskin et al, 1984;1985). Plant growth stimulating effects have been attributed to symbiotic or associative nitrogen fixation, solubilization of minerals, biological control of plant pathogens (siderophores, antibiotics), production of plant growth stimulating substances and microbial interactions leading to " niche exclusion" (Brown 1974; Kloepper, et al, 1980; Geels & Schippers, 1983; Burr & Caesar, 1984; Gaskins et al, 1984 & 1985; Schroth et al, 1984; Schippers, et al ,1985; Bakker, et al, 1986). So far, results have been highly variable and stimulating effects in green house experiments could not always be reproduced in the field (Burr & Caesar, 1984; Gaskins, et al, 1984, 1985; Schroth et al, 1984). Bennett & Lynch (1981a&b), developed a closed test tube assay for measuring root colonization capacity of bacteria under gnotobiotic conditions which proved useful for studying specific microbial interactions in the rhizosphere. Van Vurde & Schippers (1980) found that roots of wheat have two distinct zones of stimulated bacterial growth which seemed to be closely linked to a high frequency of epidermal and cortical cell lysis. However, the size of this cdonization potential differs widely among different bacterial species (Lynch & Clark, 1984). Despite relatively high numbers, bacteria were shown to cover only 4-15 % of the wheat root surface often aggregated on preferential sites (Rovira, 1956; Rovira et al, 1975; Bowen & Rovira, 1976; Bowen, 1980; Bennett & Lynch, 1981b; Lynch, 1983; Rovira et al, 1983). This might reflect limited migration on the root surface. Bowen & Rovira (1976) suggested that the dispersal of bacteria along the wheat root was due topassive transport by the root tip and elongation zone, or active migration in a film of water on the root.

2.1. Role of chemotaxis in wheat root colonization :

Scher, et al (1985) found that, chemotaxis to be the first step in bacterial seed and root colonization in soil. They observed active migration for seed colonizing, motile fluorescent*Pseudomonas*, but not for non motile mutants. Unpublished results of Ridge, cited by Bowen & Rovira (1976), supported a passive movement since *P. fluorescens* did not move more than 2-3 cm in the rhizosphere of wheat under aseptic conditions. Howie & Cook, (1985) studied the colonization of wheat roots by strains of*P. fluorescens*. It was concluded that root elongation is the primary means by which bacteria introduced on the seed surface spread in the absence of water movements along

the root. In spite the absence of active migration, *P. fluorescens* was capable of establishing itself in relatively large numbers (Burr & Caesar, 1984; Kloepper, 1980) on the root surface. Although a significant rhizosphere effect could not be demonstrated for the used *P. fluorescens* under field condition (van Elsas, et al, 1986), this observation was expected because this strain was considered as a good root colonizer (Geels & Schippers, 1983; Schippers, et al, 1985). The final high cell numbers and the relatively small inoculum size indicate that, after dispersal by the growing root tip and elongating cells, rapid growth has occurred. This would mean that a good colonizer is not only characterized by active migration, chemotaxis, or strong competitive capabilities but also by rapid growth on the root surface. Lower numbers of *B. subtilis* were expected because this bacterium seems to occur preferentially in the bulk soil (van Elsas et al, 1986). The decreasing numbers towards the root tip may indicate slow or no growth after passive dispersal by the root tip and elongating cells.

Successful establishment of bacteria on the root surface is not primarily dependent on active migration / dispersal to and on the root surface, but also on the capability to multiply rapidly on the root after passive displacement on the root tip and elongating cells. Other factors, such as physiological and plant associated environmental factors were most important for apid growth on the root surface. Further studies carried out by van Elsas, et al (1992) for the survival and root colonization by alginate encapsulated *Pseudomonas fluorescens* cells, with skim milk and bentonite clay introduced in soil showed high survival rate in soil, and in addition high number of cells colonized the wheat rhizoplane.

2.2. Biocontrol of wheat take-all by *Pseudomonas fluorescens*:

Take-all is the disease caused by pathogenic fungi *Gaeumannomyces graminis* var *tritici*, occurs worldwide in area where soil pH was slightly acidic to alkaline (approx. 6.08.5) (Cook , 1981). The pH range favorable for take-all development occurs naturally in wheat growing regions. Severity of take-all can be reduced by fertilization of wheat with ammonium forms of nitrogen (Cook, 1981; Huber et al, 1968; MacNish, 1988), which when absorbed by wheat roots reduce the rhizosphere pH through corresponding excretion of H^+ ions (Smiley, 1974 &1975). Previous studies demonstrated that seed bacterization with *P. fluorescens* 2-79 suppress take-all of wheat (Weller & Cook, 1983; Weller et al, 1985). Strain 2-79 produced phenazine-1 carboxylic acid (PCA), an antibiotic with broad-spectrum activity against fungi and bacteria (Gurusiddaiah, et al, 1986). Both genetic and chemical evidence indicate that production of PCA is the primary mechanism by which 2-79 suppresses take-all (Brisbane, et al, 1987; Brisbane & Rovira, 1988; Gurusiddaiah, et al, 1986; Hamdan et al 1991; Poplawsky, et al, 1988; Thomashow & Weller, 1988; Thomashow, et al, 1990; Weller, 1988). Thus *P. fluorescens* was found best for biocontrol of major wheat disease, Take-all. More study is needed for rhizosphere of wheat by screening newer and more potential biocontrol agents.

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C. Antimicrobial Substances Produced by Rhizobacteria : A Review.

2.4. Introduction :

Different microorganisms including both beneficial and deleterious ones heavily populate the rhizosphere region. The rhizosphere is the first line of defense for roots against attack by pathogenic fungi (Weller, 1988). Therefore there is an excellent opportunity to find rhizosphere competent bacteria in the rhizosphere, which are potential biocontrol agents. Among range of rhizobacteria including *Agrobacterium, Bacillus, Pseudomonas* and other genera with the ability to promote plant growth through biocontrol or other mechanisms, the*Pseudomonas* spp. in particular have received worldwide attention (Burr &Caesar, 1984; Defago & Hass, 1990; Schippers, 1988; Weller, 1988). It was also observed that*Pseudomonas* appeared to be most important antagonistic bacteria in the soil (Keel, et al, 1990). A successful biocontrol agent efficiently suppresses the pathogen and reduces disease incidence. Biocontrol agents act against pathogen by such various forms of antagonism, as competition, antibiosis and parasitism. Activity is not restricted to only one of these, and, indeed, an efficient biocontrol agent may affect pathogens by combination of mechanisms (Chet, 1987).

2.5. Colonization by rhizobacteria and production of antimicrobial substances :

An effective biological control agents needs to colonize the whole root system intensively, in the sense of being present at the sites where the infection takes place. This means that after introduction it becomes distributed along the root, propagates, survives and exhibits its beneficial effects for several weeks in the presence of competition from the indigenous rhizosphere microflora (Parke, 1990; Weller, 1988). Among the complete range of*Pseudomonas* strains found in the rhizosphere, only a few posses these attribute. Some*Pseudomonas* strains e.g. CHAO, found not only on the root surface but also in the root cortex, showed by indiect immuno-fluorescence staining. Small lesions in the root surface were used by strain CHAO for intrusion into the cortex (Defago et al, 1990). Root colonization is influenced by characteristics (rhizosphere competence traits) of the introduced bacteria, the rhizosphere microflora, the plant and the abiotic environment. Bacterial phenazine antibiotic production was shown to contribute to the longterm survival fluorescent *Pseudomonas* in soil (Thomashow & Pierson III, 1991). The production of specific siderophores and the utilization of a broad spectrum of siderophores constitute to the root colonizing and plant growth promoting ability of a *P. putida* strain (Bakker, et al, 1986) Chemotactic attraction of bacteria towards root or seed exudates (Bashan, 1986; Scher et al, 1985) may be a guide to infection site where exudation is enhanced. Tolerance to dry soil and low osmotic potential may help some introduced bacteria to survive (Howie, et al, 1987, Loper & Buyer, 1991). Studies with flagella negative non motile Tn5 mutants revealed that motility either enhanced (Weger, et al, 1989) or had no effect (Howie, et al, 1987) on root colonization. Cell

surface properties like aggutinability (Glandorf, et al, 1991), lipopolysaccharides (Weger, et al, 1989), and pili (Vesper, 1987) may help to support the establishment or the specificity of plant bacteria associations. Composition of root exudates may vary with plant species, cultivars and even growth stage of the plant and thus affect root colonization (Weller, 1988). Furthermore, interactions with the indigenous rhizosphere microflora and physical and chemical characteristics of the soil may drastically influence the establishment and survival of an introduced biocontrol agent (Davis & Whitebread, 1989; Weller, 1988).

2.6. The major ways of rhizobacterial antagonism :

i) Production of siderophores :

Siderophores are low molecular weight high affinity ferric ion chelaters, which are synthesized and created by many microorganisms in response to ion deprivation (Payne, 1994). Many bacteria such as *Enterobacter, Agrobacterium, Aeromonas, Arthrobacter, Acinetobacter, Mycobacteria, Paracoccus, Pseudomonas, E. coli, Salmonella* and *Rhizobium* are able to produce siderophores (Chaudhari & Chincholaker, 1998) and can suppress the growth of other bacteria. Hydroxymate and catachole type of siderophores were mainly produced by microorganisms (Payne, 1994). They suppress the pathogenic microorganisms subsequently by limited supply of iron in the rhizosphere and thereby limiting the availability of the iron necessary for the growth of the pathogens. Siderophore minus mutants and on the effects of purified siderophores or synthetic iron chelators which present evidence of an involvement of pyoverdines in the biocontrol of diseases caused by *F. oxysporum* spp., *P. ultimum* and minor pathogens (Lopar & Buyer, 1991). However, an increasing number of reports indicate that bacterial metabolites other than siderophore production, i.e. antibiotics do have a key role in disease suppression (Hass, etal, 1991; Keel, et al, 1989; Kraus & Loper, 1991; Thomashow et al, 1990).

ii) Degradation of pathogen toxins:

Toyada et al, (1988) found a non pathogenic mutant of *P. solanacearum* which was able to de-toxify fusaric acid, the wilt toxin of *Fusarium oxysporum* spp. *lycopersice* and to protect tomato plants from the wilting disease. Extracellular proteases of fluorescent *Pseudomonas* strains are suggested as inactivated hydrolysis and hydrotoxins of phytopathogenic *Fusarium* spp. (Borowicz, et al, 1991).

iii) Induction of plant defense mechanisms:

There is an increasing number of reports indicating that *Pseudomonas* might also suppress diseases by inducing resistance in the plant. Bacterial metabolites, like HCN or 24- di-acetylphloraglucinol, may also be toxic to the plant (Keel, et al, 1990; Voisard, et al. 1989). Sub-inhibitory quantities of the metabolites produced by the bacteria closely associated with the root might induce, the stress necessary to activate plant defense mechanism against the pathogen (Defago, et al 1990; Keel, et al, 1990). Bacterial influence on the plant also in the absence of the fungal pathogen (Defago, et al

1990). In the presence of *P. fluorescens* CHAO, tobacco roots were stunted and showed an increased root hair formation. Additional experiments showed that morphological changes of tobacco roots are co re-plated with the presence of functional cyanide genes in the bacteria (Defago, et al, 1990). These genes as shown above are necessary for anefficient disease suppression (Voisard, et al, 1989). Thus it seems possible that bacterial HCN might modify plant metabolism in a way that induced some defense mechanisms. Van Peer & Schippers, (1991), have investigated the possible involvement of induced resistance biological control of*F. oxysporum f. sp. dianthi* on carnation by fluorescent *Pseudomonas* spp. WCS 417. Roots were bacterized one week prior to stem inoculation with conidia of*Fusarium* disease was significantly reduced when plants were bacterized compared to plants inoculated with*Fusarium* alone. Although these systems provide evidence for an involvement of induced resistance, they cannot rule out the possibility that bacterial metabolites e.g. antibiotics, could be taken up and translocated in the plant and directly antagonise the pathogen exerting a systemic protection comparable to the action of certain pesticides.

2.8. Antibiotic production and their role in biocontrol :

Plant diseases preliminary caused by fungal and bacterial pathogens, causes severe loss of agricultural and horticultural crops every year. The loss results in reduced food supply and poor quality agricultural products, economic hardships and processes and ultimately higher price. Traditional chemical control methods are not always ecologically effective for many diseases and some chemical controls may have unwanted health and environmental risks. Biological controls involve the beneficial microorganisms such as specialized bacteria to attack and control plant pathogen and disease caused thereby. Biology offers a ecofriendly approach to the management of plant disease and incorporated with cultural and physical controls and limited chemical uses for an integrated management system. Biological control can be an important compound development of more sustainable agricultural systems (Ravera, 1998). There are some bacteria which are known to produce some lytic enzymes such as chitinase, a chitin-degrading enzyme, having a broad spectrum antifungal activity, produced by *Serratia marcescens* and used in biocontroling of *Sclerotium rolfsii* (Ordentlich et al, 1988). But the major role carried in the biocontrol of several bacterial and fungal plant pathogens was of antibiotics. Although there are many bacteria known to produce antibiotics such as *Enterobacter* spp., *Erwinia,* spp. (Chernin et al, 1996; Kernas & Mahanty, 1998), but extensive studies were carried out on antibiotic production by*Pseudomonas* and its use as biocontrol agent (Schppers, et al, 1987). Many species of*Pseudomonas* such as *P. fluorescens, P. aureofaciens, P. putida* were known to produce antibiotics such as oomycin, pyrrolnitrin, phenazine, pyoluteorin, 2-4-diacetylphlorogluconide, etc (Gutterson, 1990; Homma & Suzuki, 1989; Kraus & Loper, 1991; Thomashow & Weller, 1988; Voisard, et al, 1989). In all the strains *Pseudomonas fluorescens* was mainly used as the biocontrol agent. Following is the brief

description for major antibiotics produced by *Pseudomonas fluorescens* and description of pyrrolnitrin.

a) Oomycin A :

P. fluorescens Hv37a, isolated from the root tips of barley (Gutterson, et al, 1986) produced oomycin A, a heat stable, amphipathic molecule of 700-800 Da with a pI of 4.55 (Gutterson, et al, 1990) that is effective against *P. ultimum* in vitro (Howie, et al, 1989) and accounted for approximately 50 % of the increase in the cotton seedling emergences and 70 % reduction in pre-emergence root infection provided by Hv37 a (Howie, et al, 1991). Production of oomycin A is induced by glucose (James & Gutterson, 1986) and inhibited by amino acids (Gutterson, 1990; Gutterson, et al, 1990). Both of which are common constituents of root and seed exudates. Synthesis of oomycin A by *P. fluorescens* Hv37a, is induced by glucose, and the identification of *afu*E as the major biosynthetic locus. The major *afu* gene cluster spans approximately 15kb and includes *afu* DEFG encompassing about 9kb and encoding products respectively of 48kDa, 31 kDa, 37kDa & 145kDa.

b) Phenazine :

Phenazine antibiotics are pigmented, nitrogen-containing heterocyclic compounds produced by bacteria via the shikimic acid pathway (Turner & Messenger, 1986) and are the primary determinants in the ability of *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 to suppress to take-all a serious root and crown rot of wheat and barley caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow & Weller, 1988; Pierson III & Thomashow, 1993). Phenazine deficient mutants of 2-79 and 30-84 were reduced inability to suppress take-all and genetic complementation restore biocontrol activity to wild type levels (Thomashow & Weller, 1988; Pierson III & Thomashow,1993). By using a precisely defined set of mutants it was demonstrated that 60 % to 90 %, (Thomashow & Weller, 1988; Hamdan et al, 1991; Ownley et al, 1992) depending on the soil and assay conditions, of the suppressiveness of *P. fluorescens* 2-79 to take-all of wheat was associated with production of PCA. In studies of up to 100 days, however the ability to produce phenazine contributed positively to the persistence of 2-79 and 30-84 in soil habitats (Mazzola et, al, 1992). Populations of Phz^- mutants of both strains declined more rapidly than did those of the parental or Phz^+ complemented mutants in raw rhizosphere and bulk soils, but not in steam pasteurized soil, suggesting that the antibiotic contributes to competitiveness against indigenous microorganisms. The biocontrol activity of 2-79 was highly influenced by soil edaphic variables (Ownley, 1992; Ownley, 1991).

The cosmid clone pLSP259 carries the major phenazine biosynthetic locus from *P. aureofaciens* 30-84 and was first identified by its ability to restore production of PCA 2-hydroxyphenazine 1-carboxylic acid and 2- hydroxyphenazine. In at least five different Eco RI restriction fragments (Pierson III & Thomashow, 1993) an activator of phenazine gene expression,

PhzR resides upstream of and is divergently transcribed from*Phz BC* (Pierson III et al, 1994) in an arrangement resembling that of *afu R* and *afu E.* The phenazine biosynthetic locus from *P. fluorescens* 2-79 complimented Phz ⁻ mutants of 2-79 hybridized to sub-cloned fragments from pLSP 259, and is contained within a 12kb fragment. Mutagenesis with the transposon*Tn3 HoHo1* revealed divergently transcribed units of CA 5.0 Kb and 0.75 Kb that were required for production of PCA and were strongly and weakly expressed, respectively, under conditions favorable for PCA synthesis.

c) **Pyoluteorin :**

Pyoluteorin (Plt), is a chlorinated phenolic tetraketide with antibiotic and phutotoxic activity. It is synthesized via the serial addition of acetyl units of proline or an equivalentstarter unit derived from the tri-carboxylic acid cycle (Cuppels, et al, 1986). Synthesis in vitro is strongly influenced by culture conditions, it was produced by strain pf5 on 523 medium but not on glucose supplemented nutrient agar (Kruas & Loper, 1992). In another, study (Bencini, et al, 1983), production depended on the carbon source in minimal medium and did not occur when tryptophan was included in the medium or cultures were well aerated. The antibiotic was rapidly adsorbed and inactivated in soil, rendering it ineffective (Howell & Stipanovic, 1980). Pyoluteorin is highly inhibitory to *P. ultimum* but, not to other seedling pathogens of cotton including*Alternaria* spp, *Fusarium spp., R. solani, T. brassicola* and *Verticillium dahliae* (Howell & Stipanovic, 1980). Root and seedling fresh weights were increased 2-fold and 5-fold by treatment with *P. fluorescens* CHAO, and a Plt-overproducing derivative was more protective than the parental strain (Maurhofer, et al, 1992).

d) **Pyrrolnitrin :**

The antibiotic 3-chloro-4-(2-nitro-3-chlorophenyl)-pyrrol or pyrrolnitin is produced by a diverse number of *Pseudomonas* spp. including strains of *P. aureofaciens.* Pyrrolnitrin inhibits the growth of many different fungi and bacteria (Burkhead, et al, 1994; Nishida, et al 1965). Although Prn was first identified in 1964 (Arima, et al, 1965). Little is known regarding its mechanism of action. Pyrrolnitrin inhibits growth but dose not kill the target organism. Pyrrolnitrin (Pyr) is the most active of family of chlorinated phenylpyrrole antibiotic produced by tryptophan via series of 5 or six reactions that include chlorination of the benzene and pyrrol rings, rearrangement of the skeleton decarboxylation, and oxidation of an amino to a nitro group (Chang, et al, 1981; Mahony & Roitman, 1990). Biochemical interest has focused on the non-heam chloroperoxidase responsible for chlorination of the pyrrol ring in the penultimate biosynthetic step, the enzyme has been purified and characterized (Wiesner, et al, 1988) and the gene has been cloned and sequenced from *P. pyrrocinia* (Wolfframm, et al, 1993). Pyrrolnitrin biosynthetic capability is widely distributed among Pseudomonad's (Elander, et al, 1968), including many with biocontrol activity against plant pathogens (Voisard, et al, 1994; Krus & Loper, 1992; Homma & Suzui, 1989; Pfender, et al, 1993;

Howell & Stipanovic, 1979; Lambert, etal, 1987; Jayaswaral, et al, 1991; Janisiewicz & Roitman, 1988; Hill et al, 1994). *P. fluorescens* Pf-5 which produced Pyr or the purified antibiotic increased the immergence and survival of cotton seedlings in non sterile soil infested with *R. solani* and the antibiotic also was active in vitro against *Alternaria spp., T. brassicola* and *V. dahliae*, weakly active against, *Fusarium* spp., and not acting against *P. ultimum* (Howell & Stipanovic, 1979). Similarly, strains of *P. cepacia* with high levels of Pyr production more effectively suppress hyphal growth and damping-off of sugerbeet caused by *Aphanomyces cochliodes* than strains that produce less (Homma, 1994). Pyr was retain intracellularly by Pf5 in culture and was stable for at least 30 d after addition to nonsterile soil (Howell & Stipanovic, 1979). Both Pf5 and purified Pyr inhibited *Pyrenophora tritici-repentis*, casual agent of tan spot disease of wheat. *P. fluorescens* BL915, which was isolated from the rhizosphere of cotton and produces Pyr as well as cyanide, chitinase and gelatinase (Gaffney, et al, 1994), a Pyr^- mutant coordinately lost antagonistic activity against *R. solani* in vitro and on seedlings of cotton (Hill, et al, 1994). Introduction of an 11Kb *Eco* R1 fragment cloned from BL915 and subsequently found to encode *gacA* global regulatory locus (Gaffney, et al, 1994), not only restore the mutant to wild type activity but also caused *P. fluorescens* BL914 and BL922, both of which are Pyr^-, and ineffective against *R. solani* in vitro and on cotton, to produce the antibiotic and to inhibit the pathogen to the same extent BL915 (Gaffney, et al, 1994; Hill et al, 1994).

It was observed that genes encoded for pyrrolnitrin, in *Pseudomonas fluorescens*, are on chromosome and four gene designated as *prnA, prnB, prnC* and *PrnD*. (Hammer et al, 1997). It was also observed that *P. fluorescens* was also able to produce pyrrol derivatives. The synthesis and microbiological activities of new 1,4 and 1,5 diarylpyrrol are studied. Antimicrobial data in comparison with fungal antibiotic pyrrolnitrin confirmed an interesting antimycotic activity of 1,4 diarylpurrols. On the contrarary,1, 5- diarylpurrols showed antibacterial activity and an unexpected antimycotic activity. The position of 4-nitrophenyl group at C or C5 of the pyrrol ring influences antibacterial activity (Porretta, et al, 1985). Recently, new bacterial species identifed as *Burkholderia cepacia*, isolated from rhizosphere of tomato also showed antagonistic activity, similar to pyrrolnitrin (Sfalanga, et al, 1999).

The role of pyrrolnitrin as a biocontrol agent is not yet clear. There is controversy and it needs more investigation as mode of action of this antibiotic is not yet completely understood. However, direct interference of pyrrolnitrin or its derivatives with fungal plasma membranes has been demonstrated (Jespers, et al, 1994; Nose & Arima, 1969).

Table 1. Antibiotic production by rhizobacteria.

Antibiotic producing bacteria	Source of isolation	Antibiotic produced	Active against	References
P. fluorescens Hv37a	Root tips of barley	Oomycin A	*P. ultimum*	Howie et al, 1989 & 1991
P. fluorescens 2-79	Wheat and barley root	Phenazine	*Gaeumannomyces graminis var. tritici*	Thomashow & Weller, 1988;
P. aureofaciens 30-84	Wheat and barley root	Phenazine	*Gaeumannomyces graminis var. tritici*	Pierson III & Thomashow, 1993
P. fluorescens pf-5	Cotton roots	Pyoluteorin	*P. ultimum, R. solani*	Howell & Stipanovic, 1980
P. aureofaciens	Wheat roots	Pyrrolnitrin	*Alternaria spp., T. brassicola*	Howell & Stipanovic, 1979
P. fluorescens pf-5	Cotton seedlings	Pyrrolnitrin	*V. dahliae, Fusarium spp.*	Howell & Stipanovic, 1979, Hammer et al, 1997
P. cepacia	Sugerbeet roots	Pyrrolnitrin	*Aphanomyces cochliodes*	Homma, 1994
P. flurescens BL915	Rhizosphere of cotton	Pyrrolnitrin	*R. solani*	Hill, et al, 1994
Enterobacter agglomerans	Cotton seedlings	Pyrrolnitrin	*R. solani*	Chernin et al, 1996
Ervinia herbicola Eh1087	Rhizosphere of barley	Pyrrolnitrin	*Erwinia amylovora*	Kerns & mahanty, 1998
Enterobacter spp.	Different plant rhizospheres	Pyrrolnitrin	*Xanthomonas campestris* *P. syringae, Corynebacterium insidiosum, Clavibacterium michiganense, Erwinia carotovora, and Serratia marscens*	Chernin et al, 1996
Pseudomonas spp.	ND	1,4 & 1,5 diaryl pyrrol	*Xanthomonas campestris* *Mycobacterium tuberculosis* and *M. avim*	Porreta, et al, 1985 &1991; van Pee, et al, 1983 &2000, Di Santo, et al, 1998.

* ND: Not done.

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D. Biosurfactants : A Review.

3.0. Sufactants : definition, properties and classification :

A biosurfactant is defined as a surface active molecule produced by living cellsin the majority of cases, by microorganisms (Fiechter, 1992, Desai & Patel, 1994). The term biosurfactant has been used to refer to any compound that is synthesized by microorganisms having some influence on interfaces. In the literature, the term's surfactant and emulsifier are frequently used interchangeably. Surfactant molecules are characterized by the presence of both hydrophilic and hydrophobic (generally hydrocarbon) structural moieties. All surface active compounds that constitute the emulsion secreted by the cell to facilitate the uptake of insoluble substrate (Hommel, 1990). A typical representative of these bioemulsifiers is Emulsan (Gutnick & Mines, 1987; Gutnick & Shabtai, 1987) currently the only this bioemulsifier being marketed for industrialuse.

The evaluation of biosurfactants is carried out through surface tension measurements. The surfactant character of molecules is due to their mixed hydrophilic and hydrophobic nature. They are able to form micelles and revised micelles or to aggregateto form row-shaped micelles, bi-layers and vesicles. They accumulate at interfaces and "mediate" between phases of different polarity such as oil/water, air/water or water/solid, acting as wetting agents on solid surfaces. This dynamic process is based on the ability of the surfactant to reduce the surface tension by governing the arrangement of liquid molecules, thus influencing the formation of Hbonds and hydrophobic-hydrophilic interactions. The minimum surface tension value reached and the critical micelle concentration (CMC) needed, are parameters used to measure the efficiency of the surfactant.

Biosurfactants have many advantages over their chemically synthesized counterparts (Kosaric et, al, 1987; Cooper, 1986). They are highly specific, less toxicand biodegradable (Zajic, et al, 1977). They are effective at extreme conditions of temperature, pH and salinity (Kretschmer, et al, 1982) and are easy to synthesize from cheaper and renewable feed stocks (Desai, 1987; Desai & Desai, 1993).

Various types of biosurfactants are synthesized by a number of microbes, particularly during their growth on water immiscible substrates. Among microbes, bacteria produce a majority of biosurfactants. Biosurfactants are commonly differentiated on the basis of their biochemical nature and the producing organisms. In general, biosurfactants are microbial metabolites with a typical amphiphilic structure in which hydrophobic moiety consists of a hydrocarbon chain of a fatty acid and the hydrophilic moiety may be an ester oralcohol function of neutral lipids, or the carboxyl group of fatty acids, or amino acids, or phosphate containing part of phospholipids or carbohydrate part of glycolipids. Basically there are five major classes of biosurfactants namely: (i) Glycolipids; (ii) Phospholipids and fatty acids; (iii) Lipopeptide/ lipoproteins, (iv) Polymeric surfactants and (v) Particulate surfactants. The properties of each class of biosurfactants have been

described by Desai (1987); Desai & Desai (1993); Rosenberg, (1986); and Kosaric et al.(1987). A list of important biosurfactants their producing organisms and characteristics are given in Table 1. Biosurfactants in the lipopeptide group exhibited their antibiotic property with the surfactant activity. A list of such bacteria is given in Table 2.

3.1. Importance of biosurfactants:

Industry uses at least 10^6 tonnes /year of synthetic surfactant (Gerson & Zajic, 1978a). Many different types of surfactants are being used, but it is important to develop even more or broaden the spectrum of properties available.

- No surfactant is suitable for all the potential applications. (Cooper & Zajic, 1980).
- Nearly all surface-active compounds currently in use are synthesized chemically, with petroleum as a raw material (Greek, 1991).
- In the present conditions, biosurfactants are unable to compete economically with the chemically synthesized compounds on the market, due to high production cost (Fiechter,1992).

These results primarily by in efficient bioprocessing methodology, but also form poor strain productivity and the need to use expensive substrates. Pre-requisites for biosurfactant gaining a significant share of the market are:

i) An improved knowledge and ability to manipulate the metabolism of the producer strains, such that cheaper substrates may be used; and

ii) The improvement of process technology tofacilitate product recovery.

Improved characterization of the strain should open the way for the use of genetic manipulation of the organisms, thus enabling them to be further tailored towards optimal performance (Fiechter,1992). In addition, to the increased cost and uncertainty in the supply of petroleum, the more readily biodegradable character of biosurfactant means that they should generate fewer environmental problems in feature that will doubtlessly gain an importance in industrial process as more rigorous controls are imposed. (Fiechter, 1992).

Although many problems remain to be solved because displacement of cheap, chemically synthesized surfactants from the market will not be easy. These factors bode well for biosurfactants eventually replacing their chemical counterparts for many applications. Biosurfactants are important because they present a much broader range of surfactant types and properties than the available synthetic surfactants. (Zajic et al, 1977a, b; Margaritis et al, 1979).

3.2. Bioemulsifier and biosurfactant production by *Acinetobacter* spp. :

Acinetobacter is one of the important bioemulsifier/ biosurfactant producing bacteria. *Acinetobacter* spp. RAG-1, produced an bioemulsifier, patented by Gutnick, (1980) known as " Emulsan" is the only bioemulsifier, used by petroleum industry. Emulsans are the most efficient emulsifier discovered and wildly used in cleaning oil contaminated vessels, oil spill management

Table 1. Bioemulsifiers/ biosurfactants produced by *Acinetobacter* spp.

Organism	Type of bioemulsifier/ biosurfactant	References
A. calcoaceticus RAG-1	Lipopolysaccharide (Emulsan)	Sar, 1983; Rosenberg & Ron, 1988; Osterricher-Ravid et al, 2000.
A. radioresistens Ka53	Polysaccharide –protein complex (Alasan)	Navon-Venezia, et al, 1995; 1998; Osterricher-Ravid et al, 2000; Toren et al, 2001.
A. calcoaceticus BD4	Polysaccharide-protein complex	Kaplan, et.al, 1985
A. calcoaceticus A2	Polysaccharide-polymer (Biodispersan)	Rosenberg, et al, 1988a,b; Ron & Rosenberg, 2001.
A. johnsonii SC14	Polysaccharide-protein-lipid	Patil & Chopade, 2001

and enhanced oil recovery by chemical flooding (Gutnick & Rosenberg, 1981). Other than emulsan, *A. calcoaceticus* strains also produce different nondialyzable interfacially-active different bioemulsifiers (Gutnick et al, 1989, 1993). The bioemulsifer produced by *A. radioresistens* strain KA53 is designated as alasan or E-KA53. It has a molecular weight of about 100, 000 to 2,000,000 daltons; emulsifying activity which increases with preheating at increasing temperatures 60-90^{0}C; resistance to strong alkali while retaining increased emulsifying activity; reduced viscosity that varies as a function of pH and magnesium ions (Ron & Rosenberg, 2001).

Bioemulsifers and biosurfactants produced by *Acinetobacter* spp. have been used in different products and processes and some of them have been patented also. Bioemulsifiers produced by *A. calcoaceticus* strains have been used in shampoos, soap and/ or ameliorates, certain common dermatological problems such as acne and eczema. The skin cleasing cream and skin cleansing lotion containing these bioemulsifiers have (a) specific emulsification activity of at least about 25 units per milligram; (b) The ability to remove sebum; (c) The ability to interfere with microbial adhesion on skin or hair (Hayes et al, 1989, 1990, 1991 & 1992, US487010, CA 1266238, US4999195, CA 1300512). Bioemulsifers produced by *A. calcoaceticus* strain can be used to stabilized hydrocarbosols by surfactant package (Murphy, et al, 2000 1340969).

3.3. Physiological role of biosurfactants :

The main physiological role of biosurfactants is to permit microorganisms to grow on water immiscible substrates by reducing the surface tension at the phase boundary, thus making the substrate more readily available for uptake of metabolism (Rosenberg et al,1991). In addition to the emulsification of the carbon source biosurfactants are also involved in the adhesion of microbial cells to the hydrocarbon (Kappeli & Fiechter, 1976).

Table.2. Major types of biosurfactants with their surface tension.

Surfactant type	Microorganism	Surface Tension (ST)	References
i. Glycolipids			
Trehalose lipids	*R. erythropolis*	32-36	Kim, et al, 1990; Kristchmer etal, 1982;
	N. erythropolis	30	McDonald etal, 1981;
	Mycobacterium spp.	38	Desai & Patel, 1994
Rhamnolipids	*P. aeruginosa*	29	Mulligan and Gibbs, 1989
	Pseudomonas spp.	25-30	Sydatk et al, 1985
Sophorolipids	*T. bombicola*	33	Cooper & Paddock, 1984;
	T.apicola	30	Tullock et al, 1967; McDonald et al, 1981.
Glucose-Lipids	*Alcanivorax borkumensis*	25-30	Abraham et al, 1998; Golyshinem, 1998; Yakimov, et al, 1998a
ii.FattyAcids/Neutral Lipids			
Fatty Acids (FA)	*C. lepus*	30	Cooper et al, 1981; Cooper and Zajigic;1980
FA+ Neutral lipids	*N. erythropolis*	32	McDonald et al, 1981
iii. Lipopeptides/Lipoproteins			
Peptide –Lipid	*B. lichenlformis*	27	Desai & Patel, 1994.
Serrawettin	*S. marcescens*	28-33	BarNess& Rosenberg, 1989.
Viscosin	*P. fluorescens*	26.5	Desai & Desai, 1988
Surfactin	*B. subtilis*	27.32	Peypoux et al, 1999.
iv. Polymeric surfactants			
Lipopolysaccharide (Emulsan)	*A. calcoaceticus*	-	Shabatai & Gutnick, 1985; Rosenberg et al, 1979a
Protein –carbohydrate	*C. petrophilium*	27	Kappeli & Fiechter, 1977.
Mannan-lipid	*C. tropicalis*	-	Singh & Desai, 1989.
Polysaccharide-protein	*A. radioresistens* (Alasan)	-	Navon-Venezia, et al, 1995; 1998; Toren et al, 2001.
v. Particulate Biosurfactant			
Vesicles	*Acinetobacter* spp.	-	Sar, 1983;
Fimbriae	*A. calcoaceticus*	-	Rusansky, et al, 1987
Whole cell	*A. johnsonii* SC14	-	Patil & Chopade, 2001

- : Not done.

In experiments with *A. calcoaceticus* RAG-1, the location of Emulsan was related to growth rate : the exponentially growing cells were found to be attached to hydrocarbon whereas the slowly growing cells were free with Emulsan capsules being present in extracellular space. In general, negatively charged biosurfactant inhibit whereas, positively charged biosurfactant promote microbial adhesion to hydrophobic phases (Rosenberg, et al, 1983). Another physiological role of biosurfactant is their antibiotic effect on various microorganisms. Production and activity against different microorganisms is summarized in Table 3.

3.4. Biosynthesis of biosurfactants :

iv) Regulation of biosurfactant synthesis:

The production of biosurfactants, in general, is regulated by 3 mechanisms viz., i). Induction, ii) repression and iii) nitrogen and multivalent ions-mediated effects.

i) **Induction :** Biosurfactants are produced when microbes grow on water immiscible substrates (Hisatsuka, et al, 1981; Rosenberg et al, 1979; Beeba &Umbreit, 1971; Kaweshima, et al, 1983; Kappeli & Finnerty, 1980; Fukui & Tanaka, 1981). Addition of long chain fatty acids, hydrocarbons or glyceroids in the growth medium of *Torulopsis magnolia* caused significant increase in the sophorolipids production (Tulloch, et al 1962). Similarly, the induction of trihalolipid synthesis in *Rhodococcus erythropolis* with hydrocarbons has been reported (Rapp et al,1979; Oberbremer & Hurtig, 1989). The production of glycolipid EM by *Pseudomonas aeruginosa* SB-30 during the growth on alkanes has been reported by Chakrabarty, (1985).

ii) **Repression :** Catabolic repression is an important regulatory mechanism operating in the synthesis of biosurfactants in microorganisms. Repression of biosurfactant production by *Acinetobacter calcoaceticus* (Neufeld et al, 1983) and *Arthrobacter paraffineus* (Dunvjak, et al, 1982) on hydrocarbon substrate has been observed by organic acids and glucose, respectively. Hisatsuka et al, (1972) isolated a factor involved in N-alkene oxidation. During the growth on glucose, glycerol or palmitic acid as the substrates, this factor is not synthesized (Hisatsuka, et al, 1977). Similar results have been obtained for rhamnolipids synthesis in *P. aeruginosa* (Hauser & Karnovsky, 1958) and liposan synthesis in *C. lipolytica* (Ciriliano and Carman, 1984). Conversely, surfactin (Cooper et al, 1981; Mulligan, et al, 1989) and rhamnolipids synthesis (Parra, et al, 1989) in *B. subtilis* and emulsification of the carbon source biosurfactants are also involved in the adhesion of microbial cells to the hydrocarbon (Kappeli & Fiechter, 1976). *Pseudomonas* spp., respectively have been observed only with glucose as the carbon source, while hydrocarbon addition in the medium caused inhibition in biosurfactant synthesis.

Table.3.Summery of lipopeptide antibiotics from*Bacillus* spp.

Type of the lipopeptide	Name of organism	Antibiotic activity	References
Cyclic polypeptide			
Iturin group			
Iturin A	*B. subtilis*	Bactericidal, Fungicidal	Fiechter, 1992
Iturin C	-	-	Fiechter, 1992
Mycosubtilicin	*B. subtilis*	Fungicidal	Fiechter, 1992
	B. niger	Fungicidal	Fiechter, 1992
Bacillomycin L	*B. subtilis*	Fungicidal	Heins et al, 2000 & 2001.
Bacillomycin D			Fiechter, 1992
Bacillomycin F	-	-	Sakurai & Imanaka, 1994
Ocatapeptide group			
EM 49	*B. circulans*	Fungicidal	Hibino & Minami, 1998
333-25	*B. bungoensis*	Bactericidal	Fiechter, 1992
Polymaxin group			
A-F, K, M, P, S and T Colistin	*B. polymyxa*	Bactericidal,Gram negative	Hibino & Minami, 1998
Circulin A, B.	*B. polymyxa*	Bactericidal,Gram negative	Fiechter, 1992
Lactone group			
Esperin	*B. mersentericus*	Bactericidal	Fiechter, 1992
Surfactin, Subtilicin, Stryptolycin, ADP-1-III	*B. subtilis*	Bactericidal , Fungicidal	Fiechter, 1992; Heins et al, 2000 & 2001.
Surfactin analogue	*B. subtilis*	-	Tsuzuki, et al, 2002
Polypeptien	*B. circulans*	Bactericidal	Hibino & Minami, 1998
Brevistin	*B. brevis*	Bactericidal,Gram negative	Fiechter, 1992
Liniar lipopeptides			
Subsporin A-C	*B. subtilis*	Fungicidal	Hibino & Minami, 1998
Serexin A-D	*B. cereus*	Bactericidal, Gram positive	Fiechter, 1992
Tridecaptin A-C	*B. polymyxa*	Bactericidal	Hibino & Minami, 1998

iii) **Nitrogen and multivalent ions mediated effects :** Nitrogen and metal ion dependent regulation also plays an important role in the production of biosurfactants. Ammonium salts and urea are the preferred nitrogen sources for the production of biosurfactant by *Arthrobacter paraffineus* (Duvnjak et al, 1983). Recently a relationship between glutamine synthetase and biosurfactant production in *P. aeruginosa* RC-II has been demonstrated (Mulligan & Gibbs, 1989). Synthesis of rhamnolipid by *P. aeruginosa* is regulated by nitrogen concentration, where the exhaustion of nitrogen source stimulated (Guerra-Santos, et al, 1984; Guerra-Santos, et al, 1989; Ramana & Karanth, 1989) and the addition of nitrogen to the medium during biosurfactant production caused inhibition in rhamnolipid synthesis (Syldatk, et al, 1985; Syldatk, et al.1984). Glycolipid synthesis in nitrogen limitation condition by *R. erythropolis* (Ristau & Wagner, 1983) and Emulsan synthesis by *A. calcoaceticus* RAG-1 during the amino acid starvation (Rubinovitz, et al, 1982) are some more examples of this category. Similarly, by limiting the concentration of cations like magnesium, calcium, potassium, etc. in the medium, higher yields of biosurfactants have been documented in *Pseudomonas* spp. (Guerra-Santos, et al, 1986; Syldakt, et al, 1985). In *P. fluorescens*, iron limitation stimulates biosurfactant production (Rubinovitz, et al, 1982; Persson, et al, 1990) whereas in *B. subtilis* iron and manganese salts stimulated the biosurfactant production (Cooper, et al, 1981).

3.5. Production of biosurfactants :

Among the process parameters influencing a type and amounts of biosurfactants produced are the nature of the carbon source, possible nutritional limitations, and physical and chemical parameters such as aeration, temperature and pH. In addition a major factor is the identty of the organism or strain used for the production process. In most cases, growth on hydrocarbons induces the synthesis of biosurfactants but this is not prerequisite for all organisms (Syldatk & Wagner, 1987). The carbon source is however, an important process parameter. Changing the substrates often alters the structure of the product thus altering the properties of the surfactant. The nitrogen source and concentration as well as C:N ratio are also reported to have a major effect on biosurfactant synthesis (Ristau & Wagner, 1983; Shennan & Levi, 1987). The influence of aeration on the production of biosurfactants by *B. licheniformis* was studied and the best results are obtained under semi-anaerobic conditions. The efficiency of a range of production systems including various reactor systems and cultivation modes such as fed-batch, continuous culture, classical stirred a tank reactor (STR) and immobilized cells on calcium alginate has recently been evaluated. The control of foam formation in surfactant production process by antifoam agents is inadequate and requires either such gas exchange filters and /or mechanical foam breaker.

Integrated chemostat system equipped with modern membrane technology and coupled to powerful on line analysis is suitable not only for the production of biosurfactants but also for the

enrichment and selection of producer strain. An alternative approach for higher yield is expected to arise by using genetically engineered producer strain. Much of the efforts to date have been directed at the improvement of the economic and efficiency of bioprocesses in order to allow biosurfactants to compete successfully with chemically synthesized surface active compounds.

3.6. Genetics of biosurfactant synthesis :

The genetics of expression of surface active compounds is reviewed by Reiser, et al, (1992). In gram-negative bacteria the best studied cases include *A. calcoaceticus, Pseudomonas* and *Serratia marcescens,* and in gram positive well studied is *Bacillus subtilis* system. In *A. calcoaceticus* RA57 hydrocarbon substrate to be assigned as a function of one of the plasmids pSR4. This 20 Kb plasmid is assumed to encode a factor tightly associated with the cell surface. (Rusanky, et al, 1987). Experiments investigating the production of Emulsan using mutants and revertant of *A. calcoaceticus* RAG-1, showed that Emulsan is not the only extracellular factor for emulsification, mutants with enhanced Emulsan production were found to contain mutations affecting an emulsifier synthesis steps leading to enhance capsule production (Shabtai & Gutnick , 1986). In *Pseudomonas oleovorans,* the alkane utilizing system is encoded by the *alk* BAC gene located on the OCT plasmid (Kok et al, 1989). The biosynthetic pathway of rhamnolipid production in *P. aeruginosa* (Itoh & Suzuki,1972). Two different transferases lead to the formation of the four different rhamnolipids. The control of rhamnolipid synthesis is linked to the nitrogen metabolism of the cell (Mulligan &Gibbs, 1989). In an attempt to use cheap substrates forthe production of biosurfactants, Koch, et al, (1989) succeeded in constructing a lactose utilizing strain of *P. aeruginosa* by insertion of the *E. coli lac* ZY genes into the chromosome (Koch, et al , 1988). Various extracellular and cell bound factors have been found to affect cell surface hydrophobicity in certain *Serratia* strains (Rosenberg, 1984). Studies on mutant derivatives of *S. marcescens* NS38 lacking capacity to produce serratamolide revealed that serratamolide increases cell hydrophilicity by blocking hydrophobic sites on cell surface (Bar Ness et al, 1988).

In gram positive, Surfactin, a cyclic lipopeptide with exceptional surface activity is produced by *B. subtilis* (Arima, et al, 1968; Berheimer & Avigad, 1970). Three genes *sfp, srf* A and *com* A, required for the production of surfactin have been isolated (Nakano & Zuber, 1990). Another group of surface-active compounds whose molecular biology has been studied intensively includes the lung surfactants, which constitute a complex mixture of phospholipids, small amounts of proteins, carbohydrates and neutral lipids, and are found at the air-liquid interface in the lung alveoli. They are essential for normal respiration and their deficiency can lead to an alveolar collapse in premature babies. Two proteins SP-B (S-8 kDa) and SP-C (4.5 kDa), have been identified as human lung surfactants and their primary amino acid sequences have been derived from c-DNAs (Glasser, et al, 1987, 1988). Recently, the entire human SP-B gene was isolated and sequenced, and mapped to chromosome 2 (Pilor- Matias, et al, 1989). In case of human SP-C, two

distinct genes were identified and sequenced (Glasser, et al, 1988), and both were shown to encode an active hydrophobic region in the protein that could be responsible forthe low solubility of SP-C and its lipid association upon isolation from the lung.

3.7. Applications of biosurfactants :

The increasing interest in the potential applications of microbial surface active compounds is based on their broad range of functional properties that includes emulsification, phase separation, wetting, foaming, solubilization, de-emulsification, corrosion-inhibition and viscosity-reduction. There are therefore, many areas of industrial application where chemical surfactants could be substituted by biosurfactants in the divers fields. The striking advantages of biosurfactants over chemically synthesized surface-active compounds include their broad range of novel structural characteristics and physical properties, their production on renewable substrates, their capacity to be modified and thus tailored to meet specific requirements, and their biodegradability. Many chemical surfactants cause environmental problems due to their resistance to biodegradation and their toxicity when allowed to accumulate in natural ecosystems.

Table. 4. Applications of biosurfactants.

Area of application of biosufactants	Application	References
1. Agriculture	In pesticide as emulsifier. In bioremedation	Desai & Patel, 1994; Lemarchand & Mercier, 2000.
2. Buildingand constructions	As surfactant	Desai & Patel, 1994.
3. Food and beverage industries	As emulsifier	Marchal, et al, 1997, US5616479
4. Industrial cleaning	As surfactant	Murphy, et al, 2002, US6,369,014
5. Paper and metal industries	As emulsifier	Rosenberg, et al, 1989; Desai & Patel, 1994.
6. Textiles	As emulsifier	Horowitz & Currie, 1990; Desai & Patel, 1994.
7. Cosmetics	As emulsifier and moisturising agent	Borzeix, 1999, WO 99/62479, Hayes, M.E., CA 1300512.
8. Pharmaceutical industries	As antimicrobial agent, As emulsiifer	Neu, etal, 1990; Mintz, 1990; Lemarchand & Mercier, 2000, FR2787439; Pierce & Heilan,2001,US6,262,038.
9. Petroleum and petrochemical Industries	As cleaning agent As emulsifiying agent	Shannan & Lavi, 1987; Brown & Moses, 1988; Gutnick & Rosenberg, 1987.

In spite of all these advantages, the industrial use of biosurfactants is limited, as yet, to applications in the petroleum industry (Shannan & Levi, 1987; Smith & Collins, 1985; Brown & Moses, 1988; Singer, 1985). Much effort has been putin to enhanced oil recovery (MEOR) (Donaldson & Clark,

1982). It is estimated that only 30-50 % of the oil can be recovered from petroleum resources by conventional pumping techniques.

The only commercial industrial biosurfactant on the market was Emulsan which used in cleaning oil contaminated vessels, oil spills and MEOR. Emulsan is also used to facilitate pipeline transportation of heavy crude oil because its ability to reduce viscosity contributes to reduce transportation costs. In the food industry,surface-active compounds are used as emulsifiers in food additives for the processing of raw materials. Emulsification plays an important role in forming the right consistency and texture as well as in phase dispersion. Other applications of surface active compounds are in bakery and meat products where they influence the rheological characteristics of lower or the emulsification of partially broken fat tissue. In agriculture surfaceactive compounds are needed for the hydrophilization of heavy soils to obtain good wettability and also to achieve equal distribution of fertilizers in the soil.

A broad potential application area is the cosmetics industry where surface –active substances are found in shampoos and many skin-care products. Biosurfactants have attracted personal care market too because of their low toxicity, excellent moisturizing properties and skin compatibility (Stuwer, et al, 1987). The reports on antibiotic effects (Neu et al, 1990; Damm, et al, 1985) and inhibition of growth of AIDS virus inWBC (Mintz, 1990) have opened up new fields of their applications. The deficiency of pulmonary surfactant, a phospholipid protein complex causes respiration failure in premature infants. The gene for protein molecule of this surfactant has been isolated and cloned in bacteria. The fermentative production of this product for medical application is now possible (Lang, et al, 1989). Some other commercial applications of biosurfactants dewatering of fuel, grade peat (Cooper et al, 1986, 1988), in pulp and paper(Rosenberg, et al, 1989), coal, textile, ceramic (Horowitz & Currie, 1990), and uranium ore processing (McInnerney, et al, 1990). Many biosurfactants and their production processes have been patented, however, only Emulsan has been commercialized so far. The surfactant market is extremely competitive.

Oil industry, at the current rate cannot absorb the high cost of biosurfactants for MEOR applications. However, the cosmetics, food and pharmaceutical are low value, high value industries could easily absorb the high cost of biosurfactants.

3.7.1. Future potential :

Though biosurfactants are having tremendous applications in above explained areas, future potential of biosurfactant depends on the reduction in the cost of biosurfactants . This depends on the development of such processes for their production, in which low cost, raw materials could be used with high product yields and super active, highly specific and selective biosurfactants for specific application.

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E. Indole Acetic Acid (IAA) Production by Bacteria : A Review

3.9. Introduction :

Indole acetic acid is best known, naturally occurring plant auxin. Relatively simple organic molecules, play a crucial role in elicitation of totipotency in several types of plant cells and in the regulation of plant physiological process. Recently, diverse set of microbes including soil, epiphytic and tissue colonizing bacteria and fungi has been found to synthesize IAA (Libbert et al, 1970; Gruen, 1959; Arshad, 1991; Kaper & Veldstera, 1958, Perley & Stowe, 1966). In fact, it has been suggested that up to 80 % of bacteria isolated from the rhizosphere, can produce IAA (Prikryl, et al, 1985; Loper & Schroth, 1986; Fuentes-Ramirez, et al, 1993; Leinhos & Vacek, 1994; Kloepper, 1999; Khan, 2000). Several of these microorganisms such as *Agrobacterium tumefaciens, Agrobacterium rhizogenes, Erwinia herbicola* and two pathovars of *Pseudomonas syringae, P. savastanoi* and *P. syringae* are involved in plant pathogenesis, while others provide benefit by stimulating plant growth. This latter group includes members of the genera *Azospirillum, Pseudomonas, Xanthomonas* and *Rhizobium* as well as *Alcaligenes faecalis, Enterobacter cloacae, Acetobacter diazotrophicus, Klebsiella, Herbaspirillum seropedicae, Bradyrhizobium japonicum* and *Acinetobacter* spp. (Table 1), (Tien, et al, 1979; Kobayashi, et al, 1993, Haahtela, et al, 1990; Koga et al, 1991; Fuentes- Ramirez, et al, 1993; Fett, et al, 1987; Ernsten, et al, 1987; Sekine, et al, 1988; Huddedar et, al, 2002). It has been shown that indole-3-acetic acid (IAA) is formed from L-tryptophan via indole 3 acetamide by *Pseudomonas savastanoi* (Magie et al, 1963; Kosuge, et al, 1966). IAA is not an end product in this biosynthetic scheme but is further converted to water soluble metabolites (Kosuge, et al, 1966; Hutzinger & Kosuge, 1968). Extensive research, to isolate mutants completely deficient in IAA synthesis, failed which supports the existence of more than one route to IAA production within many of these organisms. (Glick, 1996; 1995).

It is of interest to study IAA production by bacteria not only because of the physiological effects that this phytohormone causes on plants, but also in view of the possible role in plant bacteria interactions (e.g. *Rhizobium* symbiosis). Moreover, these studies may also contribute to understand mode of action, biosynthesis of IAA in plants and analysis of transgenic plants carrying bacterial IAA biosynthetic genes (Hobbie & Estelle, 1994). In fact, it is not rare that discoveries with bacteria, with relatively simple systems can be extended to more complex ones such as plants.

4.0 Biosynthetic pathways of indole-3-acetic acid (IAA) :

Tryptophan is generally considered as a precursor of IAA because addition of this amino acid to cultures of bacteria that are known to synthesize IAA results in higher IAA production. Several biosynthetic pathways leading from tryptophan to IAA have been detected in bacteria. In the indole

Table 1. IAA production by bacteria isolated from rhizosphere.

Bacteria producing IAA	IAA production encoded on	References
Pseudomonas syringae pv. savastanoi.	Plasmid P IAA_1	Comai & Kosuge, 1980.
		Mazzola & White, 1994.
Pseudomonas syringae pv syringae	Chromosomal DNA	Fett, et al, 1987.
Agrobacterium tumefaciens	Ti plasmid	Liu et al, 1982; Inze et al, 1984.
E. herbicola	Plasmid	Clark, et al, 1993.
Bradyrhizobium japonicum	Chromosomal DNA	Ray et al, 1983; Sekine, 1988.
Rhizobium fredii	Chromosomal DNA	Sekine, 1989a; Costacura & Vanderleyden, 1995.
Azospirillium brasilense	Chromosomal DNA	Bar & Okon, 1993
Alcaligenes faecalis	Chromosomal DNA	Kobayashi, et al, 1993
E. cloacae	Chromosomal DNA	Koga et al, 1991a
E. coli	Chromosomal DNA	Koga et al, 1991a
Acinetobacter haemolyticus A19	Plasmid pUPI126	Huddedar et al, 2002

acetamide pathway (IAM), tryptophan monooxygenase converts tryptophan toIAA by oxidative decarboxylation, and IAA hydrolase hydrolyses IAM to IAA and ammonia. For*Pseudomonas* and *Agrobacterium* the genes that code for the enzymes catalyzing these reactions have been cloned and sequenced (Glick, 1995).

The indole pyruvic acid pathway (IpyA) is considered to be a major IAA pathway in plants (Schneider & Weightman, 1974), However, IpyA has been detected also in bacteria (Kaneshiro, et al, 1983; Koga, et al, 1991). IpyA arises from Prp by a transaminase reaction; decarboxylation converts IpyA to IAA aldehyde, which is further oxidized to IAA. Transaminase, whether or not specific for tryptophan are commonly present in bacteria (Liu, et al 1982), reported in *Agrobacterium tumefaciens* an α-ketoglutarate dependent. Tryptophan transaminase is involved in IAA production. Similar results were reported by Frankenberger & Poth (1988) for a rhizobacterium isolated from *Fsstuca octoflora* and by Kuo & Kosuge (1979) for *Pseudomonas savastanoi.* The gene encoding Indole-3-pyruvate decarboxylase has been cloned and sequenced from *Enterobacter cloacae* (Koga, et al, 1991) and *Azospirillum brasilense* (Costacurta, 1994). Also, TAM has been detected in some microbial cultures (Perley & Stowe, 1966) and could be an alternative precursor of IAA aldehyde. Direct conversion of tryptophan to IAA aldehyde by tryptophan side-chain oxidase has been detected by Takai & Hayaishi (1987) and by Oberhansli et al (1991) in *Pseudomonas.*

An additional IAA pathway, first described in*Crucifere* and *Brassicaceae* (Bartling, et al, 1994) from tryptophan via indole-3- acetaldoxime and IAN seems to be present in bacteria because the gene encoding for a nitrilase which catalyzes the conversion of indole3-acetonitrile to IAA has been cloned and sequenced in *Alcaligenes faecalis* (Kobayashi et al, 1993). The overview on all the biosynthetic pathways is as follows :

i) **Indole-3-acetamide pathway :**

Several decades ago, it was discovered that tumor like outgrowths appearing on some plants were induced by increased levels of IAA derived from bacteria associated with the plants. One such bacterial pathogen, *Pseudomonas syringae pv. savastanoi,* invades the tissues of oleander, olive and privat, takes up hosts constituents including tryptophan and releases biologically active compounds such as IAA, which produce the hyperplastic symptoms (Marlow & Kosuge, 1972). In *P. syringae pv savastanoi* strains isolated from oleander, a 52 Kb plasmid is necessary for IAA biosynthesis and gall formation (Comai & Kosuge, 1980). Loss of plasmid from thebacterium is associated with loss of IAA production and loss of gall induction, while reintroduction of the plasmid, restores both capabilities (Comai, et al, 1982). In *Agrobacterium tumefaciens,* the causative agent in crown gall disease, indole-3-acetic acid, is encoded within the Ti plasmid.

However, in this case the bacterial cell dose not directly produce the IAA involved in elicitation of disease, but rather *A. tumefaciens* has the unique ability to transfer a specific portion of the Ti plasmid DNA region, into the cells of dicotyledonous plants, where it is stably, incorporated into the host genome (Liu, et al, 1982). Mutagenesis of the TDNA suggested that two genes were involved in IAA biosynthesis (Inze, et al, 1984; Klee, et al, 1984; Schroeder, etal, 1984). In *E coli* "gene 1"now known as *tms*-1, encodes the enzyme tryptophan 2-monooxygenase, which catalyzes the synthesis of indole acetamide from tryptophan and indole acetamide hydrolase encoded by gene 2 known as *tms-2*, then converts indoleacetamide to IAA. Kuo & Kosuge (1970), have established that IAA biosynthesis in *P. syringae pv. savastanoi* also occurs predominantly via the indole-3-acetamide pathway. Comparison of nucleotide sequences shows that there is 54 % homology between *iaaM* from *P. syringae pv. savastanoi* and *tms*-1 from *A. tumefaciens*, and 38 % homology between *iaaH* and tms-2 (Klee et al, 1984; Gielen, et al, 1984; Yamada, et al, 1985; Follin et al, 1985). *Agrobacterium rhizogenes*, the causative agent of hairy root diseases on dicotyledonous plants possess genes for IAA biosynthesis localized in the Ri plasmid TDNA region, which is incorporated into the plant genome (Costacura & Vanderleyden, 1995). These *aux* genes are similar to the tms sequences of *A. tumefaciens*, aux –1 and tms-1 show 60 % homology, while aux-2 and tms-2 show 71 % homology, and the regulation signals are also similar to that for tms1and 2 (Gaubin et al, 1994).

Another subspecies of *Pseudomonas syringae*, the pathovar *syringae* has been found to produce IAA by the indole acetamide pathway, similar to *P. syringae pv savastanoi*, however, in this case, the genes are present on the bacterial chromosome rather than a plasmid and the direct consequences of IAA biosynthesis are unclear (Fett, et al, 1987). Rather than induced gal formation, *P. syringae pv savastanoi* causes circular brown necrotic regions surrounded by a zone of brighter yellow necrotic tissue (Mazzola & White, 1994). These symptoms of brown spot disease resulting from infection of bean *Phaseolus vulgaris* correlate with high levels of IAA.

Not all pathogenic strains of *P. syringae* synthesize IAA via the indoleacetamide pathway. A probe containing *iaaM* and *iaaH* genes from *P. syringae pv savastanoi* failed to detect these genes in *P. syringae pv syringae* strains isolated from tomato, corn and wheat and two strains of *P. syringae pv pisi.* It could be that the sequences of the *iaaM* and *iaaH* genes in these strains have diverged to the extent where sufficient hybridization can be occurred or that IAA is synthesized by another pathway (White & Ziegler, 1991). *Erwinia herbicola,* also produce IAA via the indole acetamide pathway (Manulis et al, 1991). The *iaaM* and *iaaH* genes involved were isolated from a plasmid of the *Gypsophilia* pathovar of *E. herbicola* using *P. syringae pv savastanoi iaaM* and *iaaH* genes as hybridization probes (Clark et al, 1993).

Members of the genera, *Rhizobium* and *Bradyrhizobium* induce nitrogen fixing nodules on the roots of host legumes (Sekine, et al, 1989a; Costacura & Vanderleyden, 1995), this likely occurs as

the bacteria moves to the root cortex and induce the proliferation of surrounding cortical cells through the release of auxin (Ray et al, 1983). Sekine, et al (1988), have suggested that *Bradyrhizobium japonicum* and *Rhizobium fredii*, synthesize IAA via the indole acetamide pathway. These strains were able to convert an indole acetamide analogue; naphthalene acetamide (NAM) to naphthalene acetic acid (NAA) suggesting that indole acetamide hydrolase is produced by these strains. Significant homology was found among the *Bam* gene, *iaaH* of *P. syringae pv savastanoi* and *tms*-2 of *A. tumefaciens*, especially in the core region of the genes.

Another genus of nitrogen fixing microbes, *Azospirillum*, isolated from the rhizosphere of various grasses and cereals, also has the ability to synthesize IAA. Excreted IAA appears to positively influence root system development as it was found that inoculation of roots with *Azospirillium brasilense* increase the number and length of lateral roots in wheat (Barberi, et al, 1986; Omay et al, 1993), and pearl millet (Tien, et al, 1979). It was confirmed that the plant responds, is due to bacterial IAA secretion rather than nitrogen fixation by using combinations of mutants unable to fix nitrogen or synthesize IAA (Barbieri, et al, 1986). Although the principal IAA biosynthesis route does not appear to proceed via indole acetamide (Prinsen, et al, 1993; Okon, 1994, Costacura, et al, 1994), Bar & Okon (1993), have presented evidence that this pathway did exist in *A. brasilense*. Tryptophan 2-monooxygenase activity was detected in a 56 kDa protein on a non-denaturing gel and a probe containing *iaaM* and part of *iaaH* from *P. syringae pv savastanoi* hybridized to *A. brasilense*, total DNA (Bar & Okan, 1993).

Biosynthetic pathway is as follows :

Tryptophan ⟶ Indole3-acetamide ⟶ Indole acetic acid.

ii) **Indole-3-pyruvic acid pathway :**

It is generally believed, that this is major IAA biosynthesis pathway in higher plants. Two sets of IAA biosynthesis genes were present in *A. tumefa*ciens, and that they must be located on chromosomal as well as plasmid DNA. Chromatographic and enzyme activity analysis provided evidence that the chromosomal genes were involved in the indole pyruvic acid pahway. Colonies of *Azospirillium lipoferum* for IAA deficient mutants, Abdel-Salam & Klingmuller (1987), isolated eleven mutants that produce 9-55 % of the IAA level of the wild type. Since the mutants still produce some IAA, it suggests that more than one biosynthetic pathway, or perhaps multiple gene copies for a single pathway may be present. The gene for a key enzyme in the indole pyruvic acid pathway, indole-3-pyruvate decarboxylase, which catalyzes the conversion of indole3-pyruvic acid to indole-3-acetaladehyde, has been isolated from *A. brasilense* (Costacura, et al, 1994). Sequence analysis of the 1,635 nucleotide operon reading frame shows that the 64 kDa protein shares 38 % amino acid homology with pyruvate decarboxylases from *Zymomonas mobilis* and *Saccharomyces cerevisiae. Enterobacter cloacae*, isolated from the rhizosphere of cucumbers accelerates the growth of various agriculturally useful plants. Metabolic studies suggested that this

microbe synthesize IAA via indole pyruvic acid (Koga, et al, 1991b). An IAA biosynthesis gene was derived from *E. cloacae*, chromosomal DNA and introduced in *E. coli*, for the ability to convert tryptophan to IAA (Koga et al, 1991a). Sequence analysis of the isolated*E. cloacae ipdc* gene indicates that it codes for a single, 60 KDa protein, Koga et al, (1991a). It is possible that a microbe may compensate for a defective pathway by increasing synthesis of IAA by another available route.

Biosynthetic pathway is as follows :

Tryptophan → Indole-3-pyruvic acid → Indole-3-acetaldehyde → Indole-3-acetic acid

iii.) **Tryptophan side-chain pathway :**

An IAA biosynthesis route in which L-tryptophan is converted directly to indole-3-acetaldehyde, by passing indole-3-pyruvic acid, has been demonstrated in *P. fluorescens* (Narumiya, et al, 1979; Oberhansli, et al, 1991). Indole acetaldehyde is produced from tryptophan by the action of tryptophan side chain oxidase and is subsequently oxidized to IAA by aldehyde dehydrogenase. A biocontrol strain of *P. fluorescens* (CHAO), which can suppress some fungal born root diseases including black root rot of tobacco, synthesizes tryptophan transaminase which is involved in the indole pyruvic acid pathway, as well as tryptophan side chain oxidase (Oberhansli, et al, 1991).Tryptophan transaminase activity was constant through out the exponential and stationary phases of bacterial growth, and was not significantly influenced by the addition of tryptophan to the culture media. In contrast, tryptophan side chain oxidase activity was only observed during the stationary growth phase of *P. fluorescens* CHAO (this was also observed by Narumiya, et al, (1979) in another *P. fluorescens* strain and was repressed four fold in the presence of 10mM tryptophan. In addition, in increasingly acidic media (from pH 7-pH 5.5), IAA produced by the tryptophan side chain pathway increased whereas that produced by the indole pyruvic acid pathway decreased. These observations support the theory that the pathway by which a microbe synthesizes IAA is dependent upon environmental conditions. Tryptophan side chain pathway does not play an important role in disease suppression (Oberhansli, et al, 1991). The pathway is as follows :

L-tryptophan → Indole-3-acetyladehyde → Indole-3-acetic acid

iv.) **Tryptamine pathway :**

Tryptophan can also be converted to indole-3-acetaldehyde and IAA via the intermediate tryptamine. Although this pathway appears to be widespread among plants (Taiz & Zeiger, 1991) and fungi including *Aspergillus, Penicillium* and *Rhizopus* (Frankenberger & Arshad, 1995). Very little attention has been focused on tryptamine as an intermediate in bacterial IAA biosynthesis. However, *B. cereus* has been reported to synthesize a decarboxylase, which can act on tryptophan to produce tryptamine (Perley & Stowe, 1966) and *A. brasilense* could convert tryptamine, added to the culture medium in to IAA (Hartmann, et al, 1983).

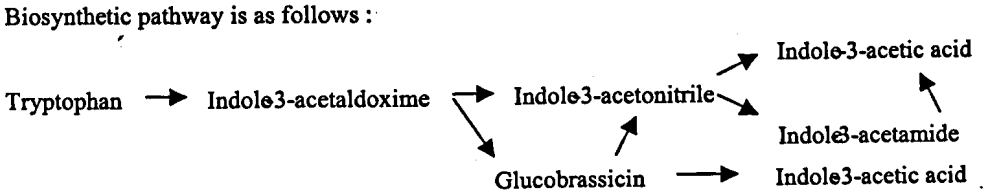
Biosynthetic pathway is as follows :

Tryptophan → Tryptamine → Indole-3- acetaldehyde → Indole-3-acetic acid.

V) **Indole-3-acetonitrile pathway :**

Although the pathway by which indole3-acetonitrile is converted to indole-3-acetic acid has been identified in higher plants belonging to the families *Cruciferae* (Cabbage), *Gramineae* (grasses) and *Musaceae* (Banana) (Kobayashi et al, 1993; Kobayashi & Shimizu, 1994; Bartel & Fink, 1994; Bartling et al, 1994), it tends to be overlooked in discussions of microbially produced IAA. Production of indole acetonitrile for IAA synthesis by the reactons, may be tryptophan dependent, involving conversion of tryptophan to indole acetaldoxime and then to indole acetonitrile either directly or via glucobrassicin (Kobayashi, et al, 1993), or may be derived from the precursor anthranilate without proceeding through tryptophan as an intermediate (Normanly, et al, 1993; Bartling, et al, 1994). Nitrilases are highly specific and are classed according to the substrate type upon which they act. Recently, nitrilase specific for indole3-acetonitrile was purified from *Alcaligenes faecalis* (Kobayashi, et al, 1993). This enzyme consists of six subunits of identical molecular weight (44 KDa each), is highly labile and functions within a narrow pH range (optimum pH is 7.5). By looking at the effects of various compound on the enzyme, it was concluded that thiol residues are likely to be present in the active site and are involved in enzyme activity (Nagasawa, et al, 1990). Interestingly, an *A. thaliana*, nitrilase gene known as *nit-2* is dramatically induced by virulent strains of *P. syringae*. A virulent strains stimulate only modest expression of *nit*-2 (Bartel & Fink, 1994). The implications of the*nit*-2 specific induction are only speculative at this point, however, it could be that auxin plays a role in plants defenseagainst pathogens or, since IAA is a virulence determinant in*P. syriange* (Comai & Kosuge, 1982), it may be that microbial IAA induces nitrilase expression and therefore increased IAA production in host plants providing a possible mechanism by which hypeplasia is induced. The occurrence of nitrile hydratases and amidases is believed to be quite common in bacteria, although those identified in various species appear to be specific for different substrates (Nagasawa, et al, 1987; Kobayashi et al, 1992; Duran et al, 1993). Although nitrile hydratases and amidases appeared to be widespread the discovery of those specific for indole acetonitrile is very recent, and much more study is required to determine their contribution in IAA biosynthesis in plant associated bacteria.

Biosynthetic pathway is as follows :



4.1. Regulation of IAA synthesis :

It was observed that IAA is synthesized by number of pathways several other factors acting in concert those will have a significant effect on the output of IAA by a microbe. The importance of location of IAA biosynthesis genes, either on a plasmid or on the bacterial chromosome in determining levels of IAA production is especially evident in *A. tumefaciens* and *Rhizogenes*. By carrying auxin biosynthesis genes on a region of the Ti plasmid which is transferred into the host plant genome, and directing their expression by plant regulation signals, these *Agrobacterium* strains can target high levels of IAA directly to the infected cells (Gaudin, et al,1994). Whereas auxin genes *P. syringae pv. savastanoi* are carried out on a plasmid, homologous genes in *P. syringae pv. syringae* are encoded on chromosomal DNA (Mazzola & White, 1994). Perhaps a consequence of this difference in location *P. syringae pv. syringae* produces much less IAA than *P. syringae pv. savastanoi.* In fact, when the IAA operon was reintroduced into *P. syringae pv. syringae* on a plasmid known to exist in three to five copies per cell IAA levels were elevated four fold (Mazzola & White, 1994).

Considering that many bacteria require tryptophan supplements to synthesize significant amounts of IAA the question arises as to whether sufficient levels are available in the rhizosphere .Two sources of exogenous tryptophan are available to *Rhizobacterium* released from protein of dying cells and from plant root exudates. Omay et al (1993), speculated that significant increase in IAA production during the stationary growth phase of *A. brasilense* may be due to tryptophan released from dying cells. However, when tryptophan was added to the culture media the time course of IAA production remained the same. Kravchenko, et al, (1991) also found that only very small quantities of tryptophan were detectable in root exometabolites compared to amounts added to synthetic media. However, it was calculated that the concentration of IAA that could be produced from available tryptophan is within the range required for maximum root lengthening, cell division in meristem tissue, cell differentiation and adventitious root formation. Tryptophan concentrations in root exudates vary with plant species, it seems reasonable to suggest that production of physiologically active levels of IAA by a rhizobacterium can beregulated by the plant with which it is associated (Glick, 1996). In laboratory cultures, addition of high concentrations of tryptophan to *Azospirillum brasilense* SP-7 caused a toxic response which included bacterial growth inhibition, a change in the colour of the culture and specific changes in transcription and protein synthesis (Bar & Okon, 1992). It was suggested that in order to reduce such a toxic levels of tryptophan *Azospirillum brasilense* SP-7 synthesizes and secrets IAA using tryptophan as a substrate (Bar & Okon, 1992). While IAA production can be enhanced by tryptophan, it can also be inhibited by anthranilate, a tryptophan precursor (Okon, 1994). A

mechanism is proposed where by these two compounds are involved in the fine regulation of IAA biosynthesis.

Effect of different factors on IAA biosynthesis :

Conditions in the rhizosphere are often quite variable, and many factors such as temperature, pH, and the availability of nutrients can affect the synthesis of IAA by bacteria associated with plant roots. Evidence is emerging to suggest that a microbe may selectively employ a particular IAA biosynthesis pathway, of the multiple pathways of which it is capable, according to its environment. For example, *Rhizobium* spp appeared to utilize the indole acetamide pathway only when associated with the host plant (Costacura & Vanderleyden, 1995). However, in the freeliving state, *Rhizobium* produced IAA via the indole pyruvic acid pathway (Kaneshiro, et al, 1983; Ernstsen, et al, 1987). The reduction in pH of an environment can also reduce auxin production per cell in some *Pseudomonas* spp. (Leinhos, 1994). There also appears to be an optimal glucose concentration for growth and auxin production, below or above which IAA levels are diminished.

Interestingly, Koga et al (1991b), have found that in *E. cloacae*, high levels of IAA produced under aerobic conditions are available for plant growth promotion. In contrast, *Azospirillum brasilense* cells under microaerophilic conditions were found to preferentially catalyze the conversion of indole pyruvic acid to IAA (Bar & Okon, 1995). In a similar manner plants may also store in an inactive form conjugated with amino acids or sugars moieties. Conversion of IAA to a conjugated form influences pool size of free IAA. Secreted free IAA may be subject to degradation by chemical or enzymatic oxidation or by metabolism by other microbes (Duveikovsky, et al, 1993), while IAA conjugates appeared to be protected (Hangarter & Good, 1981). Conjugation may provide an efficient means by which the bacteria can deliver continues concentrations of IAA to host tissues for tumor induction. In addition, it appears that the presence of some PGPR, such as *Azospirillum*, in the rhizosphere affects the metabolism of endogenous IAA in the plant (Falik, et al, 1989). It is not known, however, if this phenomenon is due to free IAA synthesis by the bacterium or by modulation of conjugated plant IAA.

4.2. Pathogenesis and growth promotion by microbial IAA :

- **Growth Promotion :**

It is now recognized that most of the diverse microorganisms, living in the rhizosphere synthesize IAA. A question that arises when considering the role of this compound in the relationship between a plant and bacteria is, why is the capacity to produce an auxin so common amongmicrobe?. It is easy to imagine that the plant growth hormone has a profound influence on the plant, but of what importance to the bacteria is its production of IAA? Of course by stimulating plant growth, these microorganisms can also increase the production of plant metabolites that they can utilize for their own growth (Gaudin et al, 1994). But other more subtle benefits may include detoxification of

tryptophan analogues which are deleterious to bacterial cells, or inhibition of the plant defense enzymes, chitinase and β-1, 3-glucanase, which would facilitate bacterial invasion (Gaffney, et al, 1990; Robinette & Matthysse, 1990).

Pathogenesis :

The outcome of the interactions between a plant and bacterium with respect to auxin depends ultimately on the amount of the hormone that is available to the plant. This in part, is contingent upon the physical relationships between the two organisms. Possible associations are i.) Bacterial IAA genes can be transfer directly in to the host plant genome. ii.) The infeting bacterium can live within the plant tissue and secret IAA in to surrounding tissues. iii.) The bacterium can live outside of the host plant, colonizing its surface such as phyllosphere or rhizosphere. Beneficial bacteria appear to excrete their effect predominantly while inhibiting the external surface of a plant (Del Gallo & Fendrik, 1994). When *Agrobacterium* spp. transform plant cells, high levels of auxin are delivered directly to the host cells (Liu, et al, 1982) and this is correlated with assimlation of plant growth to the point of gall formation (Gaudin, et al, 1994). The benefit to bacterium is a marked increase in unique, Ti or Ri plasmid encoded metabolites such a opines, which can be utilized for growth.

It is not necessary for transformation to occur in order for high concentrations of auxin to initiate gall formation in host plants. IAA biosynthesis genes in oleander strains are plasmid encoded, where as homologous genes in olive strains are found in the chromosomal DNA. Fett, et al (1987), found that although both pathogenic and non-pathogenic strains of *Xanthomonas campestris* pv. *glycines* produced IAA *in vitro*, IAA synthesis genes in pathogenic strains were encoded on a plasmid while these genes in non pathogenic strains were encoded on he chromosome. In contrast, to the induction of pathogenesis in host plants, evidence has rapidly been amassing to implicate secreted IAA in the mechanism by which rhizobacteria stimulate plant growth. Early work showed that plant growth promoting rhizobacteria (PGPR) such as *Azotobacter paspali*, secreted IAA into culture media and significantly increased the dry weight of leaves and roots of several plant species following root treatment (Barea & Brown, 1974). Barbieri et al, (1986) found that inoculations of wheat seedlings with *Azospirillum brasilense* increase the number and length of lateral roots. A similar result was seen with application of low concentrations of exogenous IAA (Omay, et al, 1993). It should be borne in mind that the size of bacterial inoculums might affect whether that bacterium either promotes or inhibits root growth, reflecting the level of bacterially produced IAA added to the plant (Okon & Morgenstern, 1987; Harari & Okon, 1989). Ernsten, et al, (1987) suggest that IAA biosynthesis may be a necessary part of root nodulation process induced by various species of *Rhizobium* and *Bradyrhizobium*. This suggests that at least two pathways for IAA biosynthesis exists in *R. leguminosarum* one pathway is constitutive and the

other is induced by tryptophan (Manulis et al, 1991). The induced pathway appears to be missing in the non-nodulating strains, intimating that this pathway might be involved in nodulation.

The idea that the pathway to IAA production might be important in determining the effet of bacteria on a plant is unexpected and interesting. Manulis et al,(1991), have found that pathogenic and non pathogenic strains of *Erwinia herbicola* pv. *gypsophilae* synthesize IAA via the indole pyruvic acid pathway. However, the only pathogenic strain, which induces crown or root gall at wound sites in gypsophilae, can produce IAA via indole acetamide. If this later pathway is inactivated, then virulence is reduced.

Plants have regulatory systems that can normally maintain IAA at nontoxic or physiologically appropriate levels (Stibon, et al, 1992). However, because of the uniqueness of the indole acetamide pathway, bacteria may override plant regulation of IAA expression, leading to induction of hypertrophic growth in the plant tissues. In contrast, PGPR such as *Enterobacter cloacae* (Koga, et al, 1991b), and *Azospirillum* spp (Costacura, et al, 1994), synthesize IAA mainly via the indole pyruvic acid pathway, which may be subject to more stringent regulation by plant metabolites. An important consideration in predicting whether bacterial IAA will stimulate beneficial growth or pathogenesis in a plant is the level of auxin synthesized by the plant itself. In plant roots endogenous levels of IAA may be suboptimal or optimal for growth (Pilet & Saugy, 1987; Taiz & Zeiger, 1991). Glick, (1996) suggests that additional input in to the IAA pool by bacteria could modify endogenous auxin to either optimal or supraoptimal levels, resulting in the induction of plant growth or pathogenesis, respectively. The auxinresponse may also depend upon the stage of plant root development. Application of low concentrations of IAA promoted growth in young maize roots, but inhibited growth in older roots (Pilet et al, 1979). This may indicate that with age plant endogenous auxin contents increases from sub-optimal to almost supra optimal levels. It should be emphasized that phytohormones such as auxin do not act alone, but rather may interact with one another in a variety of complex ways (Barendse & Peeters, 1995).

In summery, the amount of microbial IAA available to influence each plant subjects to several levels of regulation. On the part of the microbe pathway for IAA biosynthesis, the location of the biosynthesis genes on the chromosomes or a plasmid and their regulatory sequences, and the presence of enzymes involved in converting free, active IAA into a conjugated, inactive form, are involved in determining levels of IAA expression. The nature of the interaction between microbe and plant also needs consideration. Direct transformation with microbial auxin biosynthesis genes exposes plant cells to high concentrations of IAA, while secretion by bacteria living outside the cells may reduce available IAA levels, as the hormone would be subject to diffusion and degradation. It is the extent to which bacterial sources auxin modify endogenous levels of free IAA in the plant that ultimately determines whether the relationship is beneficial or pathogenic. Optimal levels enhance growth while supra optimal levels elicit a disease response.

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F. Biofertilizers : A Review

4.4. Introduction :

Biofertilizers are living fertilizer compounds of microbial inoculants or group of microorganisms which are able to fix atmospheric nitrogen or solubilize phosphorous, decompose organic material or oxidize sulphur and able to produce plant growth promoting substances like auxins, hormones etc in the soil. On application it enhances the growth of plants increase in yield and also improve soil fertility and reduce pollution (Brown, 1968; Brakel & Hlger, 1965; Denarie & Blachere, 1966; Rovira, 1965; Schank, et al, 1981; Tien, et al, 1979). Availability of fixed nitrogen in the soil a major determinant of the soil fertility and there by crop productivity. Major contributors of fixed nitrogen to soil are nitrogen fixing microorganism and chemical fertilizers. Chemical fertilizers are synthesized by industrial process such as Haber- Bosch process (Pandey & Sushilkumar, 1989). With synthetic process being highly energy intensive, the manufacturing cost of fertilizers is high. Since chemical fertilizers are easily handled and yield predictable results, they are the preferred source of N for the developed countries. Although, it is very difficult for the developing countries to afford chemical fertilizers and complete dependence of agriculture on chemical fertilizer will be unsustainable. It is now noted that excessive use of chemical fertilizers showed hazardous effects on the soil texture and they alter the soil fertility and can make the soil unfertile (Okon, 1988; Kaushik, 1985; Watanabe, 1986).

4.5. Advantages of biofertilizers over chemical fertilizers :

In contrast to chemical fertilizers, biofertilizers are eco-friendly and have definite advantages over chemical fertilizers such as :

- Chemical fertilizers supply over nitrogen whereas biofertilizers provide in addition to nitrogen certain growth promoting substances like, hormones, vitamins, amino acids, etc.
- Crops have to be provided with chemical fertilizers repeatedly to replenish the loss of nitrogen utilized for crop growth. On the other hand biofertilizers supply the nitrogen continuously through out the entire period of crop growth in the field under favorable conditions.
- Continuous use of chemical fertilizers adversely affects the soil texture where as biofertilizers when applied to soil improve the soil structure.
- The deleterious effects of chemical fertilizers are that they are toxic at higher doses.
- Biofertilizers however, have no toxic effects.
- Biofertilizers have very low production cost

4.6. Different types of biofertilizer :

In India, bacteria mostly *Rhizobium, Azotobacter, Azospirillum,* Cyanobacteria and a Mycorrhizal fungi are used as a biofertilizers. The major parameter considered for the effective biofertilization is nitrogen fixation, either symbiotic or in the non-symbiotic way and phosphate solubilization.

Following are the different types of microorganisms used for the preparation of biofertilizers based on their properties.

i.) Symbiotic nitrogen fixing microorganisms.

ii.) Non symbiotic nitrogen fixing microorganisms

iii.) Phosphate solubilizing microorganisms.

i.) Symbiotic nitrogen fixing microorganisms :

In all living organisms nitrogen is essential for the growth, since it is constitute of amino acids and bases are building blocks of proteins and nuclecic acids, respectively. An atmosphere around us contains nearly 78 % nitrogen which is in the free form, hence not available to plants.*Rhizobium* is the main nitrogen fixing symbiotic bacteria used as a biofertilizer known as *Rhizobium* biofertilizer. The ability of*Rhizobium* to fix atmospheric nitrogen and make available it to plants is the main property of this biofertilizer.*Rhizobium* in association with the legume plant fix nitrogen in nodules formed on the roots of plants. These nodules are considered as miniature nitrogen production factories in the field. There are number of species of *Rhizobium* were used for biofertilizer preparation those are listed as follows:*Bradyrhizobium japonicum, Rhizobium* spp., *R. leguminosarum, R. lupini, R. meliloti, R. phaseoli,* and *R. trifolii.* (Rai & Brahmaprakash 1993; Sekine, et al, 1988; Ray & Basu, 1992; Hartmann & Glombitza, 1967; Dullart, 1970; Trinchat & Rigaud, 1974; Basenoch-Jones, et al, 1982a; Atzorn, et al, 1988).

These species can nodulate different plants such as *Medicago indica, Melilotus* sp., *trigonella foenum-gracum, Trifolium* sp., *Pisum sativum, Vicia faba, Lens esculeata, Lathyrus* sp. *Phaseolus vulgaris, P. multifloris, Lupinus* sp. *Orniltopus, Vigina and Glycin max* (Rai & Brahmaprakash 1993) and they are selective and specific for their host plant. It was also observed that strains vary in their ability to thrive and fix nitrogen under different stresses (Hartel & Alexander, 1984). It was also observed by Bromafield & Jones (1980) those environmental factors such as soil pH, temperature, moisture etc also affect on nodulation. The genes involved in the nodulations are nod genes such as nod D1, nod D2 and nod D3 (Iyer,1990, Singh & Guar, 1995). In case of *Bradyrhizobium japonicum,* auxins such as indole-3- acetic acid and indole 3-pyruvic acid increases the number of root nodules and involved in enhanced nodulation (Kaneshiro et al, 1983; Hunter & Kuykendall, 1990). Recently, it was observed that nodulation of green gram was enhanced by co-cultivation of *Bradyrhizobium* with plant growth promoting rhizobacteria (Gupta, et al, 1998). *Rhizobium* is the most dominant and best biofertilizer among all but its scope is limited to legume plants.

v) Cyanobacteria as biofertilizer :

Cyanobacteria is a symbiotic nitrogen fixing microorganism also known as bluegreen algae (BGA). *Anabaena* has a highly specific relationship with *Azolla* and can fix nitrogen and part of nitrogen passes to the partner (Fay, 1983; Smith & Douglas, 1987). Metting (1981) recorded at

least 37 genera of *Cyanobacter* species having relation with soil. *Nostoc* showed associations with Cycad and Gunnera. Strains of *Nostoc* showed considerable promiscuity and not specialized (Quispel, 1991; Adil & Katre, 1992). The *Cyanobacteria* obtains fixed carbon from the host plant, such *Cyanobacteria* are mostly found in rice-growing areas. Recently, Hashem (2001), used *Cyanobacterial* biofertilizers for rice cultivators increase the total yield of rice. *Azolla* with its association with Anabaena were also used successfully in the rice cultivation. *Azolla* contributes in nitrogen and green manure and by floating on the water surface it causes marked reduction in weed density. It is used with corps like *Sesbania* and *Colocasia* (Kannaiyan, 1987). These biofertilizers are having limitations because of specification of plant-host interactions still they are used significantly in agriculture (Pandey & Kumar, 1989).

ii.) Non symbiotic nitrogen fixing bacteria as a biofertilizer :

There are microorganisms, which are able to fix free atmospheric nitrogen without symbiotic association with host plant. Number of bacteria such as *Azotobacter* spp, *Azospirillum* spp., *Bacillus,* spp. *Klebsiella* spp. and *Clostridium* spp. are reported for this type of nitrogen fixation but the extensively studied and used as a biofertilizers are only of genus *Azotobacter* & *Azospirillum.* Among this genus many species were reported, but in *Azotobacter, A. chroococcum* and in *Azospirillum A. brasilense* has been favored as biofertilizers. Different species of *Azotobacter,* which commonly occur in soil include *A. vinelandi, A. chroococcum, A. beijerinckii, A. agilis, A. paspali. Azotobacter* is having additional abilities to N_2- fixation, it can produce auxins such as IAA, gibberellic acid, cytokinine, B-vitamins etc, antibiotics and antifungal agents (Muller, et al, 1989; Mahmoud, et al, 1984; Apte & Shende, 1981; Tien, et al,1979; Mishustin & Shilinikova, 1972; Lakshmikumari et al, 1976; Suneja, et al, 1988 & Pandey & Kumar, 1989). Rhizosphere is very complex and dynamic environment where, number of reactions takes place at the same time. This is the region where strong competition between different microorganisms was observed for nutrients and therefore, for existence (Subba Rao, 1986). For the purpose of biofertilizer preparations mainly rhizosphere bacteria, which are plant growth promoting were screened, used and was observed to increase the productivity of crop (Narula et al, 1991,1993). *Azotobacter* biofertilizers were mainly used for wheat, maize, pearl millet, sorghum and rice. It was observed that *Azotobacter* bioinoculums increased grain yield by 8 to 43 % over the control when added with the fertilizers and when inoculated with only *Azotobacter* biofertilizers increased 0 to 72 % yield over the uninoculated controls (Pandey & Kumar, 1989; Mishustin & Shilinikova, 1969; Reichardt, et al, 1997; Hegde, et al, 1999). Extensive studies were carried out on the distribution, nitrogen fixation and its genetic pattern in *Azotobacter* (Becking, 1961; Dobeiner, 1961; 1974; Mulder & Brontonegoro, 1974; Austin, et al, 1994 ; Berger et al, 1995). In case of *Azotobacter* biofertilizers study, in India, observed the variation in the yield increase from simple to significant increase

(Austin et al,1994). Rice, cabbage and brinjal yield resulted in significant increase of yield while others showed simple increase. These results mainly depends on the ability of bacteria to fix the nitrogen (Austin et al,1994). *Azospirillum* is the another asymbiotic nitrogen fixer used as a biofertilizer (Okon, 1985). *Azospirillum* like *Azotobacter* is used for inoculations for cereal crops and vegetables. In Indian soil *Azospirillum* is present in 72 % and able to increase the yields of sorghum, wheat, pearl millet, safflower, maize, mustard, corn, panicum and setaria. *Azospirillum brasilense, A. lipoferum* and *A. amazonense* are mainly used for biofertilizer. *Azospirillum* also having ability to fix N_2, produced plant growth promoting substances such as auxins, hormones, cytokinines etc, and can produce anti fungal and antibacterial compounds (Tein, et al, 1979; Kapulnik, et al, 1982; Pandey & Kumar, 1989). It was found that*Azospirillum* applications have been shown to increase grain productivity of cereals by 5 to 27 %, of millets by 281 % and of fodder plants by 3 to 163 %. Recently,*P. vulgaris* a haemolytic bacterium isolated from rhizoplane of sunflower. Bacterization of sunflower seeds with strain of*P. vulgaris*, promote growth of sunflower. It has ability to fix nitrogen (Malik et al, 1995). The beneficial effects of both *Azotobacter* and *Azospirilla* have been related not only to their nitrogen fixing proficiency but also with their ability to produce antibacterial and antifungal compounds, hormones and siderophores (Pandey & Kumar, 1990).

iii). Phosphate solubilizing microorganisms as biofertilizers :

Phosphorus is one of the important nutrients for the growth of plant as well as microorganisms. In soil phosphate is present in the insoluble and unavailable form and only about 25 to 30% becomes available to the plants. Thus in the chemical fertilizers phosphate containing fertilizers are important such as single super phosphate. Number of soil bacteria and fungi are having ability to solubilize insoluble phosphate into soluble form by producing organic or inorganic acids such as formic, propionic, acetic, glycolic, fumaric, succinic acid, by chelating Ca ions and by producing phytase enzymes (Gaur & Ostwal, 1972; Thakkar, et al, 1993). Bacteria can able to solubilize phosphate includes *Bacillus* spp., *Pseudomonas, Xanthomonas, E. coli, Brevibacterium, Serratia, Alcaligenes, Erwinia, Nitrosomonas, Thiobacillus*, and *Flavobacterium* (Subba Rao, 1981). Some fungi are also reported to solubilize phosphate includes*Aspergillus, Glomus, Penicillium* and most important mycorrhiza (VAM) (Verma, 1993). It was observed that applications of phosphate solubilizing bacteria increases yield by 12.2 % (Gaur, 1985). A typical example is*Pseudomonas* species , also a plant growth promoting rhizobacteria. (Ostwal & Bhide, 1972; Buyer, et al, 1990; 1993; De Brito et al, 1995, Kloepper, et al, 1980; Kumar & Dube, 1991; Lugtenberg & Dekkers, 1999; Malik, et al, 1995; Marugg et al, 1985). It was also observed by Bakker et al,(1986) that yield of potato was significantly increased by phosphate solubilizing biofertilizer of*P. putida* (Bakkher & Shippers, 1987). Ghosh et al (2000), also observed effect of different biofertilizers on potato plant. Recently, it was also observed that combinations of plant growth promoting

Table. 1. Phosphate solubilizing microorganisms from soil.

Organism	Reference
Bacteria	
Bacillus spp.	Gaur, 1985; Gaur & Ostwal, 1972.
B. subtilis	Malik et al; 1995; 1999
Pseudomonas spp.	Bakker, 1986; Buyer, et al, 1990; 1993; De Brito et al, 1995; Kumar & Dube, 1991; Lugtenberg & Dekkers, 1999; Malik et al, 1995
P. putida	Bakker & Shippers, 1987; Ghosh, et al, 2000.
Xanthomonas spp.	Subba rao, 1981
E. coli	Subba Rao 1981
Brevibacterium	Subba Rao, 1981
Serratia spp.	Subba Rao, 1988
Alcaligenes spp.	Subba Rao, 1988
Erwinia spp.	Subba Rao, 1986
Nitrosomonas spp.	Subba Rao, 1988; Sundara Rao, et al, 1968
Thiobacillus thiooxidans	Subba Rao, 1988
Flavobacterium spp.	Subba Rao,1988
Kurthia spp.	Malik, et al, 1999
Achromobacter spp.	Subba rao, 1988
Aerobacter aerogenes	Sen & Pul, 1957
Acinetobacter spp.	Leinhos, 1994
Fungi	
Aspergillus spp.	Narsian et al, 1993; 1995; Gaur et al, 1980
Glomus spp.	Verma, 1993
Penicillium spp.	Verma, 1993
Mycorrhiza (VAM)	Verma, 1993
Rhizoctinia spp.	Chhonkar & Subba Rao, 1967
Cladosporium spp.	Chhonkar & Subba Rao, 1967
Candida spp.	Sethi & Subba Rao, 1968
Rhodotorula spp.	Chhonkar & Subba Rao, 1967
Phythium spp.	Sethi & Subba Rao, 1968
Actinomycetes	
Streptomyces spp.	Chhonkar & Subba Rao, 1967

potato plant. Recently, it was also observed that combinations of plant growth pomoting rhizobacteria with phosphate solubilizing bacteria in combinations such as *Klebsiella planticola* and *B. subtilis* and *Proteus vulgaris* and *B. subtilis* increased yield of sunflower and toria (Malik, et al, 1995; 1999). A new species known as *Kurthia* sp also act as a plant growth promoting rhizobacteria and increased yield of toria (Malik, et al,1999). Among fungi, phosphate solubilizing potentials of *Aspergillus awamori* and *A. aculeatus* were established and reported by Narsian et al, (1993; 1995). The most dominant fungal biofertilizer used is Mycorrhizal fungi. It is well known that these fungi are having symbiotic interactions with plant roots (Park et al, 1983; Harley, 1969). VAM are mainly in use for inoculations to plants (Bhagyaraj & Manjunath,1980). VAM are not culturable and they require host root for survival (Hepper, 1984; Powell, 1984).

Inoculations are also prepared by growing up plants in media containing spores, hyphae or VAM infected roots developed in pot culture (Powell, 1984). Seed inoculations with mycorrhizal fungi have been conducted in number of fields while soil inoculations in pits have been tried successfully for tree planting (Hall & Kelson, 1981; Mosse, 1972; 1973).

4.6. Inoculum preparation :

Field inoculations present complex problems. First is survival and viability of bacteria in the constantly changing soil environment. Second is problem of applications i.e. choice of inoculating methods (Okon,1985). To avoid these problems large study was carried out on inoculating methods.

Inoculation is the practice that introduces interested strains into the soil-plant ecosystem, and an inoculant is the formulation of the strains in to solid or liquid carriers. A good inoculant must have plant growth promoting ability, broad spectrumtowards the varieties of hosts but also with high specificity in order to be competitive for nutrients and able to promote host preferences. It must survive in inoculant formulations and must maintain its properties during the storage period. It must be tolerant to stress factors as acidity, desiccation, high temperature conditions and chemical pesticides. Flowsheet of bioinoculum preparation is given below. It is also possible to directly inoculate the soil with liquid or granulated formulations, but for technical and economical reasons, seed inoculations are mostly accepted at present. For the seed inoculation formation carriers used are mostly peat, lignite, buggas, wheat straw, coal etc. (Swtrijdom & Deschodt, 1976; Kandasamy & Prasad, 1971,Crawford & Berry; 1983; Tilak, et al, 1978). Some times some binder such as calcium carbonate is also used (Baraibar & Saito, 1987, Subba Rao, 1986).

Improvement in biofertilizer is now under study and improvement in the biofertilizers can be made by causing mutations in specific gene (s), substitution of resident gene (s), with foreign gene (s) (Pandey & Kumar, 1989). Cloning of all beneficial genes in one bacterium and production of superbug can also be possible, but the main problem with these studies is survival ofthe cloned bacterium in the complex rhizosphere environment. Protoplast fusion is a new and promising

technique in fundamental and applied genetics and can be used for the improvement of biofertilizers (Mondal et al, 1998, Bhattacharya, 2000).

Carrier based liquid bioinoculum preparation :

Mix carrier in minimum amount of water to form slurry
(sugar, gum arabis, peat etc)

↓

Mix the inoculum in the slurry
(Microbial cultures grown in broth)

↓

Add seeds to inoculum slurry

↓

Mix the seeds with inoculum slurry by hand

↓

Dry the seeds in shade on paper.

Carrier based solid bioinoculum preparation :

Mix microbial inoculum (growth in broth) with sterilized carrier (peat, lignite, baggasse, coal etc.) by hand.

↓

Add binder (calcium carbonate) in mixture.

↓

Add seeds to the mixture to form a uniform coating on it.

↓

Dry the seeds in shade on paper.

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CHAPTER 2

Studies on Occurrence and Distribution of *Acinetobacter* spp. and other Gram Negative Bacteria Isolated from Rhizosphere of Wheat.

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Studies on Occurrence and Distribution of *Acinetobacter* spp. and other Gram Negative Bacteria Isolated from Rhizosphere of Wheat.

2.1 Abstract :

Four Gram negative bacterial genera were isolated from rhizosphere of wheat . They were identified as *Pseudomonas, Acinetobacter, Moraxella* and *Serratia.* 37 *Acinetobacter* spp. isolated from rhizosphere of wheat were confirmed by chromosomal DNA transformation assay. Members of genus *Acinetobacter* were found to be one of the dominant bacteria (31.3 %) next to *Pseudomonas* (35.7 %) in the rhizosphere of wheat. Number of *Moraxella* (28 %) was found less than *Acinetobacter* and *Serratia* was present in least (4.5 %) number as compared to others in wheat rhizosphere. Percentage of these bacteria in rhizosphere of wheat was found to be : *Pseudomonas* 35.7 % , *Acinetobacter* 31.3 %, *Moraxella* 28 % and *Serratia* 4.5 %. It was observed that these four genera affect the number of each other. Interesting reciprocal interaction between number of *Acinetobacter* and *Moraxella* spp. was also observed. AMM and Holton's medium without cefsulodin was found to be the most suitable media for isolation of *Acinetobacter* spp. from rhizosphere of wheat. Maximum numbers of *Acinetobacter* spp. were found in rhizosphere soil of wheat plant than rhizoplane and non-rhizosphere soil. Growth stages of wheat plant affected the populations of these bacteria at inflorescence and fruiting stages. Number of all four genera were found to increase at inflorescence and fruiting stage of wheat plant that suggest that root exudates of wheat plant affect on natural population of rhizobacteria in the rhizosphere. Soil analysis of RS and NRS soils at each life stage of wheat plant showed clay texture and alkaline pH (7.8-8.2) of both the soils. It was observed that increase or decrease in macro and micro nutrients of RS and NRS soils of wheat plant was found to depend on life stage of wheat plant. It was also found that there was close relationship between growth stage of wheat plant, rhizosphere of wheat and rhizobacterial population in rhizosphere of wheat.

2.2 Introduction:

Rhizosphere is a very complex and dynamic environment where numerous relationships between microbial community, host plant and pathogens take place (Subba Rao, 1986). In symbiotic reactions, microorganisms produce plant growth promoting hormones for plant growth promotion as well as antibiotics, siderophores and biosurfactants for the protection of plants from plant pathogenic bacteria and fungi (Normander et al, 1999; Ordentlich et al,1987; Elad, 1996). Number of bacteria and fungi have been reported for the symbiotic reactions such as *Pseudomonas, Azotobacter, Azospirillum, Bacillus* and *Serratia* spp. (Normander et al, 1999; Ordentlich et al,1987;Subba Rao, 1986; Katznelson et al, 1948; Clark, 1949). It has been shown that rhizoplane and rhizosphere soil of many crops contain large number of microorganisms than the corresponding non rhizosphere soil (Subba rao, 1986). Very few attempts have been made to study the diversity of bacterial populations found within and around the rhizosphere, possibly because of

lack of suitable techniques for isolating a sufficient number of strains belonging to the same species (Richaume et al, 1992).

Reports on the study of gram negative bacteria, isolated from rhizosphere of plant such as *Pseudomonas, Enterobacter, Azotobacter, Azospirillum, Agrobacterium, Rhizobium* (Kleeberger, et al, 1983; Baraibar, et al, 1999; Baraibar & Galli, 1993; Kloepper & Schroth, 1981a) for the presence in different plant rhizosphere and their interactions with different plants were studied. However, there are no reports on distribution of *Acinetobacter* spp from any plant rhizosphere, except a passing report on presence of *Acinetobacter* in the rhizosphere of wheat (Kleeberger et al, 1983) but no detailed study on distribution of *Acinetobacter* and its role in rhizosphere of wheat was done.

Acinetobacter is a Gram negative, capsulated, oxidase negative, catalase positive, coccobacilli (Bouvet & Grimont, 1986 & 1987). *Acinetobacter* is ubiquitous in nature and mostly isolated from clinical sources (Dhakephalkar & Chopade, 1994). It is also an opportunistic human pathogen, which causes meningitis, respiratory and urinary tract infections (Dhakephalkar and Chopade, 1994). Now days *Acinetobacter* has been gaining attention because of its high resistance to antibiotics and heavy metals (Goldsten, et al, 1993; Deshpande & Chopade, 1994). Since *Acinetobacter* is also found in the rhizosphere of wheat and there were no detailed studies on *Acinetobacter* from plant rhizosphere, it becomes interesting to find out the probable role of *Acinetobacter* in wheat rhizosphere. The questions raised regarding the role of *Acinetobacter* are : If *Acinetobacter* spp. are found to be present in the rhizosphere of wheat then do they contribute to the plant growth promotion ? What is the mechanism of plant growth promotion by *Acinetobacter* ? Thus to answer these questions it was necessary to find the presence of *Acinetobacter* in wheat rhizosphere using percentage of bacteria in the specific area as one of the important factors. In the present study, *Acinetobacter* spp. and other gram-negative bacteria were isolated by using selective media and percentage of these bacteria was enumerated.

2.3. Materials and Methods :

2.3.1 Collection of rhizosphere samples of wheat plant :

Wheat plants were collected from Mahatma Phule Agriculture College, Shivajinagar, Pune. Samples were collected from December 1998 to March 1999. Variety of wheat plant was HD 2189. Detection of *Acinetobacter* and other gram negative bacteria were carried out in three areas such as RP (rhizoplane), RS (rhizosphere soil) and NRS (non rhizosphere soil) at the time of most significant stages of wheat plant developments. Samples were collected from three areas of wheat plants such as i.) Rhizoplane ii.). Rhizosphere soil and iii.) Non-Rhizosphere soil, at the time of different stages shown in Table 1.

Table 1. Isolation of *Acinetobacter* and other gram-negative bacteria during different life stages of wheat plant development from RP, RS and NRS areas.

Sampling number	Growth stages of wheat plant	Time (Days)
1.	Elongation stage	30
2.	Inflorescence stage	45
3.	Fruiting stage	60
4.	Ripened fruiting stage	75

* While uprooting the wheat plants sterile hand gloves were employed to avoid any body contact with wheat plant.

2.3.2. Processing of samples of wheat plant :

Wheat plants were uprooted carefully by wearing sterile gloves and soil from rhizosphere and non-rhizosphere area was collected into sterile plastic bags. Samples were immediately brought to the laboratory within 15 min. and processed immediately. For collection of bacteria, from rhizoplane, wheat roots were washed thoroughly by sterile distilled water for 6-7 times and roots were cut into 2-3 cm pieces. Isolation of *Acinetobacter* from rhizosphere of wheat was carried out as per flow sheet described in Fig.1.

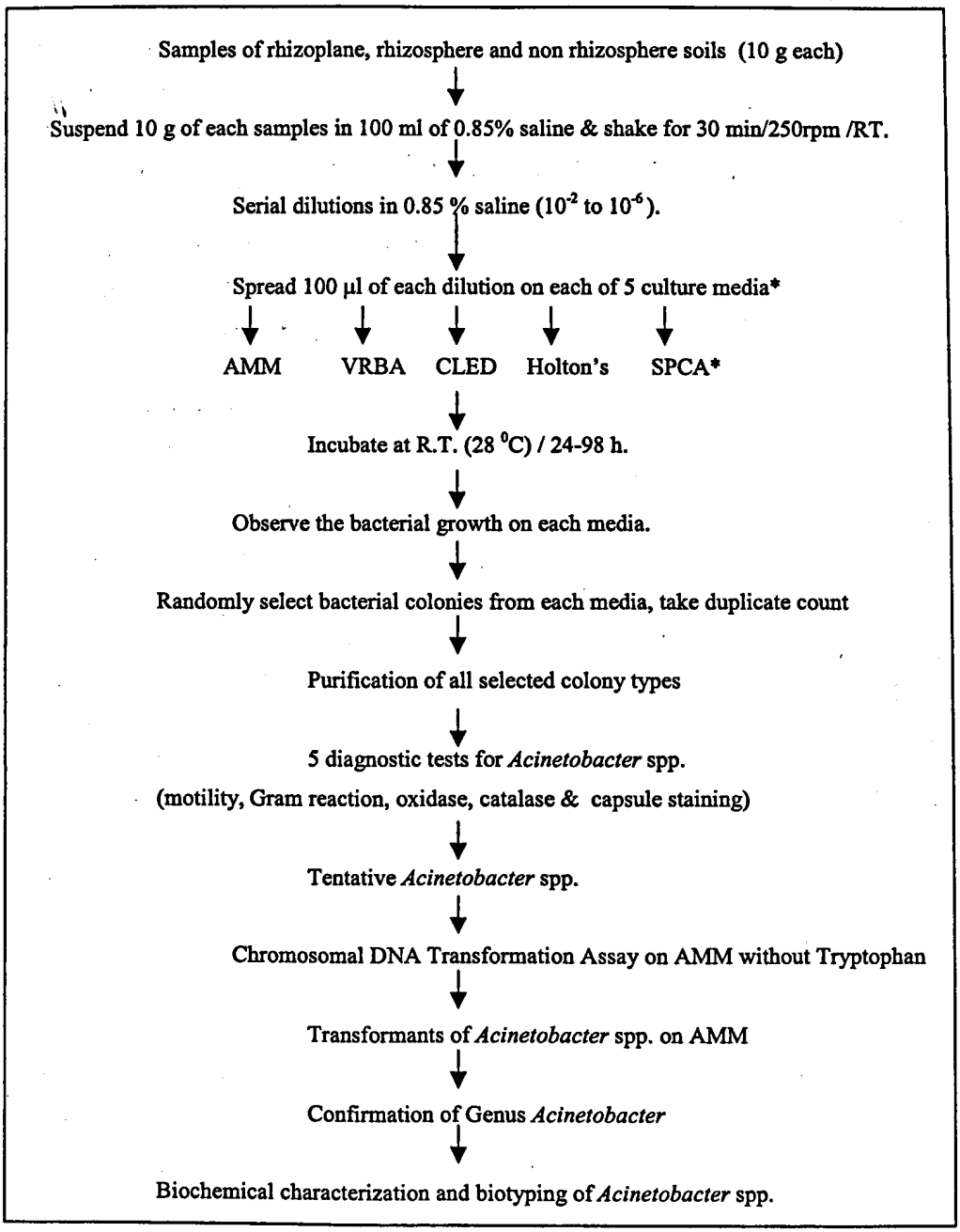
2.3.3. Cultural Media for isolation of *Acinetobacter* and other Gram negative bacteria:

Violet red bile agar (VRBA) was used as a selective media for the enumeration of all gram negative bacteria. The medium was prepared as per Kleeberger et al, (1983). Cysteine lactose electrolyte deficient agar (CLED) medium was dehydrated (Hi Media, Mumbai), *Acinetobacter* minimal medium (AMM) was prepared as per Juni (1972), and Holton's medium was prepared as per Holton's (1983) without cefsulodin antibiotic. CLED, AMM and Holton's media were used for *Acinetobacter* spp. Standard plate count agar (SPCA) was used as a common growth medium for all types of bacteria. SPCA was mainly used for counting total bacteria.

2.3.4. Characterization of *Acinetobacter* and other Gram negative bacteria :

Morphological characterization was carried out by observing colony characters and cell morphology. Other characters observed were gram staining, oxidase and catalase enzyme test, motility and capsule staining (Gerhardt, et al, 1994). All these characters were noted and preliminary identification was carried out up to genus level.

Fig.2. Flowsheet for isolation of *Acinetobacter* spp from rhizosphere of wheat.



*AMM : *Acinetobacter* minimal medium; CLED : Cystine lactose electrolyte deficient agar ; VRBA: Violet red bile agar; SPCA : Standard plate count agar. Holton's : Holton's agar (without cefsulodin).

2.3.5. Chromosomal DNA transformation assay :

Chromosomal DNA transformation assay was carried out by using *Acinetobacter calcoaceticus* BD413 trpE27, a naturally competent tryptophan auxotrophic mutant. The assay was done by using crude genomic DNA as per method of Juni (1972) as well as by purified genomic DNA as per Chen & Kuo (1993). Overnight grown *A. calcoaceticus* BD413 trpE 27 was mixed with 20 µg of crude as well as purified genomic DNA homogeneously on brain heart infusion agar (BHI). Plates were incubated at 28^{0}C. After 18-24 h incubation, growth from BHI plates was transferred to *Acinetobacter* minimal medium (AMM) without tryptophan and with 1.8 % noble agar (Difco, USA) for the growth of prototrophic transformants. The plates were incubated at 28^{0}C for 48-108 h. The colonies from AMM plates were again transferred to fresh AMM plates to confirm the growth. Those strains that were able to grow on AMM medium were confirmed as belong to genus *Acinetobacter.* All the individual transformants were stored at low temperature in fridge. *Acinetobacter calcoaceticus* MTCC (Microbial type culture collection, Chandigrah, India); 1271; *A. lwoffii* MTCC 496; *Acinetobacter calcoaceticus* MTCC 127 and *E. coli* HB101 were used as a standard positive and negative controls respectively.

2.3.6. Soil analysis :

Soil analysis for each soil sample , isolated from rhizoplane, rhizosphere and non rhizosphere soil was analyzed for its macronutrients such as total available nitrogen was estimated by KJeldahl method, (Chopra & Kanwar, 1976) total available carbon was detected by Olsans method (Chopra & Kanwar, 1976) and total available phosphate was estimated by Kanwars method. (Chopra & Kanwar, 1976). The micronutrients, such as Cu was detected by 0.1 N hydrochloric acid extraction, Fe by A.O.A.C method and Mn by nitric acid method. (Chopra and Kanwar,1976). pH was detected by colorimetric method of Kuhn's and soil type was detected by hydrometer method (Chopra & Kanwar,1976). This analysis was carried out at soil laboratory of Zuari Agro Chemicals Limited, Pune, Maharashtra, India.

2.3.7. Root mapping :

For root fingerprinting, freshly uprooted wheat roots were cut into 3-5 cm of pieces under aseptic conditions and washed with sterile distilled water (Jackson & Brown, 1966). After thorough washing, the roots were embedded (2 roots /plate) in the selective agar media as mentioned above and plates were incubated at 28^{0}C for 24-48 h. After 48 h, the roots on plates were observed for growth. The growth around the root on the medium was taken by sterile loop and suspended in 1ml of sterile (0.85 %) saline and serial dilutions were done. 100µl of each dilution was again plated out on selective agar medium such as AMM, CLED and Holton's medium (without cefsulodin) and plates were incubated at 28^{0}C up to 72 h. Colony characteristics were observed and counted.

Colonies showing characteristics matching to *Acinetobacter* spp. were isolated, purified and preliminary characterized.

2.3.8. Statistical analysis :

All the bacterial colonies were selected randomly from all media plates and counted as the square root of the mean of triplicate plates. The mean (x) values of total count and their standard deviation were subjected for their proper distribution. For the significant presence of each bacteria, anova test was done (Kulkarni, et al, 1999).

2.4. Results :

2.4.1. Characterization of *Acinetobacter* colony isolated from rhizosphere of wheat :

Wheat root samples collected from the experimental field of Mahatma Phule Agriculture College, Pune, Maharashtra, India, gave rise to the visible colonies on all five media. 4 different selective media such as VRBA, were used for isolation of gram negative bacteria and AMM, Holton's, and CLED were used for isolation of *Acinetobacter* spp. SPCA was used as a common medium for enumeration of all bacteria and fungi, present in the rhizosphere soil. Colonies were isolated from these selective media and colony morphology was studied. Following table describes the colony morphology of *Acinetobacter* strains on different selective media.

Table 2.Colony characteristics of *Acinetobacter* species on four selective media.

Colony Characters	VRBA	AMM	CLED	Holton's
Size (mm)	1-2	1-2	1-2	1-2
Shape	Circular	Circular	Circular	Circular
Colour	Faint pink-violet	White	Faint green	Faint yellow
Margin	Entire	Entire	Entire	Entire
Elevation	Low convex	Low convex	Low convex	Low convex
Consistency	Sticky	Sticky	Sticky	Sticky
Surface	Mucoid	Mucoid	Mucoid	Mucoid
Opacity	Translucent	Translucent	Translucent	Translucent

* –ve : negative; + ve : positive. VRBA: Violet red bile agar, AMM: *Acinetobacter* minimal medium, CLED: Cystine lactose electrolyte deficient agar, and Hotlon's : Holton's agar (without cefsulodin).

It was observed form Table 2, that colony size and shape of colony of *Acinetobacter* spp. was same on all media. Color of colonies on different media changes as on VRBA it appeared as Pink to violet, on AMM white, on CLED faint green and faint yellow on Holtons. This difference in colony colors was because of dyes present in VRBA, such as methyl red, in CLED bromothimol blue and phenol red in Holton's. Margin, elevation, consistency, surface and opacity of all *Acinetobacter*

colonies were same on all four media. Appearance of mucoid colony is one of the important morphological characteristics for identification of *Acinetobacter* colony.

2.4.2. Characterization of *Acinetobacter* and other Gram negative bacteria :

Colonies isolated from four selective media were purified and tested for 5 specific preliminary tests. Results of these tests were recorded and isolated gram-negative bacteria were classified into 4 different genera such as *Acinetobacter, Pseudomonas, Moraxella* and *Serratia.* Classification of these bacteria up to genus level by preliminary tests is shown in the following table.

Table 3. Preliminary characteristics of four genera isolated from rhizosphere of wheat.

Name of the test	*Pseudomonas* spp.	*Acinetobacter* spp.	*Moraxella* spp.	*Serratia* spp.
Gram reaction	Gram –ve	Gram-ve	Gram –ve	Gram -ve
Morphology	Short rods	Coccobacilli	Coccobacilli	Short rods
Motility	Motile	Non motile	Non motile	Motile
Oxidase	+ve*	-ve*	+ve	-ve
Catalse	+ve	+ve	+ve	+ve
Capsule	+ ve	+ ve	+ ve	- ve

* +ve: Positive. *-ve: Negative.

From Table 3 , it was found that, all four bacteria are gram negative. Isolates identified, as all four spp. showed difference in their preliminary characteristics such as *Pseudomonas* spp. and *Serratia* spp were motile but *Acinetobacter* and *Moraxella* spp. were non-motile. *Moraxella* spp. and *Pseudomonas* spp. showed oxidase test positive, while *Acinetobacter* and *Serratia* spp. showed oxidase test negative. Catalase test was positive for all four genera and three genera *Pseudomonas, Acinetobacter* and *Moraxella* were capsulated while *Serratia* was non capsulated. Thus these differences made them different from each other and helped in identification of bacterial species.

2.4.3. Chromosomal DNA transformation assay :

Chromosomal DNA transformation assay was carried out to confirm the isolates are from genus *Acinetobacter.* 45 tentative *Acinetobacter* isolates were tested by this assay. Out of 45, 37 isolated were confirmed as *Acinetobacter* strains.

2.4.4. Soil analysis :

Soil analysis carried out at all four growing stages of wheat plant was shown in the Table as follows :

Table 4. Soil analysis of rhizosphere and non rhizosphere soils from rhizosphere of wheat.

Location	Life stages of wheat plant (in days)	pH	Macro elements (Kg/Acre)			Micro elements (ppm)			
			N	P	K	Zn	Cu	Fe	Mn
Rhizosphere soil	0	7.8	0.16	4	88	1.31	2.21	4.98	26.5
	30	8.2	0.69	8	110	1.07	7.65	5.12	4.68
	45	8.1	0.70	6	138	1.41	15.84	17.68	8.70
	60	8.5	0.51	5.0	86	1.66	2.19	12.42	20.76
	75	8.1	0.80	12	156	0.84	6.84	11.6	5.25
Non rhizosphere soil	0	7.8	0.16	4	88	1.31	2.21	4.98	26.5
	30	8.2	0.64	11	111	1.25	4.69	8.59	4.38
	45	8.3	0.44	5	101	0.83	4.62	5.12	18.45
	60	8.1	0.70	11	157	1.35	14.2	12.45	7.05
	75	7.9	0.76	8	129	0.91	21.06	11.8	6.09

* N: nitrogen; P: phosphate; K: potassium; Zn: zinc; Cu: copper; Fe : iron; Mn: manganese; ppm : parts per million; This result is the mean of 3 experiments.

From Table 4, it is clear that pH of soil is alkaline and remains constant in first two life stages such as 0 and 30 days in RS and NRS area. For RS pH of the soil was highest (8.5) at 60 days life cycle of wheat plant that is fruiting stage of wheat plant, while for NRS pH of the soil was highest (8.3) at 45 days i.e. flowering stage of life cycle of wheat plant. In case of Micro elements such as presence of nitrogen, Phosphate and potassium was found in adequate amount in both RS and NRS soil and at all life stages of wheat plant. Micro elements such as zinc, copper, iron and manganese were also present in adequate amount in both RS and NRS soil. Some quantitative changes took place at particular stage of wheat plant in macro and micro elements in both RS and NRS soil.

2.4.5. Root mapping :

Root mapping was carried out to observe the natural colonization of *Acinetobacter* spp. to wheat roots and found that though, *Pseudomonas* colonizes 85 %, *Acinetobacter* was also able to colonize wheat root by 10 %.

2.4.6. Statistical analysis :

Statistical analysis was carried out for all isolated genera, in three areas such as RP, RS and NRS and at 5 different stages of wheat plant and on 4 selective media. Following tables will gave us the idea about effect of above factors statistically.

Table 5. Summary of ANOVA of four bacterial genera in three areas.

Source of Variations	df	RP	RS	NRS
Replicates	2	4.178**	0.936	6.769**
Media	3	22.998***	62.599***	17.098***
Area	3	51.817***	39.990***	41.283***
Media*Area	9	11.557***	22.561***	9.307***
Bacteria	3	127.035***	91.471***	95.765***
Media*Bacteria	9	2.991***	6.139***	5.729***
Area*bacteria	9	14.018***	11.969***	12.180***
Media*Area*Bacteria	27	8.297***	5.522***	9.001***
Error (C)	126	0.722	0.878	1.182

df : degree of freedom, *: 5%, **: 1%, ***: 0.1%. This is the mean of 3 experiments.

From Table 5, it was observed that number of all four bacterial genera, viz *Pseudomonas*, *Acinetobacter*, *Moraxella* and *Serratia* spp. was more in RP and RS regions as compared to NRS soil. Table 6, which shows the summary of all four bacterial genera at four life stages of wheat plant is as follows :

Table 6. Summary of ANOVA for all four bacterial genera at four life stages of wheat plant.

Source of Variations	df	Life stages of wheat plant			
		I	II	III	IV
Replicates	2	1.882*	5.763***	0.597	4.160
Media	3	80.005***	26.931***	79.139***	28.878***
Area	2	13.938***	4.578***	12.393***	17.834***
Media*Area	6	2.322***	7.148***	5.152***	9.584***
Bacteria	3	96.218***	74.461***	58.596***	116.069***
Media*Bacteria	9	16.513***	10.192***	54.535***	5.374***
Area*bacteria	6	0.676	0.728	1.953	3.395*
Media*Area*Bacteria	18	1.476**	3.231***	1.651	3.649**
Error (C)	94	0.555	0.555	1.168	1.440

df : degree of freedom, *: 5%, **: 1%, ***: 0.1%. This data is the mean of three experiments.

It was observed from Table 6, that number of all four genera, *Pseudomonas, Acinetobacter, Moraxella* and *Serratia* were maximum at inflorescence stage (flowering stage, 45 days) and fruiting stages (60 days) than all other stages. It was also observed that number of these genera was also increased at elongation stage (30 days) and ripened fruiting stage (75 days) as compared to control soil (0 days).

Table 7. Summary of ANOVA for all four bacterial genera on four selective media.

Source of Variations	df	VRBA	AMM	CLED	HOLTON'S
Replicates	2	9.503***	8.259**	0.792	0.411
Media	3	75.351***	2.369	77.516***	12.270***
Area	2	17.430***	0.348	2.733**	2.655*
Media*Area	6	12.822***	4.862**	7.331***	7.718***
Bacteria	3	67.208***	142.344***	18.508***	180.486***
Media*Bacteria	9	28.769***	8.878***	5.802***	22.096***
Area*bacteria	6	4.583***	2.538	0.760	2.231**
Media*Area*Bacteria	18	2.915***	2.369	1.153*	2.451***
Error (C)	94	0.972	1.388	0.553	0.666

*TVC on SPCA medium for three area is RP: 17,050, RS: 15,100 and NRS: 400. df : degree of freedom, *: 5%, **: 1%, ***: 0.1%. This is the mean of three experiments.

From Table 7, relation between media and bacteria was observed. it was found that all four genera were grown on a particular selective media such as maximum number of *Pseudomonas* and *Serratia* spp. were observed on VRBA medium. Maximum number of *Acinetobacter* spp. was observed on AMM and maximum number of *Moraxella* spp. was observed on Holton's medium. Following tables, shows summary of anova of interactions between bacterial genera, areas, media and life stages of wheat plant.

Table 8. ANOVA for *Pseudomonas* spp. at all stages of wheat plant.

Source of Variations	df	Sum of squares	Mean squares	F ratio	Probability
Replicates	2	0.57	0.28	0.53	0.58977
Stage	3	69.23	23.07	42.97	0.00000***
Error (A)	6	3.22	0.53	-	-
Media	3	235.03	78.34	79.20	0.00000***
Stage *Media	9	150.68	16.74	16.92	0.00000***
Error (B)	24	23.74	0.98	-	-
Area	2	8.49	4.24	5.86	0.00461**
Stage*Area	6	2.61	0.43	0.60	0.72850
Media*Area	6	11.27	1.87	2.59	0.02606*
Stage*Media*Area	18	51.39	2.85	3.94	0.00003***
Error (C)	64	46.41	0.72	-	-

Correction Factor : 4061.694, C.V. : 16,034 %, df : degree of freedom, *: 5%, **: 1%, ***: 0.1%.

Table 9. ANOVA for *Moraxella* spp. at all stages of wheat plant.

Source of Variations	df	Sum of squares	Mean squares	F ratio	Probability
Replicates	2	4.68	2.34	6.86	0.00200**
Stage	3	101.29	33.76	98.91	0.00000***
Error (A)	6	2.04	0.34	-	-
Media	3	23.28	7.76	8.17	0.00011***
Stage *Media	9	87.74	9.74	10.26	0.00000***
Error (B)	24	22.81	0.95	-	-
Area	2	15.73	7.86	11.44	0.00006***
Stage*Area	6	47.16	7.86	11.43	0.00000***
Media*Area	6	22.15	3.69	5.37	0.00015***
Stage*Media*Area	18	90.87	5.04	7.34	0.00000***
Error (C)	64	44.00	0.68	-	-

Correction Factor : 4042.655, C.V. : 15.650 %, df : degree of freedom, *: 5%, **: 1%, ***: 0.1%.

Table 10. ANOVA for *Acinetobacter* spp. at all stages of wheat plant.

Source of Variations	df	Sum of squares	Mean squares	F ratio	Probability
Replicates	2	13.01	8.50	6.96	0.00184**
Stage	3	48.62	16.20	17.34	0.00000***
Error (A)	6	5.60	0.93	-	-
Media	3	33.37	11.12	10.31	0.00001***
Stage *Media	9	289.25	32.13	29.80	0.00000***
Error (B)	24	25.88	1.07	-	-
Area	2	8.33	4.16	4.71	0.01230*
Stage*Area	6	23.12	3.85	4.36	0.00094***
Media*Area	6	22.41	3.73	4.23	0.00212**
Stage*Media*Area	18	53.80	2.98	3.38	0.00017***
Error (C)	64	56.55	0.88	-	-

Correction Factor : 3786.800, C.V. : 18.331 %, df : degree of freedom, *: 5%, **: 1%, ***: 0.1%.

Table 11. ANOVA for *Serratia* spp. at all stages of wheat plant.

Source of Variations	df	Sum of squares	Mean squares	F ratio	Probability
Replicates	2	7.67	3.83	3.03	0.05531
Stage	3	120.24	40.08	31.66	0.00000***
Error (A3	6	7.59	1.26	-	-
Media	3	379.66	126.55	89.01	0.00000***
Stage *Media	9	225.33	25.03	17.61	0.00000***
Error (B)	24	34.12	1.42	-	-
Area	2	3.02	1.51	1.27	0.28806
Stage*Area	6	29.50	4.91	4.12	0.00147**
Media*Area	6	15.56	2.59	2.17	0.05688
Stage*Media*Area	18	57.86	3.21	2.69	0.00193**
Error (C)	64	76.36	1.19	-	-

*Correction Factor : 770.781 , C.V. : 47.213 %, df : degree of freedom, *: 5%, **: 1%, ***: 0.1%.

From Table 8, it was found that maximum number of *Pseudomonas* was observed at elongation stage(30days), on VRBA medium and in rhizosphere soil. From Table 9, it is clear that maximum percentage of *Moraxella* spp. was observed at inflorescence stage (45 days) on Holton's medium and in rhizoplane area. In case of *Moraxella,* Holton's medium without cefsulodin, proved best for its isolation from rhizosphere. Table 10 indicates maximum number of *Acinetobacter* spp. was observed at inflorescence stage (45 days), on AMM and in rhizosphere area. Table 11, indicates that *Serratia* was found maximum at inflorescence stage (45 days), and in rhizosphere area on VRBA medium. Anova for four bacterial genera in the rhizosphere of wheat is as follows :

Table 12. ANOVA for 4 bacterial genera at four life stages (Colony count) .

Source of Variations	df	Sum of squares	Mean squares	F ratio	Probability
Four life stages	143	1293.24	9.04	2.97	0.00000***
Bacteria type	4	931.60	310.53	101.83	0.00000***
Error (A)	429	1308.21	3.04	-	-

*Correction Factor : 11730.322 , C.V. : 38.696 %, df : degree of freedom, *: 5%, **: 1%, ***: 0.1%.This is the mean of three experiments.

It was noted from above Table12, that all bacterial genera were significantly present in the rhizosphere of wheat.

Summary of percentage of four genera in rhizosphere of wheat at overall life cycle of wheat plant is as follows :

Table 13. Summary of percentage of four bacteria in the rhizosphere of wheat.

Bacterial spp.	Over all %
Pseudomonas spp.	35.78
Moraxella spp.	28.00
Acinetobacter spp.	31.39
Serratia spp.	4.75

* This is the mean of three experiments.

From above Table, it is clear that, *Pseudomonas* was the most dominant bacteria in the rhizosphere of wheat comprise 35.78 %. *Acinetobacter* was found the second most dominant bacteria after *Pseudomonas* comprises 31.39 %, *Moraxella* was the next dominant bacteria comprises 28% and *Serratia*, found in very less amounts of 4.75%.

2.5. Discussion :

This chapter describes the statistical distribution of Gram negative bacteria isolated from rhizosphere of wheat. Particular attention was paid to the genus *Acinetobacter* as purpose of our study to find out role of *Acinetobacter* spp. in rhizosphere of wheat. For this study, we have isolated Gram negative bacteria from rhizosphere of wheat by using selective media. We found that four bacterial genera identified as *Pseudomonas*, *Acinetobacter*, *Moraxella* and *Serratia* were present in rhizosphere of wheat. If we compare these results with previous distribution of Gram negative bacteria isolated from rhizosphere of wheat and barley, carried out by Kleeberger et al (1983), one bacterial genera was commonly found in rhizosphere of wheat was *Pseudomonas* spp. Kleeberger, et al (1983) also found *Acinetobacter* spp. present in rhizosphere of wheat but there were no detailed study on this bacteria was done by him as he focused only on *Pseudomonas* and *Enterobacter* spp.

Root mapping was carried out to observe the natural colonization of *Acinetobacter* spp. to roots of wheat. This method was employed successfully by Jackson & Brown (1966) to follow the colonization of wheat roots by *Azotobacter chroococcum*. It is one of the powerful technique to find out the presence of bacteria in rhizoplane (Penyalver & Lopez,1999). It was noted that number of all bacterial genera was more in rhizoplane and rhizosphere soil as compared to non rhizosphere soil. It was previously studied by Rovira (1956,1965 &1974) and Alexander, (1961) that there is strong relationship between plant and microorganisms present near the plant roots. It was also stated that plant root exudates play very important role in microbial growth (Subba Rao, 1986;

Rovira, 1969). These root exudates were readily used by microorganisms in the rhizoplane and rhizosphere than non rhizosphere soil (Dwivedi, et al, 1986), because of this number of all bacterial genera was found more in rhizoplane and rhizosphere soil than non rhizosphere soil.

From statistical analysis it was observed that AMM and Holton's medium found to be most suitable selective media for isolation of *Acinetobacter* spp. from rhizoplane, rhizosphere and non rhizosphere soil. Isolation of these four genera at 4 different growing stages of wheat plant and from three areas was done. It was found that there was a relation between plant growth stage and number of microorganisms around the plant root. We found that number of all four genera increased at inflorescence stage (45 days) and fruiting stages (60 days) as compared to control soil, (0 days) elongation stage (30 days) and ripened fruiting stage (75 days). Our results supported the findings of Cooper (1959); Danerfield et al, (1975 &1978) and Dwivedi et al (1986). They observed that, rhizosphere biology becomes more complex at the highest vegetative growth stage (flowering or fruiting) of the plants and number of microorganisms present in rhizosphere was maximum at these stages of plant (Timonin & Lochhead,1948; Foster & Bowen, 1982; Olsen & Bakken, 1987; Lynch,1990; Movangui, et al, 1992). This may be the reason due to which, at these two stages, we found the number of all bacterial genera increased. We also noted interactions between these four bacterial genera at each stage and found that each bacterial genera affect on the population of other. This findings supports to the findings of Newman (1978) and Dwivedi et al (1986). They also found that interactions between bacterial genera affects on the bacterial population.

We noted that there was reciprocal relationship between *Acinetobacter* and *Moraxella* spp. Whenever number of *Acinetobacter* increased, number of *Moraxella* decreased and vice versa. The reason for their reciprocal relationship may be because both are very similar to each other by morphologically, biochemically and to some extent at genetic level also (Rossau, et al, 1991). As they were similar in many aspects of their nutritional requirements and they may compete with each other for achieving the same.

Presence of each bacterium on all selective media and in three areas was observed. It was found that, maximum percentage of *Pseudomonas* was observed at elongation stage (30 days), on VRBA medium and in rhizosphere soil. This result matched with previous result of Kleeberger, et al, (1983). They also found *Pseudomonas* accounted over 50 % in wheat and barley rhizosphere. Maximum percentage of *Acinetobacter* spp. was observed at inflorescence stage (45 days), on AMM and in rhizosphere area. In case of *Acinetobacter* spp. there was no detail reports present on its occurrence and distribution in rhizosphere environment. In case of *Moraxella* spp. maximum percentage was observed at inflorescence stage (45 days) on Holton's medium and in rhizoplane area. This is also first report on distribution of *Moraxella* spp. in rhizosphere of wheat. In case of *Moraxella,* Holton's medium without cefsulodin proved best for its isolation from rhizosphere of

wheat. *Serratia* was found maximum at inflorescence stage (45 days), and in rhizosphere area on VRBA medium. It was observed that number of *Serratia* spp. was least in rhizosphere of wheat.

The percentage of 4 bacterial genera, isolated from rhizosphere of wheat in overall life cycle of wheat plant was calculated. It has been clear that, *Pseudomonas* was the most dominant bacteria in the rhizosphere of wheat comprise 35.78 %. Our results supports to the findings of Kleeberger, et al (1983) that *Pseudomonas* is the most dominant spp. in the rhizosphere of wheat. *Acinetobacter* was found the second most dominant bacteria after *Pseudomonas* comprises 31.39 %, *Moraxella* was the next dominant bacteria comprises 28% and *Serratia*, found in very less amounts of 4.75%.

Existence of rhizosphere microflora is governed by different factors such as exudation of energy rich compounds from the root system, antagonism and microbial inter competition, hyper-parasitism, edaphic factors, etc (Subba rao, 1986; Sudmen, 1969; Sivasithamparam &Parker, 1978; Scher et al, 1985; Cello et al, 1997; Seldin et al, 1998). Microorganisms growing under the influence of root are often quantitatively and qualitatively different from those inhibiting remote from this influence in soil environment (Katznelson, 1965; katznelson & Rouatt, 1957;Tyler et al, 1979; Torsvik, et al, 1990).

In the present study it was observed that *Acinetobacter* is one of the most dominant bacteria in the rhizosphere of wheat. Thus it was considered very important to find the role of *Acinetobacter* in wheat rhizosphere.

2.4 Conclusions :

- Four gram negative bacterial genera viz. *Pseudomonas*, *Acinetobacter*, *Moraxella* and *Serratia* spp. were isolated from rhizosphere of wheat were.
- *Acinetobacter* was found to be one of the dominant genera in the rhizosphere of wheat.
- AMM and Holton's medium without cefsulodin were found suitable media for the isolation of *Acinetobacter* from rhizosphere of wheat.
- Reciprocal relationship was observed between *Acinetobacter* and *Moraxella* spp.

2.5. Significance of this work :

- *Acinetobacter* is significantly present in rhizosphere of wheat. Its significant presence in rhizosphere of wheat gives us a clue that *Acinetobacter* may play an important role in rhizosphere of wheat. This is the first report on detailed distribution of *Acinetobacter* spp. from rhizosphere of wheat. Further study in this area will clarify the role of *Acinetobacter* in wheat rhizosphere is under investigation.

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CHAPTER 3

Isolation, Characterization and Biotyping of *Acinetobacter* genospecies from Rhizosphere of Wheat.

Chapter 3

Isolation, Characterization and Biotyping of *Acinetobacter* genospecies from Rhizosphere of Wheat.

3.1. Abstract :

37 *Acinetobacter* strains were isolated from rhizosphere of wheat. Strains belonging to genus *Acinetobacter* were confirmed by chromosomal DNA transformation assay. All strains were biotyped and found to be distributed into five genospecies viz. *A. calcoaceticus* (4), *A.* genospecies 3 (3), *A. baumannii* (9), *A. haemolyticus* (11) and *A. junii* (10). All these strains could not be biotyped by API 20 NE system. However, many *Acinetobacter* strains showed positive reactions for different API tests such as adipate and caprate assimilation. 28^{0}C temperature, pH 7, aeration at 250 rpm were found to be optimum physiological conditions for growth of all *Acinetobacter* spp. All *Acinetobacter* strains could tolerate 2 % NaCl (w/v) except four. *A. baumannii* A25; *A. junii* A7 and *A. haemolyticus* A19 and A22 could tolerate 4 % NaCl (w/v). 15 carbon and 13 nitrogen sources were utilized by almost all *Acinetobacter* spp. 13 organic acids, except lactic acid were utilized by almost all genospecies of *Acinetobacter*. Variation in enzyme production was exhibited by different species of *Acinetobacter*. *Acinetobacter* genospecies produced enzymes such as chitinase, urease, amylase, phosphatase, L- asparginase, xylanase, pectinase, protease, catalase and nitrate reductase. *Acinetobacter* showed high resistance to β-lactam antibiotics, moderate resistance to aminoglycoside antibiotics and sensitive to other antibiotics except chloramphenicol. *Acinetobacter* genospecies also exhibited resistance to toxic metals such as tellurium, selenium, arsenate, arsenite and lead. In general, it was found that *Acinetobacter* spp. isolated from rhizosphere of wheat showed high degree of tolerance to heavy metals than antibiotics.

Key words : *Acinetobacter*, Rhizosphere of wheat, Carbon and nitrogen source utilization, Enzyme production, Antibiotic and metal resistance.

3.2 Introduction :

Acinetobacter is ubiquitous in nature and generally found in soil, water, sewage, foods and dairy products (Cousins et al., 1977; Balows et al., 1992; Saha & Chopade, 2001). *Acinetobacter* spp. are gram-negative, oxidase negative, non motile, aerobic, coccobacilli in pairs, singles or short chains and are mostly non pigmented (Juni, 1984). Interestingly it is the only group of gram-negative bacteria, which is the normal resident of human skin, animal skin and mucous membrane (Henriksen, 1973; Patil & Chopade, 2001a; Patil & Chopade 2001b). Presence of *Acinetobacter* from soil was studied (Baumann, 1968). *Acinetobacter* is a opportunistic human pathogen and *Acinetobacter* infections are growing concern in hospitals (Bouvet & Grimont, 1987). The detailed biochemical and physiological characteristics of *Acinetobacter* spp. isolated from soil and clinical isolates were studied by Bouvet & Grimont (1986). From their biochemical analysis, *Acinetobacter*

strains were mainly identified under six genospecies, which were *A. calcoaceticus, A. baumannii, A. haemolyticus, A. junii. A. johnsonii* and *A. lwoffii.* It was observed that *A. calcoaceticus* is mainly found in soil and others were found in hospital sources. In further studies it was observed that *A. baumannii* is also present in soil like *A. calcoaceticus,* but other strains were not found in soil. 11 new strains of *Acinetobacter* spp. were unambiguously identified as *Acinetobacter* genospecies 3,6,10,11 and 12 (Bouvet & Grimont, 1987).

Acinetobacter shows plasmid mediated resistance to different antibiotics and metals. (Dhakephalkar & Chopade, 1994; Deshpande et al, 1993). Antibiotic and metal resistance is important in basic and applied research particularly with respect to plasmid genetics and the physiology and ecology of microorganisms in polluted environment / ecosystems. These are mainly used as a genetic markers. There are reports on antibiotic and metal resistance of *Acinetobacter* spp. isolated from clinical environment (Deshpande et al, 1993).

In the present study, we have carried out biochemical characterization of *Acinetobacter* spp. isolated from rhizosphere of wheat. It should be noted that, rhizosphere environment is different and complex than soil environment. Number of interactions between microorganisms and plants as well as microorganisms to microorganisms takes place and make the rhizosphere environment competitive for nutrients and hence for existence. In the present investigation, we have isolated *Acinetobacter* spp. from rhizosphere of wheat and confirmed to belong to genus *Acinetobacter* by chromosomal DNA transformation assay (Juni, 1972). For the species identification, 35 biochemical tests as per Bouvet & Grimont (1986 & 1987) and additional 48 different tests were carried out for detailed characterization of these species. This is the first report on biochemical characterization of *Acinetobacter* spp. isolated from rhizosphere of wheat. Metal and antibiotic resistance was also carried out for these strains. Different antibiotics and metal salts were tested for this purpose.

This study is important with respect to genetic study of *Acinetobacter* spp. in rhizosphere environment. Here we found that *Acinetobacter* genospecies isolated from rhizosphere of wheat were highly resistant to different antibiotics and metals. This is also, a first report on studies of antibiotic and metal resistance of *Acinetobacter* spp. isolated from rhizosphere of wheat.

3.3. Materials and Methods :

3.3.1. Characterization of *Acinetobacter* :

Morphological characterization was carried out by observing colony characters and cell morphology. Other characters observed were Gram staining, oxidase enzyme test, catalase enzyme test, motility, capsule staining, nitrate reductase and H_2S production (Gerhardt, et al, 1994).

3.3.2. Chromosomal DNA transformation assay :

Chromosomal DNA transformation assay was carried out by using *Acinetobacter calcoaceticus* BD413 trpE27, a naturally competent tryptophan auxotrophic mutant. The assay was done by using crude genomic DNA as per Juni (1972) and also by purified genomic DNA as per Chen & Kuo (1993). *A. calcoaceticus* BD413 trpE 27 overnight grown on BHI agar plate (10 μg) was mixed with crude as well as purified genomic DNA (20μg) homogeneously and grown together on brain heart infusion agar (BHI) plates at 28°C. After 18-24 h, growth of BHI agar plates was transferred to *Acinetobacter* minimal medium (AMM) without tryptophan and with 1.8 % noble agar (Difco, USA) for the growth of transformants. The plates were incubated at 28°C for 48-108 h. The colonies from AMM plates were again transferred to fresh AMM plates to confirm the growth. Those strains, which were able to grow on AMM medium, were confirmed as genus *Acinetobacter*. All the individual transformants were stored at 10°C. *Acinetobacter calcoaceticus* MTCC (Microbial type culture collection, Chandigarh, India), 1271, *A. lwoffii* MTCC , 496, *Acinetobacter calcoaceticus* MTCC 127 and *E. coli* HB101 were used as a standard positive and negative controls respectively.

3.3.3. Tolerance of *Acinetobacter* genospecies to different physico-chemical factors :

To check the tolerance of *Acinetobacter* genospecies isolated from rhizosphere of wheat were subjected to different pH, temperature, NaCl concentration and aeration conditions.

- **To check the pH tolerance :** different ranges of pH were taken such as pH 4,5,6,7,8,9,10,11 and 12. Luria broth was used, as a medium, adjusted to above pH and the tubes were incubated at 28°C for 7 days. Un-inoculated broth was kept as negative controls for each pH values.
- **Temperature studies :** Luria agar plates were prepared and all the cultures were inoculated and kept at different temperatures such as 10, 37, 41, 44 and 50°C. Plates were observed for growth up to 7 days and results were recorded.
- **Salt tolerance :** NaCl concentration was taken as 2,3,4,5 and 6 % (w/v) in Luria broth. Cultures were inoculated and tubes were incubated at 28°C for 7 days. Tubes were observed daily for growth by measuring O.D. at 660 nm and results were recorded.
- **Aeration** was carried out by subjecting the inoculated LB flasks on shaker at different aeration conditions such as 100, 150, 200 and 250 rpm for 7 days at 28°C and growth was recorded by measuring O.D. at 660 nm every day.

3.3.4. Haemolysis :

Haemolysis was carried out by preparing blood agar base (Luria agar) containing 5 % (v/v) fresh human blood (whole bood). Plates were incubated at 28°C for 24 to 48 h. After incubation plates were observed for three types of haemolysis. i) gamma haemolysis : no lysis of red blood cells

indicated by no significant change around the colony. ii.) Beta haemolysis : lysis of red blood cells with complete destruction and use of hemoglobin results in clear zone around the colony and iii.) alpha haemolysis : incomplete lysis of red blood cells with complete destruction and use of hemoglobin by organism results in greenish halo around the bacterial growth (Bouvet & Grimont, 1986 & 1987).

3.3.5. Biochemical characterization of *Acinetobacter* spp. :

For the identification of *Acinetobacter* up to species level the biochemical tests were carried out. All the tests were performed as per Bouvet & Grimont (1986 & 1987). For all these tests overnight grown cultures were used and each test recorded daily for 7 days at 28°C unless mentioned specifically.

3.3.6. Oxidation Fermentation (O/F) test :

Oxidation fermentation test was carried out as described by Collins, et al (1995).

3.3.7. Production of acid from D-glucose :

Production of acid from D-glucose was detected by two methods as described by Bouvet & Grimont (1986 & 1987).

3.3.8. Nitrate reduction :

Overnight grown culture was inoculated on oxide medium used for NO_3 reduction. Sulfanilic acid-alpha naphthylamine reagent was used for this purpose. Formation of brick red color indicates positive test. No change in color indicated negative test (Bouvet & Grimont, 1986 & 1987).

3.3.9. IMViC :

IMViC test was done as described by Collins, et al (1995).

3.3.10. Citrate utilization :

Simmons citrate agar slants were prepared and test culture was streaked on it. Slants were incubated at 28°C for 7 days. Blue color indicates utilization of citrate as a carbon source.

3.3.11. Carbon source utilization :

Carbon source utilization tests were carried out as per Bouvet & Grimont (1986 & 1987). In this method filter sterilized (Sartorious, 0.45 μm) carbon source solutions were added to pre-sterilized M 70 minimal medium without agar (Bouvet & Grimont, 1986). The final concentration of carbon sources (carbohydrates) was 0.2 % (w/v). Andrade's reagent was added to M 70 medium to detect the acid and gas production. Durham's tubes were incorporated to observe for bubble in the tubes for the gas production. Three ml M70 minimal medium was dispensed into glass tubes (diameter, 11mm & height 99 mm). The following carbon sources were used for utilization test.

i) Monosaccharides : D- glucose, D-galactose, fructose, mannose, D-ribose, rhamnose and xylose.

ii) Disaccharides : lactose, cellobiose, maltose, sucrose arabinose and millibiose.

iii) Polysaccharides : inulin and carboxymethylcellulose.

iv) Carboxylic acid: DL-lactate, acetate, citrate, D-malate, malonate, propionate, phenyl acetate, 4-amino butyrate, transaconitate and oxalate.

v) Others : sorbitol , mannitol, dulcitol, inositol, ethanol and N-acetylglucosamine.

3.3.12. Amino acids utilization :

Utilization of amino acids was also carried out by the similar procedure used for the studies on carbohydrates. Tubes containing sterile M70 minimal medium with filter sterilized amino acids at final conc. 0.1 % (w/v). Tubes were then inoculated with bacterial culture prepared in 0.85% saline, (10µl) containing 10^8cells/ml (adjusted by McFarlands scale). Tubes were incubated at 28^0C and growth was recorded daily for 7 days. Amino acids used in this study were glutamate, L-aspartate, â-alanine, threonine, tryptophan, glycine, L-leucine, tyrosine, cystine, arginine, L-ornithine and phenylacetate. Growth on the above amino acids was used for the identification of *Acinetobacter* species.

3.3.13. Nitrogen source utilization :

Nitrogen source utilization test was determined in liquid minimal medium M70 containing 0.1 % (w/v) sodium acetate but lacking $(NH_4)_2SO_4$ (Bouvet & Grimont, 1986). The following amounts of nitrogen sources were added per liter of medium : 100 mg of $NaNO_2$, 166 mg KNO_3 and 1g urea. Tubes without nitrogen source and tubes containing 0.1% (w/v) $(NH4)_2SO_4$ were used as controls (Bouvet & Grimont, 1986).

3.3.14. Biotyping by API 20 NE system :

Identification of 37 *Acinetobacter* strains was also done by API 20 NE Microtube system (API BioMerieux SA 20050 07615E-03/2000, France) intended to be used for non enteric, non fastidious, non fermentative, Gram negative, bacilli (Towner & Chopade,1987). It consisted of 8 conventional enzymatic tests (nitrate reductase, indole production, arginine hydrolysis, urease production, esculin hydrolysis, gelatin hydrolysis, and â- galactosidase production) and 12 carbon substrate assimilation tests (glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate, and phenylacetate). The test strips were inoculated as per manufacturers instructions and results were noted after 24 h incubation at 28^0C. Additional 24 h incubation was carried out for the biotypes showing ambiguous results. A 7-digit numerical profile index was then compared with the indexes listed in the catalogue supplied by the manufacturer. % identification and T indices were also taken into the consideration.

3.3.15. Organic acids utilization :

To observe the utilization of organic acids by *Acinetobacter* spp., organic acids such as malic acid, citric acid, 4 amino-butyric acid, phenylacetic acid, lactic acid, propionic acid, acetic acid, succinic acid, formic acid, n-butyric acid, oxalic acid, and tartaric acid were used. Tween 80 and tween 20

were also tested for utilization by the same procedure as organic acids. Organic acids were filter sterilized and in 0.1 % concentration were added into sterilized M70 minimal medium without agar (Bouvet & Grimont, 1986). Tubes were incubated at 28°C for 7 days. Growth in the medium was considered as a positive result. For negative control, uninoculated tubes with sterile M70 medium was used (Bouvet & Grimont ,1986).

3.3.16. Detection of enzyme production by *Acinetobacter* spp. from rhizosphere of wheat :

- **Gelatinase production :**

  For gelatinase production nutrient agar supplemented with 0.4 % gelatin was used. Plates were spot inoculated with overnight grown culture and incubated at 28°C for 48 h. Plates were then flooded with Frazier reagent (15 % (w/v) $HgCl_2$ in 20 % (v/v) HCl). Plates were observed for gelatin hydrolysis by indicating a clear zone around the spot (Bouvet & Grimont, 1986).

- **Urease activity :**

  Urease test was carried out by preparing Christensen urea agar. Surface of agar slant was inoculated with test culture and incubated at 28°C for 3-5 days. A positive test was indicated by red-violet colour. Un-inoculated negative control was kept without inoculation (Gerhardt, et al., 1994).

- **DNAse activity :**

  DNAse medium (dehydrated, Hi Media, M725) was prepared. Spot inoculation of overnight grown culture was made and plates were incubated at 28°C for 24 h. After 24 h, plates were flooded with 1 N HCl and kept for 2-5 min. Plates were observed for clear zone around the spot. *S. marcescens* was kept as positive control and *E. aerogenes* was used as negative control (Gerhardt et al.,1994).

- **Lipase activity :**

  Plates of tributyrin agar were spot inoculated with overnight grown cultures with *Acinetobacter* spp. Plates were incubated at 28°C for 2-3 days. After incubation, plates were observed for clear zone around the colony. Negative test showed absence of zone around the colony (Gerhardt et al.,1994).

- **Protease activity :**

  Overnight grown culture of organism was spot inoculated on casine agar. Plates were incubated at 28°C for 24-48 h. Positive strains showed a clear zone around the colony against opaque background. Negative test showed absence of zone around the colony (Collins et al, 1995).

- **Amylase activity :**

  Starch agar was spot inoculated with overnight grown culture of test bacteria and plates were incubated at 28°C for 7 days. After 7 days plates were flooded with 1N iodine solution. Isolates showing colorless zone against blue background were scored as a positive cultures for amylase (Gerhardt et al, 1994).

- **Pectinase activity :**

  Overnight grown culture was spot inoculated on pectin agar plates (minimal salt medium supplemented with peptone and 0.5 % pectin (Loba, India) and plates were incubated at 28^{0}C for 7 days. Subsequently plates were flooded with 1 % (w/v) cetrimide for 10 min. Pectinase positive isolates were detected by clear zone around the spot (Hankin et al, 1971).

- **Cellulase activity :**

  Carboxy methylcellulose agar (minimal salt medium supplemented with peptone and CMC) was prepared and overnight grown cultures were spot inoculated on it. Plates were incubated at 28^{0}C for 7 days. After incubation plates were flooded with 1.5 (w/v) cetryl trimethyl ammonium bromide (cetrimide) and left for 10 min. Cellulase positive cultures were detected by clear zone of hydrolysis around the spot (Hankin & Anagnostakis, 1977).

- **Xylanase activity:**

  Overnight grown cultures was spot inoculated on xylan agar (xylan 0.2 %) and plates were incubated at 28^{0}C for 7 days. After incubation plates were flooded with 0.5 % (w/v) cango red solution for 10 min and then periodically with saturated solution of sodium chloride. The isolates with xylanase activity were detected by clear zone against red background around the spot (Balkrishnan, 1993).

- **Chitinase activity :**

  For chitinase, chitin agar (Hsu & Lockwood, 1975) was prepared by minimal salt medium supplemented with 4 % colloidal chitin. Overnight grown cultures were spot inoculated on chitin agar and plates were incubated at 28^{0}C for 7 days. After 7 days incubation plates were observed for a clear zone around the spot because of hydrolysis of chitin against opaque background contributed by colloidal chitin (Hsu & Lockwood, 1975).

- **Phosphatase activity :**

  Phosphatase activity was tested for both acidic and alkaline phosphatase. Nutrient agar medium supplemented with 0.01 % phenolphthalein di phosphate (Loba Chemicals, India) was prepared. Overnight grown cultures were spot inoculated and plates were incubated for 2-5 days. After incubation, 1 drop of ammonia solution (specific gravity 0.880, 28 to 30 %) was placed in the lid of inverted petriplate and culture plate was replaced over it to allow the ammonia fumes to reach the growth. Positive test was indicated by turning the colour of colonies into red because of presence of free phenolphthalein (Gerhardt, et al.,1994).

- **L- Asparginase activity :**

  Overnight grown cultures were streaked on Luria agar with 0.2 % L- asparginase and few drops of phenol red indicator. Slants were incubated at 28^{0}C for 5 days. The color of medium changed from yellow to red due to production of ammonia around the positive strains (Benny & Kurup, 1991).

3.3.17. Antimicrobial disc susceptibility of *Acinetobacter* spp :

Antimicrobial disc susceptibility was carried out by agar dilution method as per Kirby-Bauer (Lorian, 1986). Muller-Hinton agar plates were inoculated with the overnight grown bacterial culture, of which O.D. was adjusted to 1 as per McFarland's scale. After inoculation, antibiotic discs (Hi Media) were placed over the surface of inoculated agar carefully. Inoculated plates were incubated at 28^{0}C for 18-24 h. Diameter of zone of inhibition was measured and compared with the standard chart as per manufacturers suggestions accordingly susceptibility and resistance was recorded. Antibiotics used for susceptibility were as follows : Cephalexin (Cl) , Cephazolin (Cz), Kanamycin (Km), Pefloxacin (Pf), Amikacin (Ak), Cloxacillin (Cx), Norfloxacin (Nf), Chloramphenicol (Cm), Co-trimoxazole (Q), Nalidixic acid (Na), Novobiocine (Nv), Doxicycline (Do), and Tetracycline (Tc).

3.3.18. Minimal inhibitory conc. (MIC) of antibiotics and metal salts for *Acinetobacter* spp. :

For the detection of MIC for antibiotics and metal salts, two-fold agar dilution method was carried out (Dhakephalkar & Chopade, 1994). Tubes containing 25 ml of sterile melted Muller-Hinton agar with increasing concentration of antibiotic and metals were added. The range of conc. was from 1-1024 µg/ml. Plates were dried and spot inoculated with 2 µl of overnight grown culture, 10^4-10^5 cells were added per spot. Muller-Hinton agar plates without antibiotic and metal salts were inoculated with test organism were kept as a control. Plates were incubated at 28^{0}C for 24 to 48 h. Plates were observed for growth of test organism and the concentration at which the growth of microorganism inhibits completely was termed as minimum inhibitory concentration (MIC). The lower concentration of MIC was termed as sub inhibitory concentration (SIC). *Acinetobacter calcoaceticus* BD413 and *E. coli* HB101 were used as reference strains for antibiotic and metal sensitivity. Antibiotics used for MIC were as follows :

i) â- lactam group : penicillin, ampicillin, ceftriaxone, cloxacillin, amoxicillin and cefuroxime.

ii) Aminoglycoside group: streptomycin, kanamycin, gentamycin, neomycin and amikacin.

iii) Others : nalidixic acid, chloramphenicol, tetracycline and rifampicin.

The strains not inhibited at 25 µg /ml of antibiotic were termed as resistant strains. On the other hand unlike antibiotic resistance there is no acceptable metal concentration to specify metal resistance. (Trevors et al,1985; Dhakephalkar & Chopade, 1994). Therefore strains, which were not inhibited by 10mM, were considered as resistant (Dhakephalkar & Chopade, 1994). Metal salts used for MIC were as follows : sodium arsenate, sodium arsenite, sodium tungustate, potassium tellurate, lead sulphate, vanadium pentoxide, cadmium chloride, nickel chloride, lithium sulphate, gold, mercuric chloride, platinum chloride, magnesium chloride, copper chloride, zinc chloride, antimony oxide, ferrous chloride, silver chloride , cobalt nitrate and boric chloride.

3.4. Results :

3.4.1. Isolation and characterization of *Acinetobacter* from rhizosphere of wheat:

Morphological characterization of *Acinetobacter* spp isolated from rhizosphere of wheat was done. On the basis of five preliminary tests 45 *Acinetobacter* strains were identified as belonging to the genus *Acinetobacter*. From these tests, cells were found to be coccobacilli, non-motile, oxidase negative, catalase positive and capsulated. Few strains were able to reduce nitrate and all were negative for H_2S production.

3.4.2. Confirmation of genus *Acinetobacter* by chromosomal DNA transformation assay :

Chromosomal DNA transformation assay confirmed 37 strains were genuine *Acinetobacter*, out of 45 tentative *Acinetobacter* strains. According to Juni (1972) a crude DNA preparation is sufficient for chromosomal DNA transformation assay. However, it was observed that with crude DNA, transformation rate was low whereas, pure DNA gave good results. It was observed that chromosomal DNA isolated from tentative *Acinetobacter* strains required 48-72 h to transform *A. calcoaceticus* BD413 trp E 27 (Fig.1).

3.4.3. Quantitative analysis of *Acinetobacter* spp. isolated from rhizosphere of wheat :

Chromosomal DNA transformation assay confirmed that 37 isolates were from genus *Acinetobacter*. Among these 37 strains, 15 strains were isolated from rhizosphere area (RS) of wheat plant. 12 strains were isolated from rhizoplane (RP) of wheat plant and 10 strains were isolated from non rhizosphere soil of wheat plant (Table 1).

Table 1. Chromosomal DNA transformation assay of tentative *Acinetobacter* strains isolated from rhizosphere of wheat using naturally competent auxotrophic mutant *A. calcoaceticus* BD413 trp E 27.

Source	No of tentative *Acinetobacter* strains (n = 46)	No. of strains transformed (n = 37)
Rhizoplane of wheat	12	10
Rhizosphere of wheat	23	20
Non rhizosphere soil	10	7
Pseudomonas putida 1313 [a] (control)	1	0

Pseudomonas putida 1313 [a] : Standard strain taken as a negative control.

3.4.5. Biochemical characterization and identification of *Acinetobacter* :

Biochemical tests were carried out for the species identification of *Acinetobacter* genospecies confirmed by chromosomal DNA transformation assay. The standard biochemical tests were described by Bouvet & Grimont (1986 & 1987), were followed for this identification and compared with their results. Results obtained by Bouvet & Grimont methods are described in Table 2. API 20 NE was also done. According to the standard tests 37 *Acinetobacter* spp. were divided into 5

Fig.1

Fig.2

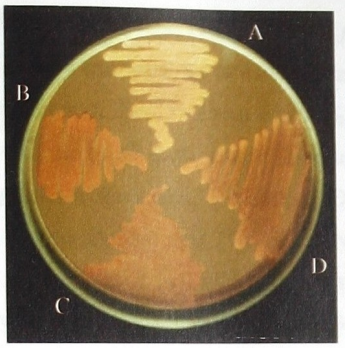


Fig.3

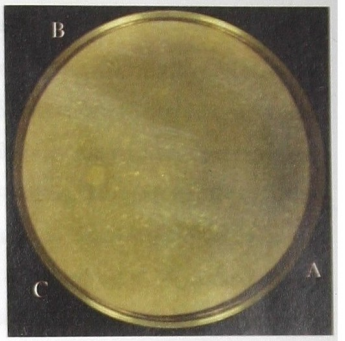


Fig.4A

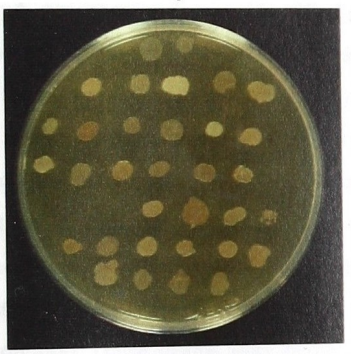


Fig.4B

Fig.1. Chromosomal DNA transformation assay of *Acinetobacter* genospecies isolated from rhizosphere of wheat.
A : Standard *A. calcoaceticus* MTCC1271; B: *A. calcoaceticus* BD413 trp E27 a naturally competent strain; C&D : growth of prototrophic transformants on *Acinetobacter* minimal medium (AMM) agar plate; E&F: Purified genomic DNA of tentative *Acinetobacter* spp.

Fig.2. Pigment production by *Acinetobacter* genospecies.
A: Yellow pigment producing *Acinetobacter junii* A4; B: Brown pigment producing *Acinetobacter baumannii* A18; C & D: Pink pigment producing *Acinetobacter* genospecies 3 A15 & A28.

Fig.3. Chitinase enzyme production by *Acinetobacter* spp. isolated from rhizosphere of wheat.
A. B&C: *Acinetobacter* genospecies producing chitinase enzyme.
A: *A. haemolyticus* A19; B: *A. junii* A7 and C: *A. baumannii* A25.

Fig.4. Antibiotic and metal resistance of *Acinetobacter* genospecies.
A : Resistance of *Acinetobacter* genospecies to chloramphenicol (MIC>1024 µg/ml.).
B : Resistance of *Acinetobacter* genospecies to tellurium (MIC> 1024 µg/ml).

different genospecies. These are group I.) *Acinetobacter calcoaceticus* (4); II.) *A.* genospecies 3 (3); III.) *A. baumannii* (8); IV.) *A. haemolyticus* (12) and V.) *A. junii* (10).

- **Group I. :** *A. calcoaceticus* as they can grow up to 41^{0}C and not above. Gelatin hydrolysis and haemolysis tests were negative. None of them able to produce H_2S and indole. They can form acid from glucose and citrate was utilized by all these strains. â- xylosidase test was also positive and they can utilize DL-lactate, glutarate, malonate, L-histidine, D-malate, L-aspartate, â- alanine, ethanol, L- arginine and DL- 4 aminobutyric acid (Table 2). None of them were able to utilize transaconitate, phenylalanine and oxalate. 2 % NaCl (w/v) was tolarated by all *A. calcoaceticus* strains. Brown pigment production was observed in *A. calcoaceticus* A2. These strains were named as A2, A3, A5 and A35.
- **Group II :** *A.* genospecies 3 were identified as they can grew up to 41^{0}C but unable to grow at 44^{0}C and above. Gelatin was not hydrolyzed and human blood was not haemolyzed. Nitrates were reduced but variation occurred with A15. Citrate was utilized. All carbon sources were utilized by most of the strains only oxalate and L- ornithine was not utilized by any *A.* genospecies 3 strain. Acid was not produced from D-glucose. Phenyl acetate was not utilized by A25, but variation occurred with A12 strain. DL lactate, L-malonate, glutarate, L-phenylalanine, L-histidine, ethanol, tween 80 and tween 20 were utilized by all strains as sole source of energy (Table 2). All strains could tolarate 2 % NaCl (w/v). Two strains of *A.* genospecies 3, A15 and A28 exhibited pink pigment production. These strains were named as A12, A15 and A28.
- **Group III :** *A. baumannii* were identified as all isolates grew at 44 ^{0}C and also at 41 and 37^{0}C. Nitrate was reduced to nitrites but A32, A18, A30 and A33 did not reduced nitrate to nitrite. Gelatin was not hydrolyzed, but in case of strains A13, A30 and A33 it varies. Human blood was not haemolyzed but this also varied in case of A13, A30 and A33. Acid was produced from D-glucose by only 3 strains A16, A18 and A13. Citrate was utilized by all strains. Phenylacetate was not utilized by 3 strains A16, A30 and A33. Following compounds were utilized by these strains : ethanol, tween 80, tween 20 ,DL- lactate, L-malonate, L- histidine, L- aspartate, L-leucine, ethanol, L-arginine, β-alanine, and 4-amino butyric acid (Table 2). Oxalate was not utilized by any *A. baumannii* strain but all other carbon sources were utilized by one or other *A. baumannii* strains. 25 % *A. baumannii* strains were able to tolerate 4 % NaCl (w/v) and 2 % NaCl (w/v) was tolerated by all strains. Four *A. baumannii* strains showed pigment production among which 3 produced brown pigment A1, A16 and A18 and A23 produced pink pigment. These strains were named as A1, A13, A16, A18, A23, A30, A32 and A33.
- **Group IV :** *A. haemolyticus* were identified as all isolates were hydrolyzed human blood. Gelatin was also hydrolyzed, except A24, A27 and A34. Citrate was utilized by all strains. These strains grew up to 41^{0}C and unable to grow at 44^{0}C except 2 strains A19 and A36. Acid was not produced

from D-glucose except 1 strain A11. Nitrate was not reduced to nitrite in complex media except 4 strains A19, A21, A25 and A24. DL- lactate and glutarate were utilized by all strains. Following compounds were utilized by maximum isolates as a sole source of carbon and energy those were : phenylalanine, malonate, L-histidine, L-leucine, L-tyrosine, L-arginine, β-alanine, ethanol, tween 80, tween 20 and 4-aminobutyric acid. Two compounds were not utilized by maximum strains were phenylacetate and transaconitate (Table 2). Three strains A19, A22 and A25 were able to tolerate 4 % NaCl (w/v) and all strains were able to tolerate 2 % NaCl (w/v). Out of four, three strains were able to produce brown pigment which were A11, A17, A19 and A34 produced yellow pigment. These are named as A11, A17, A19, A21 A22, A24, A29, A34, A36 and A37,

Group V : *A. junii* and isolates were identified, as no strain was able to hydrolyze sheep/human blood. Acid was not produced from D-glucose by all the isolates. Citrate was utilized by all the strains. Gelatin was hydrolyzed by 5 strains A4, A5, A6, A9 and A31. All strains can grow at 37 and 41^{0}C and 8 strains (A4, A7, A6, A8, A9, A10, A14 and A31) grew at 44^{0}C. Nitrate was not reduced to nitrite in complex media by all isolates except 3: A7, A14 and A26. Glutarate, malonate, â -alanine, L-arginine, ethanol, tween 80 and tween 20 were utilized by all the isolates as a sole source of carbon and energy. Others such as phenylacetate were utilized by only one isolate A14, DL-lactate, phenylalanine, L- histidine, D-malate, L- aspartate, L-leucine, L-tyrosine and 4 aminobutyric acid were utilized by maximum isolates. None of them could utilize transaconitate and oxalate (Table 2). One strain A9, tolerate 4 % NaCl (w/v) and all others were able to tolerate 2 % NaCl (w/v). Seven strains exhibited pigment production. Out of seven, six strains A10, A7, A8, A14, A20 and A9 produced brown pigment and A4 produced yellow pigment. These isolates were named as A4, A6, A7, A8, A9, A10, A14, A20, A26 and A31.

None of *Acinetobacter* strains produced indole and H_2S in complex medium. *Acinetobacter* spp. can utilize broad spectrum of carbohydrates without production of acid and gas. Table 2, describes utilization pattern of *Acinetobacter* strains isolated from rhizosphere of wheat. 57 carbon sources were tested against 37 strains of *Acinetobacter* and only L- histidine, of those was utilized by all strains and vise versa. L- histidine was utilized by all strains of five genospecies. All isolates were grown at 2% NaCl (w/v) and only 4 strains were grown at 4 % NaCl (w/v). These were A7, A19, A22 and A25. All strains were checked at different pH ranges from 5-10 showed that none of them grown at pH 5 and all grew from pH 6-10 with optimum pH 7 and 8.

Table 2. Phenotypic characterization of *Acinetobacter* genospecies isolated from rhizosphere of wheat.*

Characteristics	Group I	Group II	Group III	Group IV	Group V
	A. calcoaceticus	*A.* genospecies 3	*A. baumannii*	*A. haemolyticus*	*A. junii*
	(N =04)	(N = 03)	(N = 09)	(N = 11)	(N = 10)
Gram Staining	Gram-ve	Gram-ve	Gram-ve	Gram-ve	Gram-ve
Morphology	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli
Motility	Non motile	Non motile	Non motile	Non motile	Non motile
Oxidase	-	-	-	-	-
Catalase	+	+	+	+	+
Capsule	+	+	+	+	+
Growth at :					
44^{0}C	00	00	09	10	08
41^{0}C	04	03	09	11	10
37^{0}C	04	03	09	11	10
28^{0}C	04	03	09	11	10
Pigment production	01	02	05	04	07
Acid from Glucose	00	02	02	00	00
Gelatin Hydrolysis	00	00	02	09	05
Haemolysis	00	00	03	11	00
Citrate (Simmons)	04	03	09	11	10
NO_3- reduction	02	02	04	04	04
Utilization of :					
DL – Lactate	04	03	09	11	07
L – Phenylalanine	00	02	05	07	05
Phenyl acetate	01	01	04	03	01
Malonate	04	03	09	10	10
L – Histidine	04	03	09	09	09
D – Malate	04	03	08	10	09
L – Aspartate	04	03	08	09	09
L – Leucine	01	02	06	06	08
L – Tyrosine	01	02	05	06	07
β- Alanine	04	03	09	10	10
L – Glycine	04	02	09	10	09
Trans-Aconitate	00	02	04	02	00

Table 3 Continued....

Characteristics	Group I	Group II	Group III	Group IV	Group V
	A. calcoaceticus	*A.* genospecies 3	*A. baumannii*	*A. haemolyticus*	*A. junii*
	(N =04)	(N = 03)	(N = 09)	(N = 11)	(N = 10)
D- Glucose	03	03	06	09	08
L – Tryptophane	02	02	04	06	05
Na – Acetate	04	03	09	10	10
Oxalate	00	00	00	04	00
Ethanol	04	03	08	10	10
L – Arginine	04	03	09	09	10
L – Ornithine	02	00	06	07	10
DL 4-Aminobutyrate	01	03	09	09	05
Tween 20	04	03	09	11	09
Tween 80	04	03	09	11	10
Other tests :					
Tolerance to					
NaCl 4 %(w/v)	02	01	01	02	01
NaCl 2 %(w/v)	04	03	09	11	10
Indole	00	00	00	00	00
Methyl Red	03	02	01	04	02
Voges Proskauer	00	00	00	00	00
Triple sugar iron test	04	03	09	11	10
Adipate	02	02	07	10	08
Caprate	1	03	06	09	07

*This was done as per Bouvet & Grimont (1986 & 1987). Besides these some additional tests were done.

- : negative, + : positive, 00: negative result, N : total number of strains.

3.4.6. Biotyping of *Acinetobacter* genospecies by API 20 NE system :

All 37 strains, isolated from rhizosphere of wheat were tested for API 20 NE system. None of these isolates were matched with the characters cited in the profile index list of API 20 NE catalogue (6th edition, 1997). Thus API 20 NE system was not suitable to identify the isolates, but it is important to note that some of API 20NE tests such as adipate, caprate assimilation and ONPG tests were showed positive results for some *Acinetobacter* strains isolated from rhizosphere of wheat.

3.4.7. Utilization of different sugars by *Acinetobacter* spp. :

Additional sugars than Bouvet & Grimont (1986 & 1987) were tested for their utilization, as these additional sugars were the root exudates of wheat plant. Table 3 showed utilization of these sugars by five different *Acinetobacter* spp. isolated from rhizosphere of wheat and two standard strains of *A. calcoaceticus*. It was observed that all sugars were utilized by all strains of *A.* genospecies 3. Sucrose, lactose, rhamnose, D- mannitol, raffinose, galactose and dulcitol were utilized by all *A. calcoaceticus* strains, and 50 % strains utilized D-glucose, D-xylose, D-fructose, D-maltose, arabinose, millibiose and inuline. All *A. baumannii* strains utilized sucrose, lactose, D-mannitol, D-maltose and raffinose. 88 % strains were able to utilize D-xylose, D-fructose, arabinose and inuline, and 75 % strains were able to utilize D-glucose, rhamnose, D-ribose, galactose, mellibiose and dulcitol. All *A. haemolyticus* strains utilized sucrose, lactose, D- mannitol, D-xylose, D-maltose and arabinose. 91 % strains were able to utilize D-fructose, raffinose and inuline and 75 % strains utilized D-glucose, rhamnose, D- ribose, galactose, millibiose and dulcitol. All *A. junii* strains utilized sucrose, lactose, D-mannitol, D-xylose, and galactose. 90 % strains were able to utilize rhamnose, D-maltose, arabinose, raffinose, and inuline. 80 % strains were able to utilize D-glucose, D-fructose, galactose and 70 % strains utilized D-ribose and mellibiose. Thus overall, different strains of *Acinetobacter*, from all genospecies were able to utilize all sugars which shown in Table 3.

3.4.8. Utilization of different amino acids by *Acinetobacter* spp. :

Different amino acids utilization was tested for all *Acinetobacter* strains, as some amino acids are root exudates of wheat plant. All strains of *A. calcoaceticus* could utilize L- glutamic acid, valine, L-arginine, β-alanine, L- glycine and L- aspartic acid, 75 % strains utilized L- histidine. 50 % strains were able to utilize L-cystine and 25 % strains utilized L- lysine, L- threonine, L- leucine and tyrosine. None of *A. calcoaceticus* strain was able to utilize phenylalanine. All *A.* genospecies 3 strains utilized valine, β-alanine, tyrosine, L- aspartic acid, phenylalanine and L- histidine. 67 % strains were able to utilize L-arginine, L-lysine, L- leucine, L- glutamic acid and L-glycine. 33 % strains utilized L-cystine and none of *A.* genospecies 3 strains was able to utilize L- threonine. It was noted that different strains of *A. baumannii*, *A. haemolyticus* and *A. junii* were able to utilize

Table 3. Sugar utilization by *Acinetobacter* genospecies isolated from rhizoplane, rhizosphere and non rhizosphere soil of wheat plant*.

Utilization of Sugars	*Acinetobacter* genospecies					Standard Strain*
	A. calcoaceticus (N=04)	*A.*genospecies3 (N= 03)	*A baumannii* (N=09)	*A. haemolyticus* (N=11)	*A. junii* (N=10)	*A calcoaceticus* (N=02)
Sucrose	4	3	9	11	10	2
Lactose	4	3	9	11	10	2
D-Glucose	2	3	6	9	8	1
Rhamnose	4	3	6	9	9	2
D-Mannitol	4	3	9	11	10	1
D-Ribose	4	3	6	9	7	2
D-Xylose	2	3	7	11	10	1
D-Fructose	2	3	7	11	8	1
D-Maltose	2	3	9	11	9	1
Arabinose	2	3	7	11	9	1
Raffinose	4	3	9	10	9	2
Galactose	4	3	6	9	10	2
Melibiose	2	3	6	9	7	1
Dulcitol	4	3	6	9	8	2
Inuline	2	3	7	10	9	1

* 0.2 % sugar concentration was used in M 70 minimal medium: total number of strains. Standard strain
** :*A. calcoaceticus* MTCC 1271 and *A. calcoaceticus* MTCC 1425.

different amino acids. Table 4 shows utilization of amino acids by *Acinetobacter* spp. isolated from rhizosphere of wheat.

3.4.9. Organic acid utilization of *Acinetobacter* spp. isolated from rhizosphere of wheat :

Organic acid utilization was specially tested for *Acinetobacter* spp. isolated from rhizosphere of wheat because, some organic acids were the root exudates of wheat plants, like sugars and amino acids. Utilization of these organic acids as a nutrient by *Acinetobacter* spp. can play an important role in existence of *Acinetobacter* in wheat rhizosphere. Citric acid was utilized by strains of *A. baumannii* A23, *A. calcoaceticus* A35 and *A. junii* A14, acetic acid was utilized by strain of *A. calcoaceticus* A5, *A. haemolyticus* A37 and *A. junii* A6. Phenylacetic acid was utilized by *A.* genospecies 3 A12, *A. baumannii* A13 and A18, *A. haemolyticus* A11and A37 and *A. junii* A14. Propionic acid was utilized by *A. junii* A6 and *A. haemolyticus* A37. Transaconitic acid was utilized only by one strain of *A. haemolyticus* A34. All other organic acids such as succinic acid, malic acid, fumaric acid, oxalic acid, formic acid, tartaric acid, 4-amino butyric acid and n-butyric acid were utilized by different strains of *Acinetobacter* genospecies and at the same time, none of *Acinetobacter* strain was able to utilize lactic acid. Results are shown in Table 5.

3.4.10. Enzymes produced by *Acinetobacter* spp. isolated from rhizosphere of wheat :

It was found from Table 6 that that all 37 strains were able to produce L- asparginase except one *A. baumannii* A31. Phosphatase was produced by all *Acinetobacter* strains except one strain of *A. baumannii* A33 and one strain of *A. haemolyticus* A22. Lipases were produced by two strains of *A. calcoaceticus* A2 and A35, four strains of *A. baumannii* A13, A16, A25 and A33, four strains of *A. haemolyticus* A11, A21, A24 and A27. Gelatinase was produced by three strains of *A. baumannii*, A25, A30 and A33, by six strains of *A. haemolyticus* A11, A19, A21, A29, A36 and A37, and by five strains of *A. junii* A4, A6, A7, A9 and A31. Chitinase was produced by two strains of *A. haemolyticus* A19 and A22, one strain of *A. baumannii* A25 and one strain of *A. junii* A7. Protease was produced by three strains of *A. baumannii* A1, A30 and A33, three strains of *A. haemolyticus* A19, A21, A27 and by one *A. junii* A7. Amylase was produced by one *A. calcoaceticus* A35, two *A. haemolyticus* A22 and A27, one *A. baumannii* A32 and two *A. junii* A26 and A31. Urease was produced by two *A. calcoaceticus* A2 and A5, two *A.* genospecies 3 A12 and A15, seven *A. baumannii* A1, A13, A16, A18, A23, A25, A30 and A32, six *A. haemolyticus* A11, A17, A22, A27, A34 and A37 and six strains of *A. junii* A8, A9, A10, A14, A20 and A26. Pectinase was produced by one strain of *A. calcoaceticus* A3, one *A. baumannii* A1, one *A. haemolyticus* A11 and one *A. junii* A9. Xylanase was produced by two *A. haemolyticus* A19 and A22 and one *A. baumannii* A32. Cellulase was not produced by any strain of *Acinetobacter* isolated from rhizosphere of wheat. Thus overall it was found that all *Acinetobacter* spp. showed presence of

Table 4. Amino acids utilization by *Acinetobacter* genospecies isolated from rhizoplane, rhizosphere and non rhizosphere soil of wheat plant *.

Utilization of:	*Acinetobacter* genospecies					Standard Strain*
Amino acids	*A. calcoaceticus* (N=04)	*A.* genospecies 3 (N= 03)	*A. baumannii* (N=09)	*A. haemolyticus* (N=11)	*A. junii* (N=10)	*A. calcoaceticus* (N=02)
L- glutamic acid	04	02	08	09	10	02
L- valine	04	03	08	11	10	01
L- arginine	04	02	09	11	10	01
β-alanine	04	03	08	10	09	02
L-lysine	01	02	07	07	06	00
L- cystine	02	01	02	03	05	01
L-threonine	01	00	06	07	10	01
L-leucine	01	02	06	09	08	00
L- glycine	04	02	09	10	09	02
Tyrosine	01	03	05	06	06	00
L- aspartic acid	04	03	08	08	09	02
Phenylalanine	00	03	06	06	05	01
L- histidine	03	03	09	09	09	01

* 0.1 % concentration of amino acids were used in M70 minimal medium.
00 : not utilized; N: total number of strains;
Standard strains ** : *A. calcoaceticus* MTCC 1271 & 1425.

Table 5. Organic acids utilization by *Acinetobacter* genospecies isolated from rhizoplane, rhizosphere and non rhizosphere soil of wheat plant *.

Organic acids utilized	*Acinetobacter* genospecies					Standard Strain*
	A. calcoaceticus (N = 4)	*A.*genospecies3 (N = 3)	*A. baumannii* (N = 9)	*A. haemolyticus* (N = 11)	*A junii* (N = 10)	*A. calcoaceticus* (N =2)
Citric acid	00	00	01	01	01	00
Malic acid	00	00	00	00	02	00
Phenylacetic Acid	00	01	02	02	01	00
Lactic acid	00	00	00	00	00	00
Acetic acid	01	00	00	01	01	01
Propionic acid	00	00	00	00	01	00
Succinic acid	01	02	01	04	02	01
Fumaric acid	02	01	02	05	04	01
Oxalic acid	04	03	09	10	10	00
Formic acid	04	03	09	02	09	00
Tartaric acid	04	03	06	10	09	00
4 amino butyric acid	02	03	09	10	04	01
n-butyric acid	04	03	06	12	10	01
Trans aconitic acid	00	00	00	01	00	00

* 0.1 % organic acids were used in M70 minimal medium. 00 : not utilized, N: total number of strains;
Standard strains** : *A. calcoaceticus* MTCC 1271 & 1425.

different enzymes (Table 6). Fig.3. shows chitinase enzyme production by *Acinetobacter* genospecies.

3.4.11. Antibiotic disc susceptibility of *Acinetobacter* spp. from rhizosphere of wheat :

Antibiotic disc susceptibility test carried out for these *Acinetobacter* strains showed maximum resistance to β-lactam antibiotics such as penicillin, ampicillin, ceftriaxone, cloxacillin, amoxicillin and cefuroxime than aminoglycoside antibiotic such as streptomycin, kanamycin, gentamycin, neomycin and amikacin. To the aminoglycoside antibiotics, moderate resistance was observed. In case of other antibiotics tested such as nalidixic acid, chloramphenicol, tetracycline and rifampicin, *Acinetobacter* was found sensitive to nalidixic acid, tetracycline and rifampicin but resistant to chloramphenicol.

3.4.12. Antibiotic resistance of *Acinetobacter* from rhizosphere of wheat :

MIC for different antibiotics revealed that resistance of *Acinetobacter* spp. to β-lactam antibiotic was high (MIC 512-1024 μg/ml). Among β-lactam antibiotics such as Pn, Cf, Ct, Cx, Ax and Ap, maximum resistance was observed to Ct and Pn (MIC 1024 μg/ml). Moderate resistance was shown by aminoglycoside antibiotics such as Kn, Sm, Am, Gm, Tb, and Am (MIC 28-512 μg/ml). In others such as Rif, Nal, Tc and Cm, *Acinetobacter* strains were sensitive to Rif, Nal and Tc (MIC 8-32 μg/ml) but resistant to Cm (MIC 1024μg/ml). These results matched perfectly with antibiotic susceptibility test. Fig. 4.A, B and C, shown the graphical representation of these results.

- **Metal resistance of rhizosphere *Acinetobacter* :**

Metal ions were divided into three groups on the basis of metal resistance of *Acinetobacter* spp. isolated from rhizosphere of wheat. Group I metal ions are those to which *Acinetobacter* spp. showed high resistance, this includes, Pb, Li, Te, Mg, B, Mi, W, and As^{4+} (MIC 512-1024μg/ml). Group II metal ions showed moderate resistance includes, Cd, Pt, Cu, Zn, Sb, Co and As^{3+} (MIC 28-512 μg/ml). Group III includes such metal ions to which all *Acinetobacter* spp. are sensitive and not grown at high concentrations, these includes, Hg, Ag and Au (MIC 8-32 μg/ml). Fig.4B. shows resistance of *Acinetobacter* spp. to tellurium (MIC 1024μg/ml). Fig. 5. A, B & C, shows the graphical representation of these results.

Table 6. Enzymatic activity of*Acinetobacter* genospecies isolated from rhizosphere of wheat *.

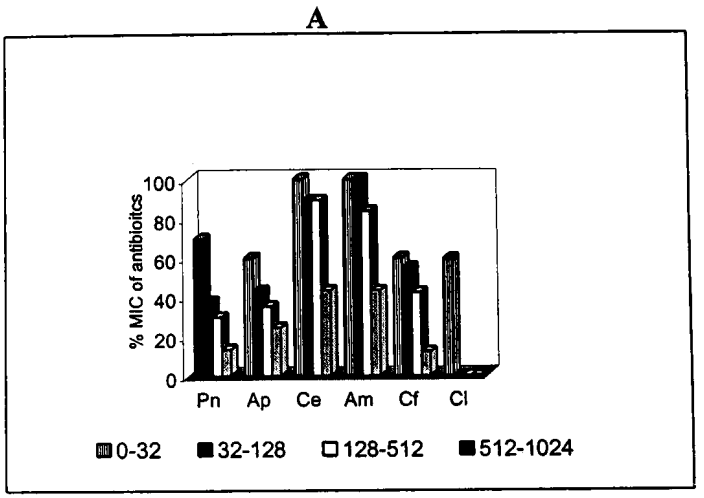
Enzymes tested	*Acinetobacter* genospecies					Standard Strain*
	A. calcoaceticus (N = 04)	*A.*genospecies3 (N = 03)	*A. baumannii* (N =09)	*A. haemolyticus* (N = 11)	*A junii* (N =10)	*A. calcoaceticus* (N =2)
Gelatinase	00	00	03	06	05	00
Lipase	02	00	04	04	06	01
Protease	00	00	03	03	02	01
Amylase	01	00	01	02	02	00
Protease	00	00	03	03	01	01
Amylase	01	00	01	02	02	00
Pectinase	01	00	01	01	01	00
Cellulase	00	00	00	00	00	00
Xylanase	00	00	01	02	00	00
Chitinase	00	00	01	02	01	00
Phosphatase	04	03	07	11	10	01
L-Asparginase	04	03	08	11	10	02
Urease	02	02	08	06	06	00
β- galactosidase	03	02	08	11	09	02
Oxidase	00	00	00	00	00	00
Catalase	04	03	08	11	10	02
Haemolysis	00	00	03	11	00	00

00 : not produced.

Standard strains ** : *A. calcoaceticus* MTCC 1271 & 1425.

Fig.4.A. Resistance of *Acinetobacter* spp. isolated from rhizosphere of wheat to different antibiotics.

A : Beta-lactam antibiotics.



B : Aminoglycoside antibiotics.

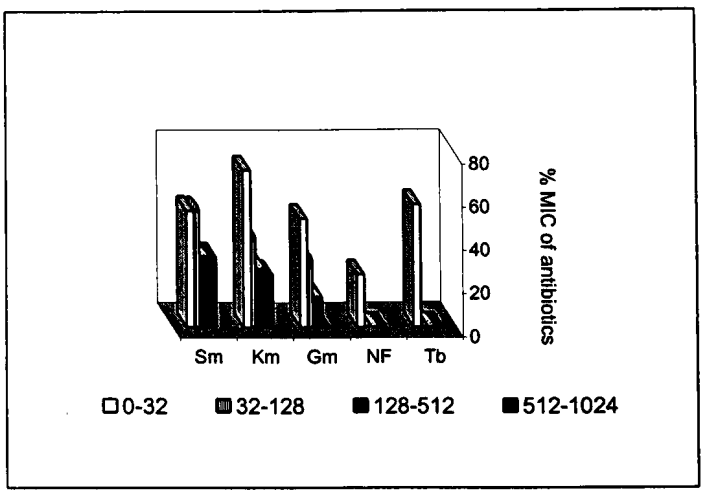


Fig.4. Resistance of *Acinetobacter* spp isolated from rhizosphere of wheat to different antibiotics.

C: Other antibiotics.

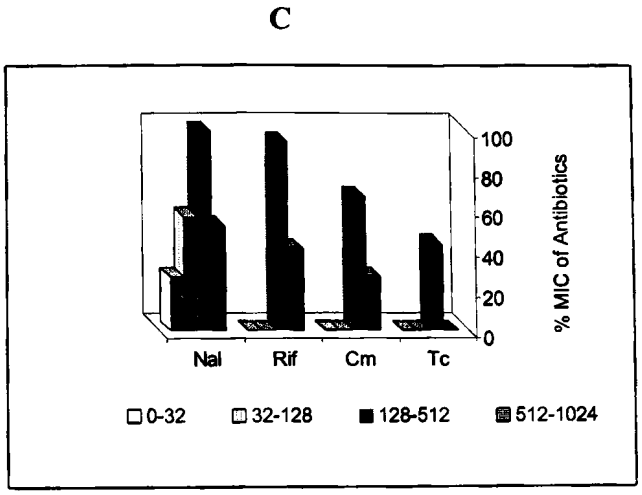


Fig. 5 B. Metal resistance of *Acinetobacter* spp. isolated from rhizosphere of wheat.

(Concentration in μg/ml).

A: Group-I metal ions.

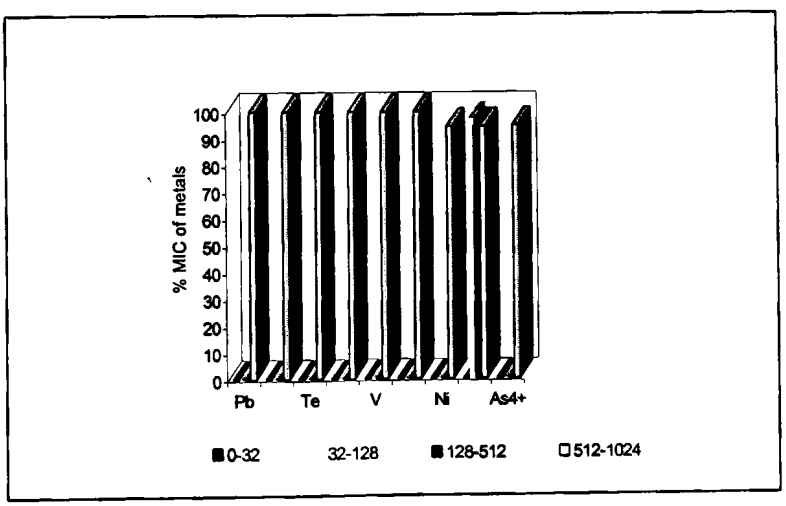
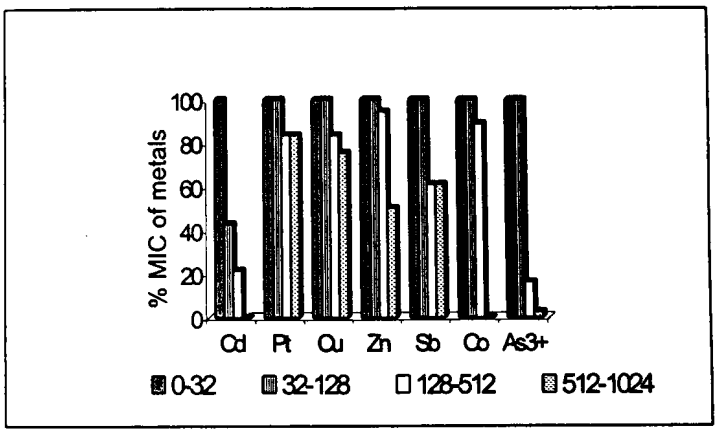


Fig.5B. Metal resistance of *Acinetobacter* spp. isolated from rhizosphere of wheat (concentration in μg/ml).

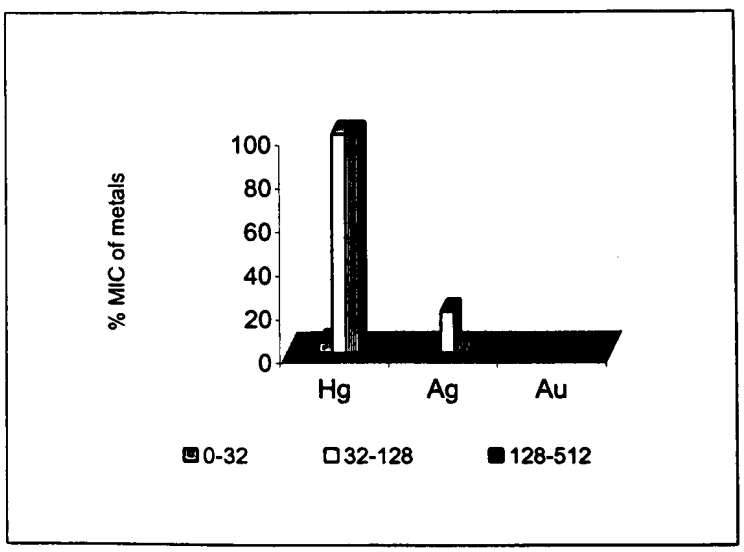
Resistance to B: Group- II metal ions

B



Resistance to C: group III metal ions.

C



3.5. Discussion :

There are number of reports on presence of *Acinetobacter* in soil (Bauman, 1968; Naik & Chopade, 1994) but its presence in the rhizosphere was neglected. There is only one passing report on presence of *Acinetobacter* in rhizosphere of wheat (Kleeberger, et al, 1983). Chapter 1, describes detailed distribution of *Acinetobacter* in rhizosphere of wheat and from this it is clear that *Acinetobacter* is second most dominant gram negative bacteria in the rhizosphere of wheat. From five preliminary tests, 45 tentative *Acinetobacter* spp. were isolated from three areas of wheat plant such as rhizoplane, rhizosphere and non-rhizosphere soil. These preliminary tests found reliable for identification of *Acinetobacter* spp. from other gram-negative bacteria from rhizosphere of wheat.

By employing chromosomal DNA transformation assay 37 *Acinetobacter,* were confirmed as genuine *Acinetobacter.* This assay is very specific for the identification of *Acinetobacter* genus discovered by Juni (1972). Chromosomal DNA transformation assay is based on the fact that *Acinetobacter* is naturally competent for genetic transformation and it has unique ability of chromosomal transformation within the genus only. In *Acinetobacter* an enzyme anthranilate synthetase, playing an important role in tryptophan operon, has a unique structure containing two sub units, a and b and both are required for functional enzyme (Crawford, 1980). No other bacterium in the microbial world has anthranilate synthetase having two subunits. The assay was done using naturally competent tryptophan auxotrophic mutant of *Acinetobacter calcoaceticus* BD413 trpE27. Therefore, the strains, which belong to genus *Acinetobacter*, could only able to transform the mutant to prototroph and could grow on *Acinetobacter* minimal medium (AMM) without tryptophan. Thus, 37 strains confirmed were able to transform trpE27 mutant to prototroph and were able to grow on AMM.

After confirmation of genus *Acinetobacter,* all 37 strains were studied for its biochemical characterization as per Bouvet & Grimont (1986 &1987). Biochemical characterization done by Bouvet & Grimont was of clinical and few soil isolates, and out of 299 only 253 strains were identified as *A. baumannii* by employing these biochemical tests. It was observed that in soil mostly *A. calcoaceticus* and *A. baumannii* were present, however from our results we noted that five genospecies of *Acinetobacter* were present in the rhizosphere soil and those include *A. calcoaceticus, A. baumannii, A.* genospecies 3, *A. junii* and *A. haemolyticus*. Percent distribution of these five strains showed number of *A. haemolyticus* (32.4 %) was maximum, next was *A. junii* (27.1 %) than *A. baumannii* (12.6 %), *A. calcoaceticus* (10.8 %) and *A.* genospecies 3 (8.1 %).

Biochemical test was showed positive results to all 37 strains those exhibit growth of all strains at 28, 37 and 41^{0}C, were able to grow on Simmon citrate medium, and all were able to produce pigment, all could utilize malonate, tween 20 and tween 80. Indole and Voges Proskauer

test (VP) were negative for all, could tolerate 2 % NaCl w/v and showed triple sugar ion test positive. These results were matched with previous findings of Bouvet & Grimont (1986 & 1987).

Members of genus *Acinetobacter* had been reported as nutritionally versatile (Baumann, 1968). This observation consistent with our results. Besides the comparison of all phenotypic and biochemical characteristics of *Acinetobacter* genospecies (Bouvet & Grimont 1986 & 1987; Dhakephalkar & Chopade,1994) all strains were tested for API 20NE microtube system for biotyping, but none of the strain was biotyped by this system. Rhizosphere *Acinetobacter* showed positive reactions for some of API 20 NE tests such as adipate, caprate, ONPG and NO_3. It should be noted here, that API 20 NE system is mainly composed for identification of clinical isolates and not for environmental (Towner & Chopade, 1987). Therefore here we observed variability in results.

Tolerance of different physico-chemical factors was tested for *Acinetobacter* strains isolated from rhizosphere of wheat. It was observed that 28^{0}C temperature and neutral to alkaline pH such as pH 7 to pH 10 was suitable pH for the growth of *Acinetobacter*. Temperature of rhizosphere was noted while sampling which was found to be in the range of 22-28^{0}C, because of this 28^{0}C may be the optimum temperature for growth but still all strains were able to grow up to 41^{0}C. The similar results were also observed by *K. pneumoniae* strains isolated from rhizosphere of rice (Rangarajan et al, 2001). They were also able to grow at 41^{0}C. As compared to this, it should be noted that only specific genospecies of *Acinetobacter* could grow at 41 and 44 ^{0}C, as this is one of the distinguishing characteristics of *Acinetobacter* genospecies (Bouvet & Grimont, 1986 & 1987). Soil analysis of the sampling field showed alkaline pH, thus these strains could grow at high alkaline pH such as pH 12. It was previously found that pH of the soil change because of influence of root exudates. (Rovira, 1965, 1969). This may be one of the reason for their growth at alkaline pH. It was found that aeration increased the growth of *Acinetobacter* spp. this showed highly aerobic nature of *Acinetobacter*.

It was observed that as NaCl concentration affect on growth of *Acinetobacter* and as the concentration increased the growth of *Acinetobacter* spp. decreased. It was observed that 2 % salt concentration is tolerated by all strains while 4 % NaCl concentration was tolerated by only 4 strains of *Acinetobacter*. Rangarajan et al (2002), reported that *Pseudomonas* spp. isolated from rice rhizosphere tested for tolerance of NaCl from 0.5 to 2 M. He observed that maximum 1.5 M NaCl was tolerated by *Pseudomonas* spp. however 2.0 M NaCl found inhibitory to all strains. Our results showed high tolerance to NaCl as compared to *Pseudomonas* spp. Thus it is more beneficial as these *Acinetobacter* spp. can be used successfully in the soil where NaCl concentration is high up to 2 % w/v and four of them can be used up to 4 % (w/v).

All 37 strains were isolated from rhizosphere of wheat and hence tested for utilization of different carbon , nitrogen and organic acids as these are the content of root exudates secreted by wheat plant (Subba Rao , 1986). Some amino acids are present in the root exudates of wheat plant such as leucine, valine, amino butyric acid, glutamine, alanine, aspargine, serine, glutamic acid, aspartic acid, cystine/ cysteine, glycine, phenylalanine, threonine, tyrosine and lysine. It was found that utilization of these amino acids was different among different species of *Acinetobacter*. One or other strain of *Acinetobacter* utilized maximum amino acids. All strains of *A. calcoaceticus* could utilize L- glutamic acid, valine, L- arginine, β-alanine, L- glycine and L- aspartic acid. All others were utilized by one or other strains of *A. calcoaceticus*. None of *A. calcoaceticus* strain could able to utilize phenylalanine.

All strains of *A.* genospecies 3 could utilize, valine, β-alanine, tyrosine, L-aspartic acid, phenylalanine, and L-histidine. Other amino acids were utilized by one or other strains of *A.* genospecies 3. None of *A.* genospecies 3 can utilize threonine. In case of *A. baumannii*, all strains could utilize L- arginine, L- glycine, and L- histidine. Other amino acids were utilized by one or other strains of *A. baumannii*. All strains of *A. haemolyticus* were utilized valine and L- arginine, others were utilized by one or other strains of *A. haemolyticus*. In case of *A. junii*, L- glutamic acid, valine, L- arginine, and L- threonine were utilized by all strains of *A. junii* however; others were utilized by one or other strains of *A. junii*.

If compare these results with four different *Pseudomonas* and *Enterobacter* spp. isolated from wheat and barley rhizosphere, Kleeberger, et al (1983) observed that pattern of utilization of these four strains for each amino acid is different. Amino acids utilized were DL- arginine, tryptophan, β-alanine, and L-valine. It was found that all these amino acids were utilized by *P. fluorescens. P. putida, P. stutzeri* and *Pseudomonas* spp. Tryptophan and valine was not utilized by *P. stutzeri* and *Pseudomonas* spp. and DL- arginine was not utilized by *Pseudomonas* spp. In case of *Enterobacter agglomerans* I, *Enterobacter agglomerans* II, *Enterobacter cloacae* and *Citrobacter freundii*, isolated from rhizosphere of wheat and barley, were unable to utilize lysine however, utilization of ornithine was observed by *E. agglomonas* I, *E. cloacae* and *Citrobacter freundii. Serratia* spp. were also found to utilize lysine as well as ornithine. Recently it was observed by Overbeek & Elsas (1995) that proline, a root exudate of wheat rhizosphere induced promoter activity in *P. fluorescens* mutants in wheat rhizosphere. Thus overall, it should be noted that utilization and presence of different amino acids as root exudates by plants influence rhizobacteria. It should be noted that we have tested broad range of amino acids than previously reported data and found that, compared to other rhizobacteria *Acinetobacter* spp. isolated from rhizosphere of wheat were able to utilize more amino acids excreted by wheat plant as a root exudates.

Different sugars, which are generally present in root exudates of wheat plant such as glucose, rhamnose, arabinose, xylose and raffinose (Subba Rao, 1986). Very interesting results were obtained about utilization of sugars. All strains of *A.* genospecies 3 were able to utilize all sugars. In case *A. calcoaceticus*, sucrose, lactose, rhamnose, D- mannitol, D-ribose, raffinose, galactose, and dulcitol were utilized by all *A. calcoaceticus* strains and other sugars were utilized by one or other strains of *A. calcoaceticus*. All *A. baumannii* strains could utilize sucrose, lactose, D-mannitol, D- maltose and raffinose, others were utilized by one or other strains of *A. baumannii*. All *A. haemolyticus* strains can utilize, sucrose, lactose, D- mannitol, D- xylose, D- maltose and arabinose, others were utilized by one or other strains of *A. haemolyticus*. In case of *A. junii*, all strains could utilize, sucrose, lactose, D- mannitol, D- xylose, and galactose, others were utilized by one or other strains of *A. junii*. Overall sucrose, lactose, and D- mannitol were utilized by all 37 strains of *Acinetobacter* isolated from rhizoplane, rhizosphere and non rhizosphere soil of wheat.

It is previously found that (Kleeberger, et al, 1983; Subba Rao,1986; Rangarajan, et al, 2001) *Pseudomonas* spp, *Enterobacter* spp and *Serratia* spp were able to utilize sugars as they are one of the important root exudates. Sugars utilized by them include sucrose, lactose, dulcitol, sorbitol, m-inositol, trehalose, glucose and arabinose. We have tested more number of sugars than previously reported and found that, *Acinetobacter* spp. isolated from rhizosphere of wheat were also able to utilize almost all sugars.

Different organic acids which are root exudates of wheat plant such as, tartaric acid, oxalic acid, malic acid, acetic acid, citric acid, succinic acid, fumaric, butyric, propionic and valeric acid (Subba Rao, 1986) were also tested for utilization. As compared to amino acid and sugar utilization, oxalic acid was the only organic acid, which was utilized by all 37 *Acinetobacter* genospecies. All of them utilized less organic acids and lactic acid was not utilized by any *Acinetobacter* genospecies. Organic acids utilized by all strains of *A. calcoaceticus* were oxalic acid, formic acid, tartaric acid and n-butyric acid. Citric acid, malic acid, phenylacetic acid, lactic acid, propionic acid, and transaconitic acid were not utilized by any *A. calcoaceticus* strains. All *A.* genospecies 3 strains could utilize oxalic acid, formic acid, tartaric acid, 4 aminobutyric acid and n butyric acid. None of *A.* genospecies 3 strain could utilize, citric acid, malic acid, lactic acid, acetic acid, propionic acid, and transaconitic acid. In case of *A. baumannii* all strains utilized, fumaric acid, oxalic acid, and 4 aminobutyric acid. Malic acid, lactic acid, acetic acid, propionic acid and trans aconitic acid were not utilized by any *A. baumannii* strain. All strains of *A. junii*, utilized oxalic acid and n-butyric acid. Lactic acid and trans aconitic acid were not utilized by any *A. junii* strain. There are passing reports stated that all rhizobacteria can utilize organic acids (Subba Rao, 1986; Rovira & Harris, 1961, Rangarajan, et al, 2001) but no detailed study on organic acid utilization by rhizobacteria was done. However, there was no detailed study on role of utilization of

organic acid by rhizobacteria was done. Thus organic acids utilization study for rhizosphere *Acinetobacter* was carried out by us for the first time, no report is present on this type of study.

It was previously noted that, carbon exudates from plants have importance as energy substrates for microbial colonization (Subba Rao, 1986). It was also proved that root exudates are complex mixtures of many compounds that vary to some extent with plant species, plant age, physical stress and biological stress (Franzel, 1960; Rovira, 1969; Gaskin et al, 1984). Simple sugars have been reported frequently in root exudates and in rhizosphere soil (Schonwitz & Ziegler, 1982; Bachmann & Kinzel, 1992). It was also proved that microbes increased root exudation and root growth (Rovira, 1962 & 1965a; Gardner et al, 1983; Scher et al, 1985). Secondary chemical modification of root exudates within the rhizosphere undoubtedly helps to determine species composition of microbial communities. Recently it was observed by Overbeek & Elsas (1995) that proline, an amino acid acts as an inducer, for the promoter activity of *P. fluorescens* in wheat rhizosphere. Thus in view of this background, utilization pattern for root exudates by *Acinetobacter*, may help to clear the role of root exudates on growth pattern of rhizobacteria.

Fifteen different enzymes were detected for their production by *Acinetobacter* from rhizosphere. It was observed that all 37 *Acinetobacter* showed oxidase enzyme test negative, which supports to its genus characterization. All *A. calcoaceticus* strains showed catalase, urease, phosphatase and L-asparginase enzyme test positive. None of the strain produced cellulase. In case of amylase enzymes, 1 *A. calcoaceticus*, 1 *A. baumannii*, 2 *A. haemolyticus* and 2 *A. junii* produced amylase. In the previous investigation, Bouvet & Grimont (1986 & 1987) reported that out of 49 strains tested, no strain produced amylase, however, Dhakephalkar & Chopade (1994) reported that 73 % isolates of *Acinetobacter* were produced amylases.

In our study we found that out of 37, 4 strains of *Acinetobacter* species were showed chitinase production. Chitinase is one of the important enzyme produced by rhizobacteria mainly *Pseudomonas*, *Serratia* spp., *Enterobacter* spp. and has important role in biological control (Gaffney et al, 1994; Chernin, et al, 1995; Chet, 1969).

It was reported by Vernica (1969), that soil microorganisms such as *Serratia*, *Pseudomonas* and *Enterobacter* spp. were able to produce phosphatase , lipase, urease, protease, oxidase, catalase and chitinase. In our study we found that 17 strains out of 37 can produce lipases, which has ability to act on interfaces. All other enzymes were detected from one or other species of *Acinetobacter*. There were no detailed enzymatic studies were carried out on rhizosphere *Acinetobacter*. However, this is the first report on enzymatic studies of *Acinetobacter* from rhizosphere of wheat. From above results it can be concluded that maximum enzymes were detected from different *Acinetobacter* species may also possess genes, encoding for different

enzymes like lipase, protease, chitinase, gelatinase, nitrate reductase, phosphatase, L-asparginase, pectinase, xylanase, β-galactosidase, DNAse and urease. Presence of genes encoding for these enzymes enable *Acinetobacter* spp. to utilize wide array of compounds and can protect the bacteria from different pathogens and helps the bacteria to exist in adverse conditions and become dominant bacterial flora of rhizosphere of wheat as rhizosphere is the most competitive environment. Thus overall, *Acinetobacter* from rhizosphere of wheat has versatile nature for utilization of nutrients. It utilized maximum sugars and amino-acids. It is also able to utilize different organic acid. These bacteria produced different enzymes. Thus, these all test showed ability of this bacterium to become one of the dominant bacteria in the rhizosphere of wheat.

Antibiotic susceptibility test and MIC carried out for different antibiotics revealed that, most of the *Acinetobacter* strains were found to be resistant to β-lactam antibiotics. However moderate type of resistance was exhibited to the all aminoglycoside antibiotics. The majority of isolates were sensitive to the other antibiotics except chloramphenicol. Rifampicin and nalidixic acid were the most toxic antibiotics, inhibiting growth of all *Acinetobacter* strains even at low concentrations. The similar observations were found to be with environmental isolates by Dhakephalkar & Chopade (1994); Deshapande et al (1994); Shakibaie, et al, (1999). Recently antibiotic resistance of rhizosphere *Pseudomonas* spp. was studied by Rangarajan (2002). Antibiotics used were ampicillin, chloramphenicol carbenicillin, erythromycin, kanamycin, rifampicin, streptomycin, trimethoprim and tetracycline. It was found that most of the strains were resistant to erythromycin, ampicillin, carbenicillin and trimethoprim and sensitive to chloramphenicol and rifampicin. The contrast result was observed by Dhakephalkar & Chopade (1994), they observed that all soil isolates exhibited resistance to chloramphenicol. Thus our result perfectly matched with the previous results of Dhakephalkar & Chopade (1994).

We observed that *Acinetobacter* spp. isolated from rhizosphere of wheat was found to high level of tolerance to different heavy metals. 100 % rhizosphere isolates showed resistance to metal ions of group -I. 60-90 % of isolates displayed high resistance level to group II metal ions. All isolates were sensitive to group -III metal ions. Burd et al (1998), observed that *Pseudomonas* spp., were genetically modified and resistant to high concentrations of arsenate, arsenite, nickel, cobalt and zinc, which are used in bioremediation. If we compared these *Acinetobacter* spp. for the heavy metal resistance it is very clear that they can play important role in bioremediation and in removal of environmental pollution. The important point should be noted that, there was no need of genetic modification for these strains. This high resistance exhibited by these *Acinetobacter* spp may be because; heavy metals are ubiquitous in nature in wide range of concentrations (Dhakephalkar & Chopade, 1994). Some metal ions are essential for the growth of microorganisms, while others are tolerated at extremely low concentrations. At high concentrations

they proved to be toxic because they denature structural and functional proteins (Gadd & Griffiths, 1978; Meers & Chow, 1990). They have potential to influence the economy of microorganisms but as pollutants most can severely disrupt ecosystem functioning. However, the demarcation between essentiality and toxicity is not distinct and usually depends upon active concentrations of heavy metals that is assessable to biological systems, which ultimately may become a threat to public health (Rai, et al, 1981; Bergogne & Towner, 1996). All the *Acinetobacter* spp. tested in present investigation could produce exopolysaccharides. These exopolysaccharides may trap the metal ions and prevent them from entering in to the cells and thereby rendering them harmless to the cell. This property may have potential biological application for the *Acinetobacter* spp. in removal of metals ions from polluted environments. Therefore it has to be investigated, from where these isolates have acquired the gene/s encoding antibiotic and metal resistance. The interesting result from this study is, high level of metal resistance shown by rhizosphere *Acinetobacter* spp than antibiotics.

3.6. Conclusions :

- Five different genospecies such as *A. calcoaceticus*, *A.* genospecies 3, *A. baumannii*, *A. haemolyticus* and *A. junii* were in rhizosphere of wheat.
- *Acinetobacter* spp. from rhizosphere of wheat were able to utilize most of the amino acids, sugars and organic acids excreted by wheat plant similar to root exudates.
- Enzymes produced by *Acinetobacter* isolated from rhizosphere of wheat were phosphatase, L-asparginase, pectinase, chitinase, protease, xylanase, β-galactosidase and amylase.
- *Acinetobacter* from rhizosphere of wheat exhibit high metal and antibiotic resistance.

3.7. Significance of this work :

- *Acinetobacter* is one of the important rhizobacteria present in the rhizosphere of wheat. It has ability to utilize different amino acids, sugars and organic acids as well as able to produce enzymes which indicated that it plays an important role in rhizosphere of wheat. *Acinetobacter* spp. Isolated from rhizosphere of wheat exhibited high antibiotic and metal resistance and can be useful in the study of gene transfer of *Acinetobacter* to other microorganisms. Studies on *Acinetobacter* from different rhizosphere will improve our knowledge of ecology of *Acinetobacter* in rhizosphere.

3.6. References :

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CHAPTER 4

Plasmid pUPI126 Encoded Broad Spectrum Antimicrobial Activity Exhibited by Pyrrolnitrin Antibiotic Produced by *Acinetobacter haemolyticus* A19 Isolated from Rhizosphere of Wheat.

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Plasmid pUPI126 Encoded Broad Spectrum Antimicrobial Activity Exhibited by Pyrrolnitrin Antibiotic Produced by *Acinetobacter haemolyticus* A19 Isolated from Rhizosphere of Wheat.

4.1. Abstract

Acinetobacter spp. identified as *A. haemolyticus* A19 showed antibiotic as well as chitinase production. Antibiotic produced by *A. haemolyticus* A19 was inducible by co-cultivation with *K. pneumoniae* with an optimum proportion of 2:1 and was extra cellular. pH 7, 28^{0}C temperature and 2 % of NaCl (w/v) were the most suitable environmental conditions for the production as well as activity of antibiotic. Antibiotic was produced at early stationary growth phase (48 h) of *A. haemolyticus* A19. It has a very broad spectrum of activity and showed antimicrobial activity against plant as well as human pathogenic bacteria and fungi. Antibiotic was purified by extracting with ethylacetate followed by column chromatography. Further purification was done by preparative thin layer chromatography. Yield of antibiotic was found to be 15 mg/lit. The antibiotic was active at very low concentrations such as 50 μg /ml and is water soluble. It is stable at room temperature up to 7days. ^{1}H-NMR analysis revealed this antibiotic as a pyrrolnitrin. It was found that pyrrolnitrin production in *A. haemolyticus* A19 was encoded by plasmid pUPI126 of molecular weight 40 Kb. Plasmid pUPI126 was self transformed to *E. coli* HB101 at a frequency of 5 x 10^{-5} per μg of DNA. It is also conjugally transformed to *E. coli* HB101 rif^r mutants at a frequency of 5.9 x 10^{-8} per recipient cells. Plasmid pUPI126 was 100 % stable in *Acinetobacter* and 95 % in *E. coli* HB101. Transconjugants as well as transformants showed antibiotic production. This is the first report of plasmid mediated pyrrolnitrin production in the genus *Acinetobacter.*

Key Words : *A. haemolyticus*, Rhizosphere of Wheat, Pyrrolnitrin, Plasmid pUPI126, Broad antimicrobial spectrum, ^{1}H-NMR, Stability of plasmid pUPI126, Conjugation, Transformation, Chitinase

4.2. Introduction :

Rhizosphere is a complex environment where number of interactions between plant and microorganisms takes place (Subba Rao, 1986). Recently ability of certain antagonistic bacteria to protect plants from soil borne fungal pathogens is well established (Howell & Stipanovic, 1980; Keel, et al, 1990; Schippers et al, 1987; Schroth & Hancock, 1982; Weller, 1988). Biocontrol is strongly correlated with the production by the bacterial antagonists of fungal factors such as antibiotics, hydrolytic enzymes and siderophores (Bakker et al. 1986; Howell & Stipanovic, 1980; Keel, et al, 1990; Thomashow & Weller, 1988; Vincent et al,1991). Number of rhizobacteria such as *Pseudomonas, Serratia, Burkholderia* exhibited antagonistic against phytophatogenic bacteria and fungi due to the production of secondary metabolites showing strong antibiotic activity (Keel, et al, 1989 & 1992; Burkhed, et al, 1994; Gaffeny, et al , 1994; Itoh et al, 1992 & 1994; Sfalanga, et al, 1999) . Many *Pseudomonas* species are able to produce antibiotics such as Phenazine, 2-4-diacetylphlorouglucinol, oomycin, pyoluteorin and pyrrolnitrin (Arima et al, 1964; Thomashow &

Weller, 1988;Keel, et al, 1989 & 1992; Gutterson et al, 1986; 1990; Howell & Stipanovic, 1980; Roitman, et al. 1990; Hill et al, 1994). One of these antibiotics is pyrrolnitrin or 3-chloro-4-[2'-nitro-3'- chloropheny] pyrrol (Hammer, et al, 1997;Chernin et al 1996; Arima et al, 1964).

Pyrrolnitrin is mainly an antifungal antibiotic produced by soil or rhizosphere bacteria such as *P. fluorescens* , *P. putida*, *P. cepacia* , *P. pyrrolnitri and Burkholderia cepacia* (Burkhed, et al, 1994; Gaffney, et al , 1992; Itoh et al, 1992 & 1994; Sfalanga, et al, 1999). This antibiotic was first discovered from *Streptomyces pyrrocinia* (Di Santo, et al, 1998). Pyrrolnitrin also shows activity against some plant pathogenic bacteria such as *Xanthomonas campestris; P. syringae; Corynebacterium insidiosum; Clavibacterium michiganense; Erwinia carotovora* and *Serratia marcescens* (Chernin, et al, 1996). Later on derivatives of pyrrolnitrin have been discovered such as 1,5 diarylpyrrol, 1,4 diarylpyrrol, bromoderivaties of pyrrolnitrin. (Scalzo, et al ,1988; Porreta et al, 1985 & 1991; van Pee & Ligon, 1998 & 2000). These derivatives exhibit antibacterial activity. This antibiotic is mainly against plant pathogen and used as a biocontrol agent for plant diseases (Duffy & Defago 1999). Recently this antibiotic and its derivatives showed antibacterial activity against *Mycobacterium tuberculosis* and *M. avim* (Di Santo et al, 1998). It shall be noted that *Mycobacterium* is one of the most resistant pathogen to most of the antibiotics and causes tuberculosis in humans. Mainly arylpyrrole derivatives and pyrrolnitrin showed appreciable inhibiting activity against these two species of *Mycobacterium.* Antimycobacterial potency correlate with presence of halogens in the phenyl ring and nitro group at position 3 of pyrrole (Di Santo et al, 1998).

Acinetobacter is ubiquitous in nature and found in soil, water, food, and on skin of healthy humans (Baumann,1968, Saha & Chopade 2001, Patil & Chopade 2001). It is a gram negative, oxidase negative, catalase positive, non-motile and capsulated coccobacilli. *Acinetobacter* is known as a important nosocromial human pathogen (Chopade et al, 1994, Deshpande & Chopade, 1994, Shakibaie et al, 1999) and show high degree of metal and antibiotic resistance. The antibiotic produced by *Acinetobacter haemolyticus* A19, isolated from rhizosphere of winter wheat (HD 2189), also produce indole-3 acetic acid. It is known that this antibiotic has been found in various *Pseudomonas* species as one of the secondary products of tryptophan metabolism (Roitman et al, 1990a).

The present chapter describes the pyrrolnitrin production by *A. haemolyticus* A19, its inducible nature, purification and its broad-spectrum antimicrobial activity. The producer strain and purified antibiotic have been shown to be very effective as antagonists in vitro against many fungi, bacteria and yeast. The important observation about this antibiotic is that antibiotic production is plasmid pUPI126 encoded which is self-transferable *to E. coli* HB101.

4.3. Materials and Methods

4.3.1. Bacterial strains used :

Acinetobacter spp. isolated from rhizosphere of wheat and identified as *A. haemolyticus* A19 was used and other standard fungal and bacterial pathogens used included : *E. coli* NCIM 2810; *S. typhimurium* NCIM 2501; *K. pneumoniae* NCIM 2719; *P. vulgaris* NCIM 2027, *S. dysentery* type II; *V. cholerae* (AFMC, Pune); *P. aeruginosa* NCIM 2036; *Xanthomonas campestris* NCIM 2954; *Xanthomonas malvacerum* NCIM 2310; *Enterobacter aerogenes* NCIM 2340; *P. syringae* NCIM 5102; *Alternaria solani* NCIM 887; *Alternaria* spp NCIM 1280; *Heliminthosporium* spp. NCIM 1079; *Ustilago maydis* NCIM 895; *Rhizoctonia solani* 1060; *Fusarium* spp. NCIM 895; *Fusarium oxysporium* NCIM 1072; *Fusarium moniliforme* NCIM 1100; *Fusarium proliferatum* NCIM 1102; *Aspergillus parasiticus* 898, *Trichoderma* spp. NCIM 1059; *T. viride* NCIM 1195; *Cladosporium herbarium* NCIM 1112; *Cladosporium* spp NCIM 1082; *Candida albicans* (B.J. M. C., Pune); *Cryptococcus neoformans* (AFMC, Pune).

4.3.2. Isolation of *Acinetobacter* spp. from rhizosphere of wheat :

Acinetobacter spp. was isolated as described in chapter 2 (section 2.3.4 &in chapter 3, section 3.4).

4.3.3. Detection of antimicrobial spectrum of antibiotic produced by *Acinetobacter haemolyticus* A19:

Acinetobacter haemolyticus A19 was first tested for chitinase activity (Chernin et al., 1995). After chitinase test, *A. haemolyticus* A19 was tested for antibiotic production by agar well method on Luria agar. Number of plant and human pathogenic bacteria were tested against *A. haemolyticus* A19. The plates were incubated at 28^{0}C for 24 h, and cell suspension was prepared in 0.85 % saline and O.D. was adjusted to 0.1 at 660 nm. The sensitive strains of bacteria were spread on Luria agar and fungi on potato dextrose agar. O. D. of sensitive strains was adjusted to 0.05 at 660 nm. Agar well method was used to detect the antimicrobial activity. Standard strains used for checking antimicrobial activity are described in section 4.3.1.Plates were incubated at 28^{0}C for 24 h for bacterial cultures and 96 h for fungal cultures. Zone of inhibition was measured in cm.

4.3.4. Detection of siderophore production :

To confirm that the compound produced by *A. haemolyticus* A19 was indeed an antibiotic, siderophore production was tested. Siderophore production was detected by 3 chemical assays : i.) Ferric perchlorate assay ii.) Csaky assay and iii.) Arnow assay.*A. haemolyticus* A19 was grown in Luria broth for 24-48 h at 28^{0}C and the culture supernatant was used for siderophore detection as described below :

4.3.4.1. Ferric perchlorate Assay : 0.5 ml of culture supernatant was mixed with 2.5 ml of ferric perchlorate reagent. (5 mM Fe $(ClO_4)_3$ in 0.1 M $HClO_4$). Orange to purple color was developed. The absorbance was measured at appropriate wavelength such as 526 nm (Payne, 1994).

4.3.4.2. **Csaky Assay :** This assay was done as per the method described by Payne, (1994). In brief : 1ml culture supernatant (18 h) of *A. haemolyticus* A19 was hydrolyzed with 6N H_2SO_4 in boiling water bath for 6 h or at 130^{0}C for 30 min. Solution was then buffered by adding 3 ml of 3.5 % sodium acetate solution followed by addition of 1ml sulfanilic acid solution (1.3 g sulfanilic acid dissolved by heating in 100 ml 30 % acetic acid followed by 0.5 ml iodine solution). After 3-5 min., excess iodine was removed with 1 ml sodium arsenite solution (2g Na_3ASO_4 in 100 ml distilled water) and 1ml á naphthylamine solution (3g á naphthylamine dissolved in 100 ml of 30 % acetic acid) was added. Water was added up to 10 ml and allowed to develop colour for 20-30 min. Purple color was considered as a positive result. Absorbance was measured at 526 nm. (Payne, 1994).

4.3.4.3. **Arnow Assay :** 1 ml of culture supernatant (18 h) was mixed as follows : 1ml HCl (0.5N) + 1 ml nitratemolybdate (10 g sodium nitrate and 10 g sodium molybdate dissolved in 100 ml of water) produced yellow color, addition of 1ml of 1 N NaOH, color changed to red which was stable at least 1 h. Absorbance was measured at 510 nm. (Payne, 1994).

4.3.5. **Detection of acid production :**

Acid production was tested as per method described by Casida (1988) using Luria agar. The plates were incubated at 28 ^{0}C for 48 to 72 h and observed for zone of inhibition in cm.

4.3.6. **Time course of antibiotic production :**

Time course for antibiotic production was carried out in 100 ml of Luria broth in 250 ml conical flask at 28^{0}C with shaking at 150 rpm. *A. haemolyticus* A19 was grown in the same medium and 1ml of 12 h culture (O.D. 0.1 at 660 nm) was inoculated into the growth medium. Samples were tested for antibiotic production after each 6 h interval by employing agar well method. Growth of bacteria was measured at 660 nm and zone of inhibition was measured in cm after each 6 h.

4.3.7. **Extra and intra cellular antibiotic production :**

Following experiment checked the extra cellular or intra cellular antibiotic production. *A. haemolyticus* A19 was grown in 50 ml of Luria broth , after 48 h the broth was centrifuged at 10,000 rpm for 10 min at 4^{0}C and supernatant as well as cell pellet was checked for antimicrobial activity by agar well method. Plates were incubated at 28^{0}C for 24–48 h. After 48 h, plates were observed for zone of inhibition. To check the intracellular nature of antibiotic, the cells were grown in LB broth for 18 h. After 18 h, cells were subjected for lysis by 1% SDS and cells were also lysed by ultrasonicator (REMI, 450) at different time intervals such as from 5 seconds to 15 min. Cell lysis was confirmed by employing protein estimation as per Lowry et al (1951). After the treatment with 1% SDS, and ultrasonicated cell suspension was centrifuged at 10,000 rpm for 10 min at 4^{0}C and supernatant was tested for antimicrobial activity by agar well method.

4.3.8. **Effect of different environmental factors on antibiotic production :**

Antibiotic production by *A. haemolyticus* A19 was tested for effect of different physico-chemical factors as described below :

4.3.8.1. Effect of pH : Effect of pH on antibiotic production was tested within the range of pH 5,6,7,8,9,10,11 and 12. Luria agar was adjusted to the different pH as above and inoculated with 12 h culture of *A. haemolyticus* A19, against sensitive strains and agar well method was used. Plates were incubated at 28°C and after 48 h, plates were observed for zone of inhibition, which was measured in cm.

4.3.8.2. Effect of temperature : To observe the effect of different temperatures on antibiotic production, Luria agar plates were inoculated with 12 h culture with producer and sensitive bacteria by agar well method. Plates were incubated at different temperature such as 10, 28, 37, 44 and 50°C. After 48 h incubation at above temperatures, plates were observed for zone of inhibition, which was measured in cm.

4.3.8.4. Effect of salt concentration : Effect of salt concentration was carried out by using different concentration of NaCl such as 2,3,4 and 5 % (w/v) in Luria agar. Plates were inoculated by 12 h old culture with producer and sensitive bacteria by agar well method. Plates were incubated at 28°C. After 48 h incubation, plates were observed for zone of inhibition, which was measured in cm.

4.3.9. Induction of antibiotic by co-cultivation :

A. haemolyticus A19 was inoculated with a sensitive bacteria *K. pneumoniae* NCIM 2719 in different proportion such as 2:1, 1:1 and1:3 proportions in 250 ml Luria broth in 500 ml conical flasks. Flasks were incubated at 28°C at 150 rpm. for 24-48 h. After 48 h, cells were separated by centrifugation at 10,000 rpm for 20 min. at 28°C and supernatant as well as cell pellet was checked for antibacterial activity by agar well method. *A. haemolyticus* A19 and *K. pneumoniae* NCIM 2719 were inoculated separately and used as controls. Plates were incubated at 28°C for 24-48 h. After 48 h plates were observed for zone of inhibition, which was measured in cm.

4.3.10. Extraction of antibiotic :

As the antibiotic produced by *A. haemolyticus* A19 was inducible in nature, *A. haemolyticus* A19, and *K. pneumoniae* NCIM 2719 were inoculated in 100 ml of Luria broth in 2:1 proportion respectively, in 250 ml conical flask. Flask was incubated at 28°C for 24-48 h at 150 rpm. After 48 h, 1000 ml broth was centrifuged (Remi, RM12C, India) at 10,000 rpm for 15 min. at 28°C to remove the cells. After centrifugation, supernatant containing antibiotic was extracted with ethyl acetate in 1:1 proportion for three times. pH was adjusted to 7 at each step of extraction. After extraction, the organic phase was collected and ethyl acetate was removed by subjecting the sample to rota evaporation at 40°C and 190 rpm for 1 h (Buchi, Switzerland). The concentrated residue (20mg) was collected and dissolved in 2 ml of ethyl acetate. This residue was tested for antimicrobial activity against sensitive bacteria and fungi by agar well method. Plates were incubated at 28°C and observed for zone of inhibition which was measured in cm.

4.3.11. Purification of antibiotic :

Column chromatography and preparative TLC of the extracted residue was carried out for purification of antibiotic. This is described as follows:

4.3.11.1. Separation of compounds in residue by column chromatography :

5 ml of residue obtained by extraction was loaded on a column of silica gel G (35cm x 1.5 cm, glass) and the column chromatography was carried out in chloroform and methanol system (90:10). After each 5 min. samples were collected separately and for each sample TLC was carried out by using thin layer chromatography plates (Merck, 60 gel). TLC was carried out in chloroform methanol system (10:1). The bands were observed for single spot under UV at 270 & 336 nm and or by spraying iodine which gives yellow color spot (Koga et al, 1991).

4.3.11.2. Preparative thin layer chromatography :

Preparative TLC was carried out on silica plate (200 x 200 cm) obtained from Merck, India. The purified sample obtained from column chromatography was loaded on the TLC plate. The TLC was carried out in chloroform : methanol system (90:10) and the plate was observed for band under UV at 270 and 336 wavelength. R_f value was measured and the band was eluted and purified by filtration. The pure compound then evaporated on rota evaporator (Buchi, Switzerland) under vacuum pressure for 6-8 h. The powder obtained after purification was again tested for TLC to check purity of compound. The purified sample was subjected to ^{1}H- NMR for the structural analysis.

4.3.12. Structural analysis of antibiotic by ^{1}H-NMR :

The pure antibiotic sample was subjected to ^{1}H- NMR for its structural analysis. 5 mg of the antibiotic sample was dissolved in 2-3 ml of $CdCl_3$ and analyzed by mercury ^{1}H- NMR (300 MHZ, Vavion, USA) and peaks were identified for antibiotic. The purified antibiotic was tested for the antimicrobial activity against range of bacteria as well as fungi.

4.3.13. Plasmid isolation and agarose gel electrophoresis :

To observe presence of plasmid/s in *A. haemolyticus* A19, plasmid isolation was carried out by method of Kado & Liu, (1980) and also by Sambrook et al (1989). 0.7 % agarose gel electrophoresis in TAE buffer was carried out. 1Kb DNA ladder was used as a standard. Gel was stained with 0.05 lg/ml of ethidium bromide. Plasmid bands were observed under gel documentation system (Alpha Imager, 2000, Alpha Innotech Inc., USA).

4.3.14. Conjugal transfer of plasmid pUPI126 to *E.coli* HB101 :

Conjugation between *A. haemolyticus* A19 (donor) and rif^r mutant of *E. coli* HB101(recipient) was carried out by the membrane filter technique (Deshpande & Chopade, 1994). Overnight cultures of donor (2 ml, 1:10 diluted) and recipient (2 ml) were mixed and filtered on to a Sartorious membrane filter (Germany, 0.45μM) and incubated on Luria agar plates at 37^{0}C for 24 h. Growth was resuspended in 2 ml of 0.85 % saline serially diluted and 100μl of each dilutions was spread

on Luria agar plates containing 100µg/ml of tellurium, 100µg/ml of rifampicin and 100µg/ml of chloramphenicol. The plates were incubated at 37°C up to 3-4 days and observed for growth of transconjugants. The transconjugants were selected, purified and were tested for antibiotic production against bacteria by agar spot method. Plates were incubated at 28°C for 24 h and observed for zone of inhibition.

4.3.15. Transformation of plasmid pUPI126 to *E. coli* HB101 :

Plasmid DNA from *A. haemolyticus* A19 was isolated by employing various methods. The quantification of pure DNA was done by employing absorbance spectra at 260/280 nm by spectrophotometer (Shimadzu, Japan) and by standard high DNA mass ladder (GIBCO, BRL).

- **Preparation of Competent cells :**

The transformation was carried out by preparing competent cells of *E. coli* HB101 rifampicin resistance mutant using $CaCl_2$-$MgCl_2$ method as described by Sambrook, et al (1989). In brief : 3 ml of ml overnight grown culture of recipient strain in LB broth (as a standing culture) at 37 °C was used. This culture was diluted to 1 :10 in sterile LB broth. Transferred the early-log-phase culture to a chilled centrifuge tube. Kept chilled with crushed ice for 10 min. Centrifuged at 10000 rpm at 4°C for 10 min. Supernatant was removed and pellet was added in to 5 ml ice-cold 10mM Tris –HCl (pH 7.6) and 50 mM $CaCl_2$. The suspension was held in ice for 15 min and than centrifuge at 10000 rpm for 10 min at 4 °C. Supernatant was removed and the prepared competent cells were suspended in 10mM Tris–HCl (pH 7.6) and 50mM $CaCl_2$ buffer and used immediately for transformation.

- **Transformation of plasmid pUPI126 to *E. coli* HB101 :**

In brief :1 µg (50 µl) of plasmid DNA was mixed with 100 µl of competent *E. coli* HB101 cells (10^8 cells/ml) in a 1.5 ml of microfuge tube and incubated on ice for 30 min. The microfuge tube was immediately transferred to water bath adjusted to 42° C for 2-3 min. and immediately kept on ice for 5 min. Finally 850 µl of double strength LB broth was added to above suspension and incubated at 37 °C for 1h in a water bath. Cultures were serially diluted into sterile Luria broth (upto 10^{-8}) and 100 µl of appropriate dilutions including undiluted solution was spread on selective LB agar medium supplemented with 100µg/ml of chloramphenicol, and 100µg/ml of tellurium. The plates were incubated at 37°C for 24-48 h. The number of transformant colonies were counted and transformation frequency was determined by calculating number of colonies grown on selective agar medium (as transformants) as per recipient or number of transformants per µg of DNA. At the same time competent cells without plasmid DNA was used as control and plated on selective and non selective agar medium. Te^r and Cm^r were selected as markers for transformation. Transformants were purified and presence of plasmid pUPI126 was confirmed by plasmid isolation and agarose gel electrophoresis (Sambrook, et al, 1989).

4.3.16. Stability of plasmid pUPI126:

Stability of plasmid pUPI126 in *E. coli* and *Acinetobacter* was determined as per method described by Chopade, et al (1985). In brief : *Acinetobacter haemolyticus* A19, transformants and transconjugants were grown in LB and LB with potassium telluride (100µg/ml) and chloramphenicol (100µg/ml) separately. Overnight grown culture was serially diluted and 100µl of each dilution was plated on plain LB agar and LB containing potassium telluride or chloramphenicol or LB containing both. Isolated colonies were checked for production of an antibiotic compound.

4.4. Results :

4.4.1. Isolation and characterization of *Acinetobacter haemolyticus* A19 from rhizosphere of wheat:

A strain isolated from rhizosphere of wheat showed Gram negative, nonmotile, oxidase negative, catalase positive, capsulated, coccobacilli characters was considered as tentative *Acinetobacter* spp. Chromosomal DNA transformation assay for this strain showed positive results confirmed that the strain belonging to genus *Acinetobacter*. This strain was identified as *A. haemolyticus* A19 by Bouvet & Grimont (1986 & 1987) system. Biochemical characteristics of *A. haemolyticus* A19 are shown in Table 1.

4.4.2. Antibiotic production by *Acinetobacter haemolyticus* A19 isolated from rhizosphere of wheat:

A. haemolyticus A19 exhibited good antifungal as well as antibacterial activity and also chitinase activity. The spectrum of antibiotic produced by *A. haemolyticus* A19 is shown in Table 2. It was found that spectrum of antibiotic is very broad as it includes human and plant pathogenic bacteria as well as fungi. This is interesting to note that the bacteria isolated from rhizosphere exhibit antibiotic producing ability against human pathogenic bacteria and fungi in addition to plant pathogenic bacteria and fungi (Fig. 1A & B).

Siderophore production in *A. haemolyticus* A19 was tested to confirm whether the antimicrobial activity exhibited by the bacteria is indeed because of antibiotic not by siderophore. Siderophore production checked by ferric perchlorate assay, Csaky and Arnow assaygave negative results indicating absence of siderophore production in *A. haemolyticus* A19. Some bacteria can produce acid and inhibit the growth of other bacteria. Acid production test also showed negative result in *A. haemolyticus* A19. Hence it was confirmed that the inhibition exhibited by *A. haemolyticus* A19 was neither because of acid production nor by siderophore. Thus these results indeed supports the antibiotic production by *A. haemolyticus* A19.

4.4.3. Time course of antibiotic production :

Time course for antibiotic production was observed throughout the growth phase of the organism, which showed that maximum antibiotic, was produced at (48 h) stationary phase. Fig. 6 shows the

time course of antibiotic production and growth phase of *A. haemolyticus* A19. It is clear from Fig. 6, that antibiotic production started from 6 h onward which corresponds to log phase and it slowly increased up to 48 h and at 48 h, in stationary phase maximum antibiotic production was detected, after 48 h it again decreased.

4.4.4. Extra and intra cellular location of antibiotic production :

Experiment carried out for checking extra and intra cellular activity showed that the supernatant of the culture did not exhibit the zone of inhibition, while the cells or cell pellet showed good antibacterial as well as antifungal activity. From this result it was concluded that this may be an intracellular antimicrobial activity. For intracellular activity, protein estimation of the lysed cells was carried out. The cells were treated with SDS and ultrasonicator for different time intervals and lysis was achieved. Cell lysate showed the presence of protein as time increases. The supernatant obtained after centrifugation, tested for antimicrobial activity, showed negative results, this indicates that there was no intracellular antibiotic production by the bacteria.

4.4.5. Effect of different environmental factors on antibiotic production :

Different physico-chemical factors were tested to check its effects on production of antimicrobial compound produced by *A. haemolyticus* A19.

4.4.5.1. Effect of pH : Antimicrobial activity checked at different pH showed that at acidic pH such as pH 5, there was no activity at pH 6, 7 and 8 activity was detected. From pH 9 activity was increased up to pH 11 and at pH 12 again activity was decreased. Thus at alkaline pH 11, the maximum antibiotic activity was detected. Fig.7a shows antibiotic activity at different pH.

4.4.5.2. Effect of temperature : Antimicrobial activity at different temperature was studied. It showed maximum activity at 28^{0}C and after that at 37 ^{0}C, however, activity was less. At temperature 10 as well as 44 and 50^{0}C very less activity was detected. It means the optimum temperature for this antibiotic is 28^{0}C. Fig. 7b shows effect of different temperatures on antibiotic activity.

4.4.5.3. Effect of NaCl : Observed at different concentrations showed that 1 and 2 % NaCl (w/v) was best for antibiotic activity. Fig.8. shows effect of different NaCl concentrations on antibiotic production.

4.4.6. Production of antibiotic by co-cultivation method :

It was found that this antibiotic in general, was not produced by *A. haemolyticus* A19 intra or extra cellularly. It was observed that this antibiotic is produced by *A. haemolyticus* A19 in presence of sensitive organism. This proved inducible nature of this antibiotic. Different proportions of producer and sensitive organisms were tested in order to get maximum antibiotic production and 2:1 (producer : sensitive) proportion was found to be best for maximum antibiotic production. Antimicrobial activity tested for cell pellet as well as supernatant showed more activity in supernatant than pellet. It means this antibiotic is inducible and after induction it remains extra cellular in nature.

4.4.7. Extraction and purification of antibiotic :

Fig. 1A

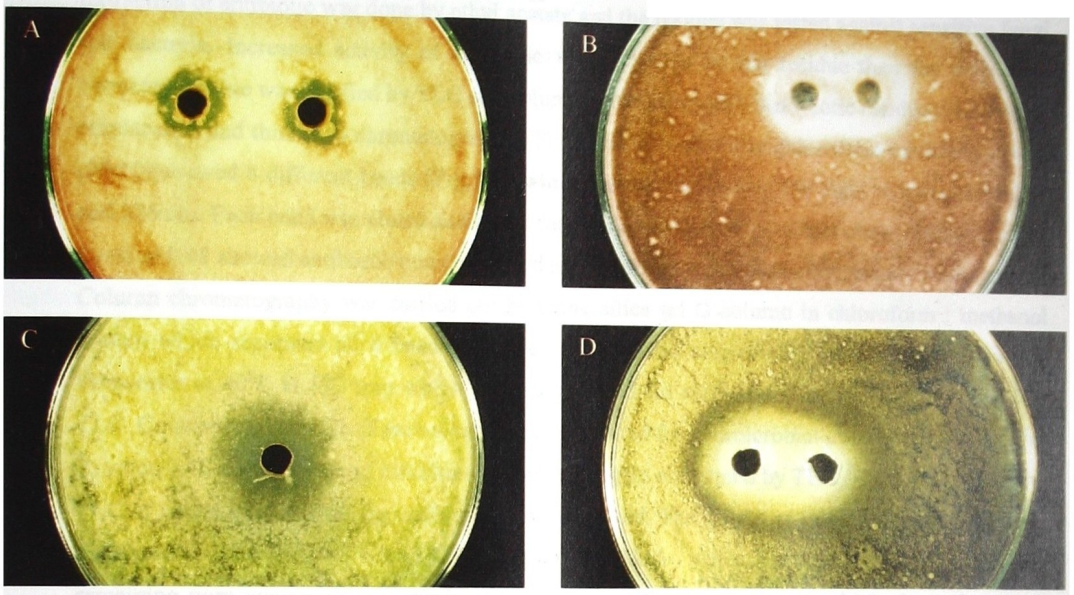


Fig. 1B

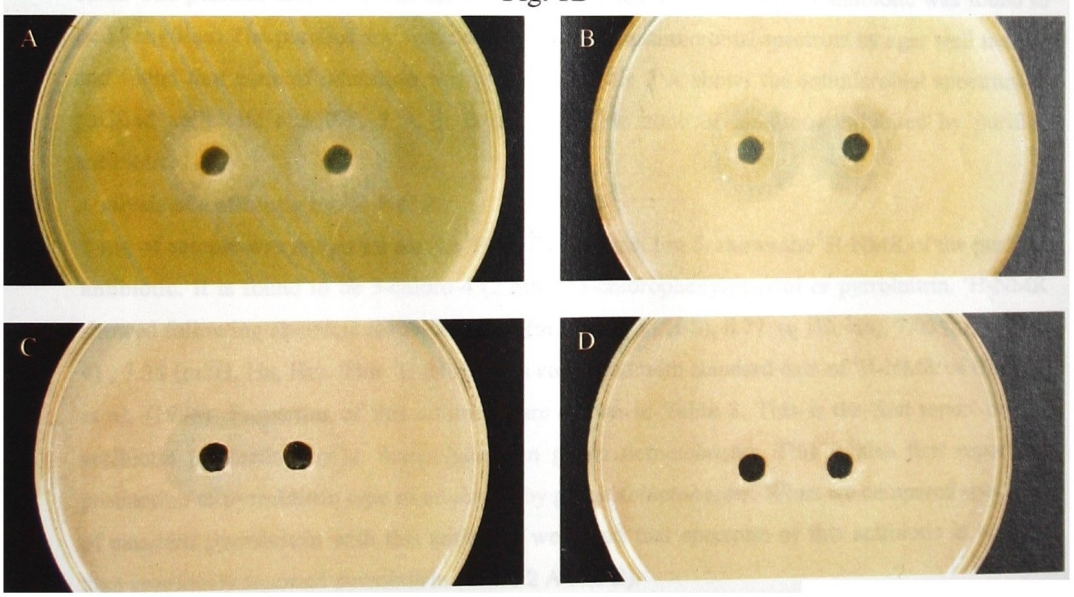


Fig.1. Antibiotic production by *A. haemolyticus* A19 isolated from rhizosphere of wheat.
A: Antifungal activity of *A. haemolyticus* A19 against plant and human pathogenic fungi.
A: *F. oxysporum* (NCIM 1072); B: *A. niger* (B. J. M. C. Pune);
C: *Cladosporium* spp. (NVIM 1082); D: *Helminthosporium* spp.(NCIM 1079).

B: Antibacterial activity of *A. haemolyticus* A19 against plant and human pathogenic bacteria.
A: *X. campestris* (NCIM 2954); B: *X. malvacerum* (NCIM 2301); C: *S. typhimurium* (NCIM 2501);
D: *K. pneumoniae* (NCIM 2719).

Extraction of antibiotic was done by ethyl acetate and the residue was tested against sensitive fungi and bacteria. Increased activity of antibiotic was exhibited by the residue than the cells. The extracted residue was purified by TLC and column chromatography. Antibiotic residue dissolved in ethylacetate and thin layer chromatography (TLC) was carried out in chloroform methanol system (90:10) showed 8 different bands (Fig.2A.) which were visualized by UV light at wavelength 256 and 336 nm. Each band was eluted and tested for antibacterial activity, it was observed that band of R_f value 0.48 showed antibiotic compound and hence further separated by column chromatography. Column chromatography was carried out by using silica gel G column in chloroform : methanol system which separated all 8 bands. Sample collected and concentrated by evaporation on rota evaporator at 40^0C at 190 rpm which was again tested for single band or purified compound by TLC. Concentrated sample of each single band was tested for antimicrobial activity and the band of R_f value 0.48 showed zone of inhibition, which was again confirmed by TLC (Fig.2 B). Preparative TLC of eluted band containing antibiotic was carried out. The band was scratched and collected in a clean test tube and 4-5 ml of chloroform was added. After filtering and evaporating, the remaining pure compound was again checked for purification by TLC, which showed a single band. The purified antibiotic was off-white in color. The yield of purified antibiotic was found to be 15 mg/liter. The purified antibiotic was checked for antimicrobial spectrum by agar well method and found that zone of inhibition was increased. Table 2 A shows the antimicrobial spectrum of purified antibiotic and Fig. 5 A & B shows visible zone of inhibition exhibited by purified antibiotic.

4.4.8. Analysis of antibiotic by ^{1}H-NMR :

5 mg of sample was subjected for the ^{1}H-NMR analysis. Fig.5. shows the ^{1}H-NMR of the purified antibiotic. It is found to be 3-chloro-4 (2 nitro –3-chlorophenyl) pyrrol or pyrrolnitrin. ^{1}H-NMR showed following chemical shifts: ä 6.2-6.6 (m, 2H, H-2 , H-5), 6.77 (q 1H, H6), 7.03 (m, 1H, H-4) , 7.38 (m2H, Ha, Hc). This ^{1}H-NMR was compared with standard data of ^{1}H-NMR of Chernin et al, (1996). Properties of this antibiotic are shown in Table 3. This is the first report on the antibiotic production by *A. haemolyticus* in genus *Acinetobacter*. This is also first report on production of pyrrolnitrin type of antibiotic by genus *Acinetobacter*. When we compared spectrum of standard pyrrolnitrin with this antibiotic we found that spectrum of this antibiotic is broader than previously reported pyrrolnitrin (Table 2 A and B).

4.4.9. Involvement of plasmid in antibiotic production :

The plasmid was isolated from *A. haemolyticus* A19 that is shown in Fig.4. The molecular weight of this plasmid pUPI126 was 40 Kb. Plasmid pUPI126 was conjugative with rif^r mutant of *E. coli* HB10 and transconjugants were isolated within 5 days from the selective medium. The frequency of conjugation was found to be 5.9 x 10^{-8}. *E. coli* HB101 (pUPI126) transconjugants were tested for antibiotic production against sensitive bacteria. It was found that transconjugants exhibited zone

of inhibition against sensitive bacteria. This confirmed that antibiotic producing genes were present on plasmid pUPI126 and it is a conjugative plasmid.

4.4.10. Transformation of plasmid pUPI126 to *E. coil* HB101 :

Transformation of plasmid pUPI126 in *E .coli* HB101 rif^r mutant was carried out. Transformation frequency was found to be 5 x10^5 per ìg of DNA. The cont rol plate without DNA did not form any colony on LB agar containing chloramphenicol, rifampicin and tellurium. Plasmid isolation of *E. coli* HB101 rif^r transformants showed presence of plasmid pUPI126 in it and had the same molecular weight (40 Kb). *E. coli* HB101 (pUPI126) transformants also exhibited antibiotic production. Thus it confirmed that plasmid pUPI126 encoded the genes for antibiotic production and plasmid pUPI126 is transformable to *E. coli* HB101.

4.4.11. Stability of plasmid pUPI126 in *Acinetobacter* and *E. coli* HB101 :

It was found that plasmid pUPI126 is very stable (100 %) in its original host *A. haemolyt*icus A19 and plasmid pUPI126 was found to be 95 % stable in *E. coli* HB101.

Fig.2A

Fig.2B

Fig.4

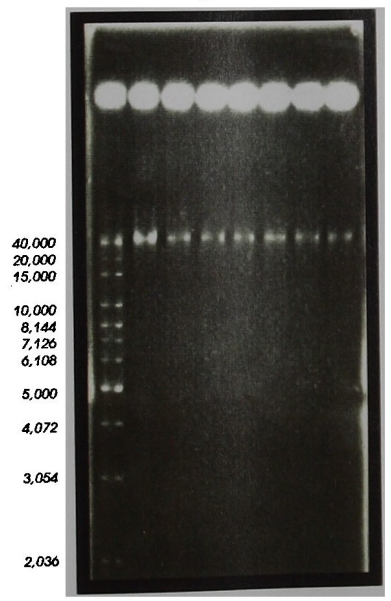


Fig.2A. TLC of extracted antibiotic produced by *A. haemolyticus* A19.*

A 19 : *A. haemolyticus* A19.*

*: Solvent system used for TLC : Chloroform : Methanol (90:10)

Fig.2B. TLC of purified antibiotic produced by *A. haemolyticus* A19.*

*: Solvent system used for TLC : Chloroform : Methanol (90:10)

Fig.4. Plasmid pUPI126 in *A. haemolyticus* A19, in transconjugants and transformants of *E. coli* HB101

Lane 1 : 1 Kb DNA ladder; Lane 2 & 8 : *A. haemolyticus* A19;

Lane 3 & 4 : transconjugants of *E. coli* HB101

Lane 5,6 & 7 : transformants of *E. coli* Hb101

Fig. 3. ^{1}H-NMR* analysis of purified antibiotic from *A. haemolyticus* A19. * : ^{1}H-NMR : 300 MHz; Varian; USA.

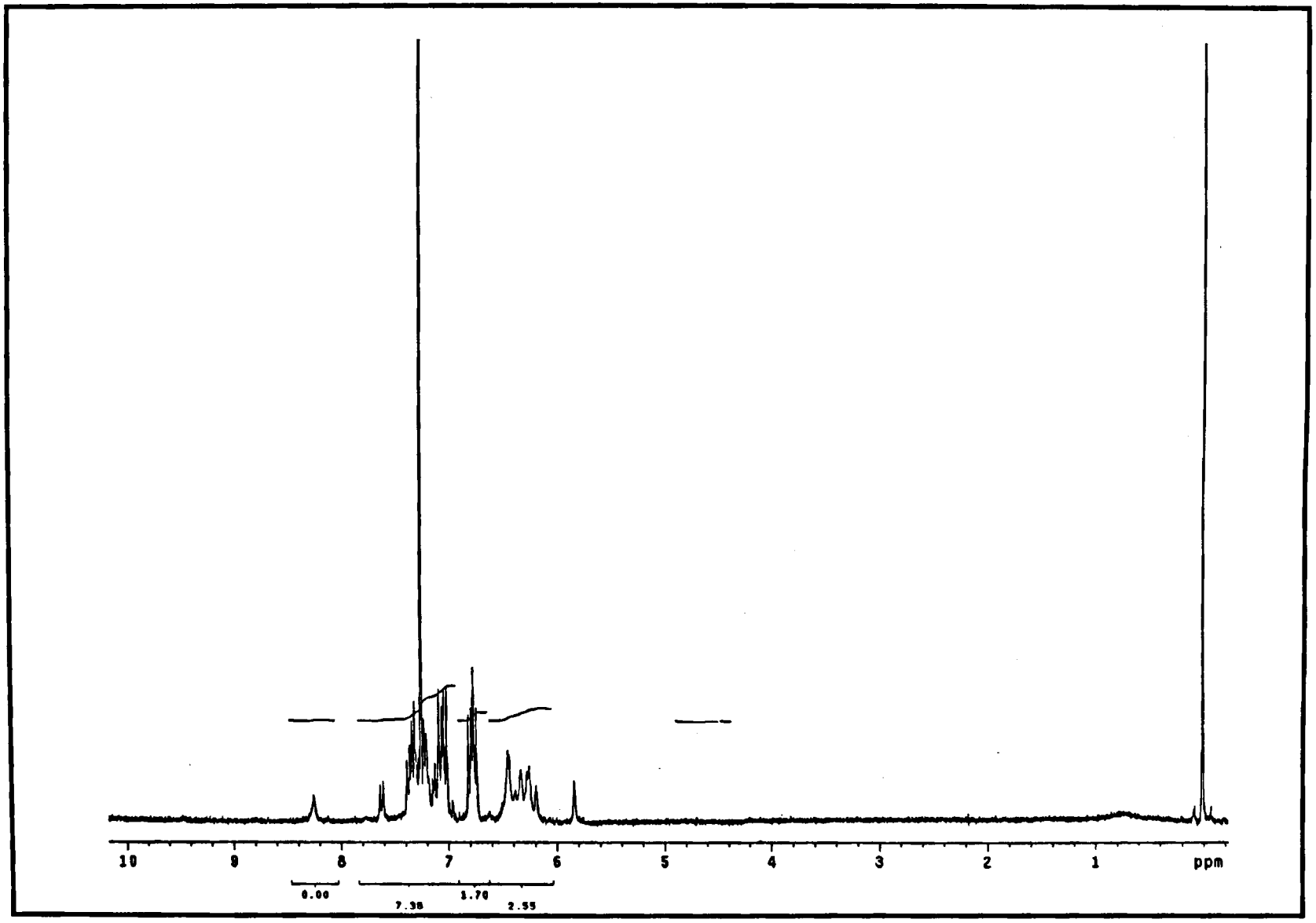


Fig. 5A

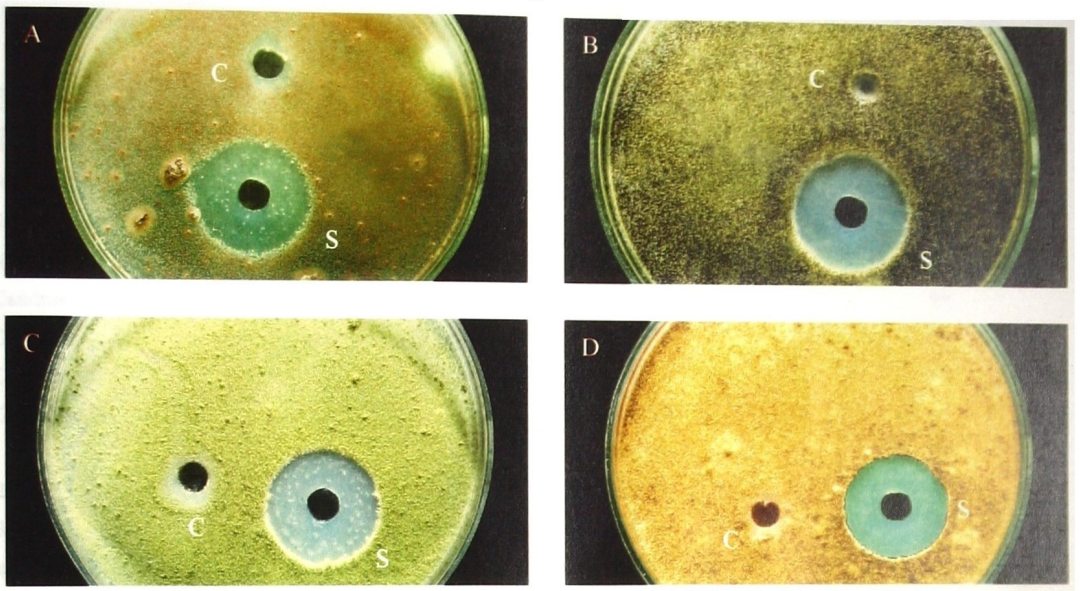


Fig. 5B

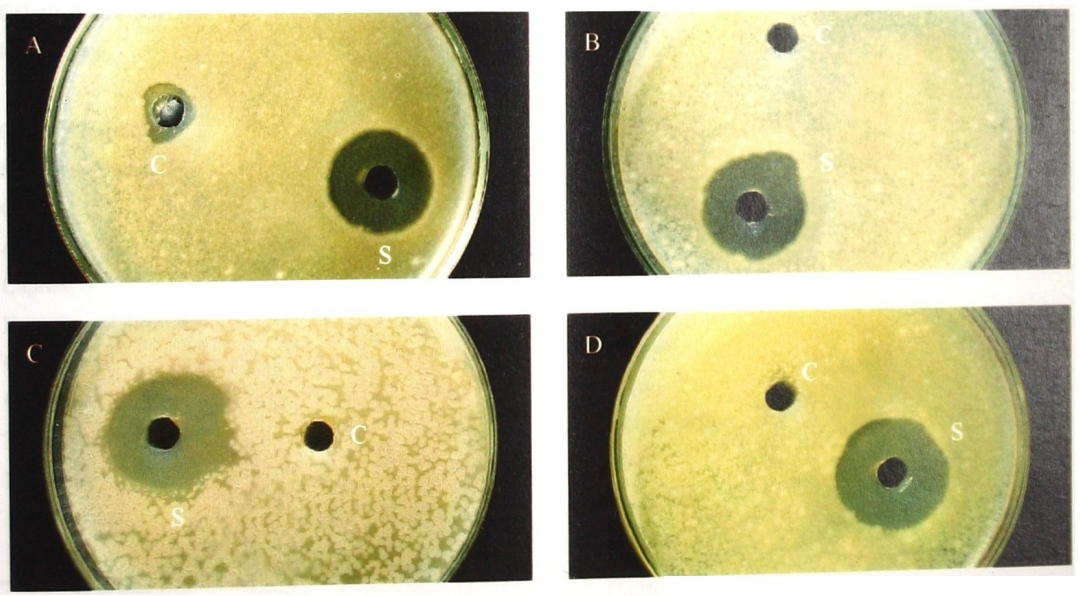


Fig.5 : Antimicrobial activity of purified pyrrolnitrin from *A. haemolyticus* A 19.
A : Antifungal activity of pyrrolnitrin from *A. haemolyticus* A 19.
A : *F. oxysporum* (NCIM 1072); B : *A.niger* (B.J.M.C. Pune);
C : *A. fumigatus* (AFMC, Pune); D: *A. Cladosporium* spp. (NCIM 1082).
C : Control; S : Sample
B : Antibacterial activity of *A. haemolyticus* A 19 against plant and human pathogenic bacteria.
A : *X. campestris* (NCIM 2954), B: *S. typhimurium* (NCIM 2501);
C : *Bacillus* spp. (NCIM 2012); D: *E. aerogenes* (NCIM2340)
C : Control; S : Sample

Table.1. Morphological, physiological and Biochemical characteristics of *A. haemolyticus* A19.

Characteristics	*A. haemolyticus* A19	Characteristics	*A. haemolyticus* A19
Gram staining	Gram negative	**Utilization of :**	
Morphology	Coccobacilli	DL-lactate	++
Size	2-3mm	L-phenylalanine	++
Motility	Non motile	Phenylacetate	++
Catalase	Positive	Malonate	++
Oxidase	Negative	L- histidine	++
Capsule	Capsulated	D-malate	++
Growth at :		L-aspartate	--
		L-leucine	--
28^0C	++	L-tyrosine	++
37^0C	++	β-alanine	++
44^0C	++	Transaconitate	++
Acid from glucose	++	L-tryptophan	++
Gelatin hydrolysis	++	Ethanol (100%)	++
Haemolysis	++	L-arginine	++
	β-haemolysis		
Citrate (Simmon's)	++	L-ornithine	++
Nitrate reductase	++	DL-4-aminobutyrate	++
ONPG	++	Tween 20	++
Adipate	--	Tween 80	++
Caprate	--	Tripal sugar iron test	++
Enzyme production:		**Enzyme production :**	
Chitinase	++	Proteases	++
Cellulase	--	Lipase	--
DNAse	++	β-galactosidase	++
Pectinase	--	Urease	--
Amylase	--	Phosphatase	++
Xylanase	++	L-Asparginase	++

*++: Positive, --: Negative

Table 2A. Activity of purified antibiotic produced by *A. haemolyticus* A19 against plant and human pathogenic bacteria and fungi. (This is the mean of three experiments).

Name of the pathogen	Zone of inhibition in diameter (cm)
Plant Pathogenic Bacteria :	
E. aerogenes NCIM 2340	1.5
Xanthomonas campestris NCIM 2954	2.0
X. malvacearum NCIM 2310	2.0
P. syringae NCIM 5102	1.0
P. putida NCIM 2102	1.1
Bacillus spp	2.0
Human Pathogenic Bacteria	
Kl. Pneumoniae NCIM 2719	2.0
P. vulgaris NCIM 2027	2.0
V. cholerae (AFMC, Pune)	1.2
S. dysentery type II (AFMC. Pune)	1.1
P. aeruginosa NCIM 2036	2.0
E. coli NCIM 2810	0.7
Staphylococcus aureus NCIM 2603	0.5
Salmonella typhimurium NCIM 2501	1.5
Human Pathogenic Fungi	
Aspergillus niger (B.J. M. C)	2.0
Aspergillus flavus NCIM 556	1.3
Penicillium notatum (AFMC)	1.2
Penicillium citrinum NCIM 925	1.3
Cryptococcus neoformans (AFMC)	1.1
Candida albicans (B.J. M. C.)	2.0
Plant Pathogenic Fungi	
Helimenthosporium spp. NCIM 1079	2.0
H. gramineum NCIM 1070	1.7
Fusarium spp. NCIM 895	2.0
Fusarium monilliforme NCIM 1100	2.0
A. fumigatus NCIM 898	2.0
Fusarium oxysporum NCIM 1072	1.7
Fusarium proliferatum NCIM 1102	1.5
Aspergillus parasiticus NCIM 898	1.0
Trichoderma spp. NCIM 1059	1.5
Trichoderma viridae NCIM 1195	1.5
Alternaria spp. NCIM 1280	1.3
Alternaria solani NCIM 887	1.2
Ustilago maydis NCIM 895	1.5
Gonoderma lucidum NCIM 1091	1.5
Trametes serialis NCIM 1182	1.5
Alternaria brassicola NCIM 1045	1.6
Cladosporium herbarium NCIM 1112	1.5
Cladosporium spp. NCIM 1082	1.2
Rhizoctonia solani NCIM 1060	1.3

Table 2B. Antimicrobial spectrum of standard pyrrolnitrin antibiotic produced by different rhizobacteria.

Fungi	Bacteria	References
Alternaria spp.	*Agrobacterium tumefaciens*	Corelli et al., (1987).
Aspergillus niger	*Clavibacterium michiganense*	Corelli et al., (1987).
Botrytis cinerea	*Corynebacterium insidiosum*	Corelli et al., (1987).
Fusarium oxysporium f. sp. *meloni*	*Erwinia carotovora*	Corelli et al., (1987).
Fusarium oxysporum f. sp. *vasinfectum*	*Pseudomonas syringae pv. syringae*	Chernin et al., (1996).
Penicillium expansum	*Pseudomonas syringae pv. phaseolicola*	Chernin et al., (1996).
Pythium ultimum	*Pseudomonas syringae pv. lachrimans*	Chernin et al., (1996).
Rhizoctonia solani	*Serratia marcescens*	Chernin et al., (1996).
Rhizopus sp.	*Xanthomonas campestris pv. cucurbitae*	Chernin et al., (1996).
Selerotium rolfsii	*Xanthomonas campestris pv. malvecerum*	Chernin et al., (1996).
Candida albicans	*Xanthomonas campestris pv. vesicatoria*	Chernin et al., (1996).
Candida spp.	*Mycobacterium tuberculosis*	Di Santro et al., (1998).
Candida pseudotropicalis	*Mycobacterium avim.*	Di Santro et al., (1998).
Penicillium notatum	*Streptomyces* spp.	El-Bana & Winkelmann. (1998).

Table 3. Properties of antibiotic produced by *A. haemolyticus* A19.

Color	:	Off white/pale yellow
Yield	:	15mg/lit
Nature	:	Off-white crystalline powder
Chemical composition	:	Pyrrolnitrin
Solubility	:	Soluble in water, chloroform, ethyl acetate, methanol.
Activity	:	Active at 50 μg/ml conc.
Stability	:	Stable at room temperature (7 days)
Spectrum	:	Broad; human and plant pathogenic bacteria and fungi .
Storage	:	At 10^0 C.

* This data is mean of 5 experiments.

Fig. 6. Time course for antibiotic production by *A. haemolyticus* A19

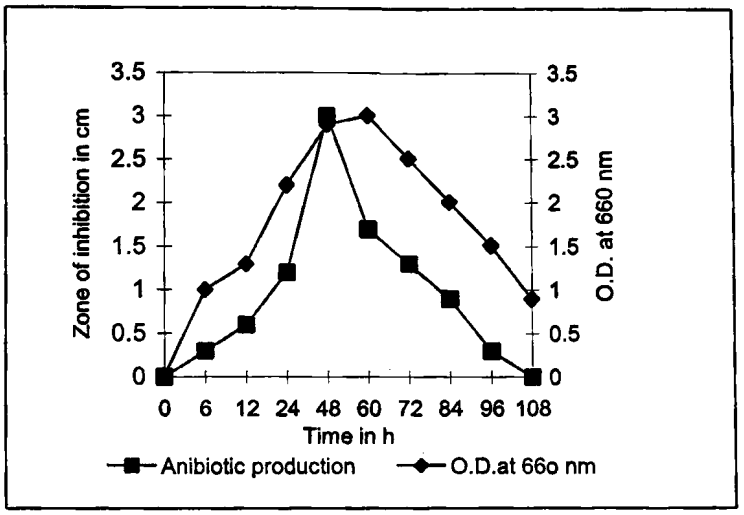


Fig.7a.Effect of different temperatures on antibiotic activity exhibited by *A. haemolyticus* A19.

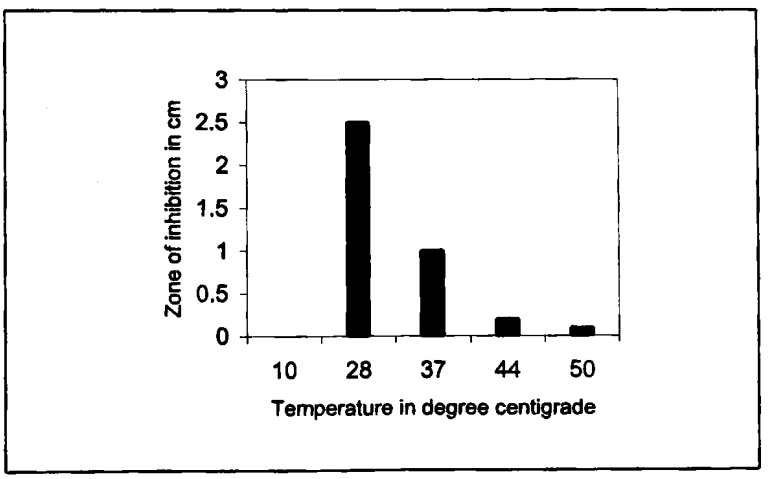


Fig.7b. Effect of different pH on antibiotic activity exhibited by *A. haemolyticus* A19

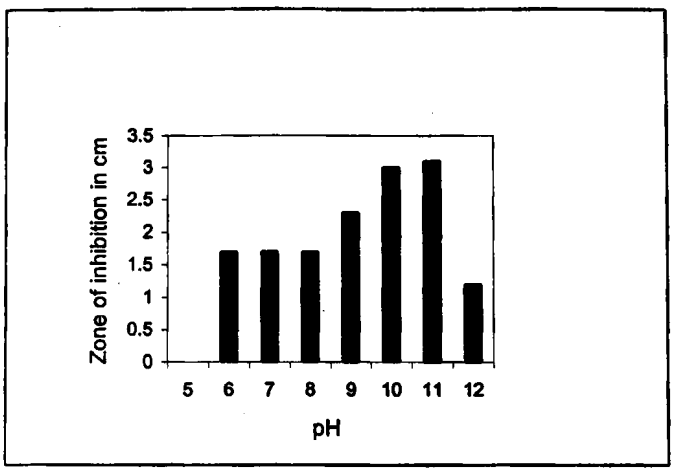
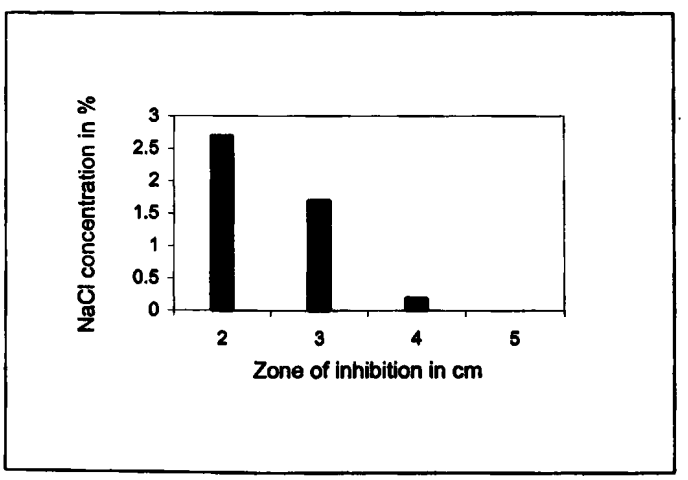


Fig.8. Effect of NaCl concentration on antibiotic activity exhibited by *A. haemolyticus* A19.



4.5. Discussion :

Pyrrolnitrin is one of the broad spectrum antibiotic produced by many soil bacteria and mainly by *Pseudomonas* spp (Arima, et al, 1964; Hwanga, et al, 2002). *Enterobacter agglomonas* is also reported to produce pyrrolnitrin by Chernin et al (1996). In the present study we observed pyrrolnitrin antibiotic production by *Acinetobacter haemolyticus* A19 isolated from rhizosphere of wheat. It also exhibited chitinase enzyme production. Therefore it can be concluded that as this genus *Acinetobacter* has the capacity to produce antibiotic, chitinases and also able to produce IAA, a plant growth promoting auxin, (Huddedar, et al, 2002; Leinhos, 1994). Thus *Acinetobacter* spp. has ability to become dominant and can be dominant in the rhizosphere and can stimulate plant growth. *A. haemolyticus* A19 showed maximum antibiotic production at 48 h at 28^{0}C under a given set of conditions. This time period coincides with early stationary growth phase of *A. haemolyticus* A19. Growth for extended period (60-72h) caused sharp decrease in the antibiotic production and at 108 h antibiotic production was not detected at all. There are many reports about the antibiotic production in the stationary phase as antibiotic is a secondary metabolites (Reichnbach, et al, 1988). In case of *Pseudomonas* and *Enterobacter* the growth phase for pyrrolnitrin production was also stationary phase (Carweight , et al, 1995; Hammer & Evensen, 1993; Reichnbach, et al, 1988).

A. haemolyticus A19 exhibited chitinase activity. Chitinases are known to act on chitin and mainly against fungi (Hsu & Lockwood, 1975; Chernin, et al, 1995). Previously we considered that antifungal activity showed by *A. haemolyticus* A19 is because of chitinases. *A. haemolyticus* A19 antibiotic producer, was tested for siderophore and acid production, as siderophore production is known from genus *Acinetobacter* (Marie, et al. 1998). This confirmed that the antimicrobial activity is indeed only because of antibiotic and not because of siderophore and or acid production. In most of the cases, antibiotics are extracelluar (Hacene, et al, 2000; Paradker, et al, 1998; Gupte & Naik, 1999, Kearns & Mahanty, 1997).

It was noted that this antibiotic is inducible in nature and can get induced by sensitive bacterium and comes out of cells. Antibiotic induction took place using *K. pneumoniae* and extracted with ethyl acetate. The most important observation revealed that after induction the crude extract also showed antifungal activity when tested with 23 different plant and human pathogenic fungi. This result confirmed beyond doubt that antifungal activity is not only because of enzyme chitinase but it is due to an antibiotic. Purification of this antibiotic was carried out by extracting it with ethylacetate and separation with TLC in chloroform methanol system. R_f value of pyrrolnitrin from *A. haemolyticus* A19 (0.48) in comparison with standard pyrrolnitrin (0.48) is very similar (Chernin et al, 1996, Howell & Stipanovic, 1979). ^{1}H-NMR values when compared to the Chernin, et al (1996) was very similar. Thus ^{1}H-NMR clearly confirmed the structure of the antibiotic.

It is important to note that pyrrolnitrin is known to be a powerful fungicide and it is not unique to only genus *Pseudomonas*. There are several reports on the use of pyrrolnitrin as a biocontrol agent against several fungal diseases (Carweight, et al, 1995; Hammer & Evensen, 1993; Homma & Suzui, 1989; Homma et al, 1989; Howell & Stipanovic, 1979; Janisiewicz, et al, 1991, Jayaswal et al, 1990). However, there are some reports which showed incapability of pyrrolnitrin in biocontrol such as mutants of *Pseudomonas fluorescens* and *P. cepacia* have been described which are deficient in pyrrolnitrin production , yet not different from the wild type strains in their biocontrol activity against fungal diseases. (Kraus & Loper, 1992, McLoughlin, et al, 1992). It has previously shown by Chernin et al, (1996) that *E. agglomerans* strain IC1270 was able to reduce the incidence of root rot disease caused by *R .solani* in cotton and at the same time Tn5 mutants of these strains , one of which is deficient in chitinolytic enzyme production but still posses antibiotic activity and other of which is deficient in both of these activities , were equally unable to protect cotton against root rot caused by *R. solani* (Chernin et al, 1995). These controversies in observation raised question whether pyrrolnitrin is indeed act as a biocontoling agent against microbial pathogens in the rhizosphere. Recently once again it is proved that pyrrolnitrin has an important role in plant protection (Hwanga, et al 2002; Sfalanga et al, 1999).

Mode of action of pyrrolnitrin is not yet clear but direct interference of pyrrolnitrin or its synthetic derivatives with fungal plasma membranes has been demonstrated (Jespers et al, 1994; Nose & Arima, 1969). The combination of chitinases, cell wall degrading compounds and antibiotics, which have direct effect on membranes has been shown to be important for antagonism in many diseases (Lorito et al, 1994; Utkhede & Gaunce, 1983). Hill et al (1994), reported that biosynthesis of antifungal agent including pyrrolnitrin was synthesized and regulated by *lemA* and *gacA* genes. Recently, it was noted by Hammer et al, (1997), that in *Pseudomonas fluorescens* four gene were encoded for the pyrrolnitrin synthesis. 32-kb genomic DNA fragment from this strain that contains gene involved in the biosynthesis of pyrrolnitrin. Marker-exchange mutagenesis of this DNA with Tn5 revealed the presence of 6.2-kb region that contains genes required for the synthesis of pyrrolnitrin. In *Acinetobacter haemolyticus* A19, we found that, the genes encoding for production of pyrrolnitrin is plasmid pUPI126 encoded and it is a self transferable plasmid. The antibiotic produced by *A. haemolyticus* A19 can be considered as an advantage as it has also ability to produce chitinase and IAA. One important fact should be noted that tryptophan, is the first substance for the pyrrolnitrin production. Biosynthetic pathway starts from tryptophan and produces pyrrolnitrin. (Hammer, et al, 1997). The additional characters showed by *A. haemolyticus* A19 were Cm^r and Te^r resistance. Acquiring high resistance for these antibiotics and metals *Acinetobacter* can be used in genetic engineering as a selective marker. One most important result we revealed that plasmid pUPI126 is self conjugative, which is very important in case of rhizosphere where the gene transfer mainly takes place through conjugation specially crops such as

wheat and sugarbeet, provide conditions conductive to conjugal plasmid transfer between bacterial inhabitants (Smit et al, 1998; Lilley, et al, 1994; Elsas, et al, 1988). Soil is rich medium for the transfer of plasmids in microorganism (Naik, et al, 1994). It was also noted that plasmid pUPI126 is transformable which could well provide a means of genetic adaptation to changing environmental positions to their hosts. Pyrrolnitrin produced by *A. haemolyticus* A19 is plasmid encoded but in contrast in *Pseudomonas* genes for pyrrolnitrin production was chromosomal encoded (Hammer et al, 1997). In this background it should be noted that pyrrolnitrin production in *A. haemolyticus* A19 is more important and also has many other capabilities described above and hence can be potentially used for biocontrol purpose by cloning the gene of pyrrolnitrin production. This is the first report in the genus *Acinetobacter* on the production of pyrrolnitrin type of antibiotic. This is also a first report on the production of plasmid-encoded pyrrolnitrin production in the genus *Acinetobacter* and in other microorganisms.

The present report provides the first piece of evidence that *Acinetobacter* is able to produce pyrrolnitrin like *Pseudomonas* and *Enterobacter*. We have also found that the same strain can also produce indole acetic acid and chitinase enzyme activity, has high metal and antibiotic resistance and having different enzymes such as gelatinase, protease, xylanase, phosphatase, L-asparginase, and DNAse, which also can help the bacteria to interact with other microbial community in rhizosphere of wheat. *Acinetobacter* spp. also have been reported for phosphate solubilization (Leinhos & Vocek, 1994) and can promote plant growth. *A. haemolyticus* A19 also has ability to solubilize phosphate (data not shown), hence can stimulate plant growth. This antibiotic display broad spectrum of antagonistic activity and can be used in agriculture as a biocontrol agent for plant protection as well as stimulation. Because of ability to produce antibiotic *A. haemolyticus* A19 can grow very well and compete with other soil bacteria, fungi and yeast and even with antibiotic producing micro-organisms. *Acinetobacter haemolyticus* A19 producing this antibiotic can colonize the roots of various crop plants and produce antifungal metabolites such as pyrrolnitrin, present a real alternative to the application of chemical fungicides. Pyrrolnitrin from *Pseudomonas* spp. had used already against clinical antifungal agents for the treatment of skin mycoses (Tawara et al, 1989; Umio, et al, 1986), and phenylpyrrole derivative of pyrrolnitrin has been developed as an agricultural fungicide (Gehmann et al, 1990; Nevill, et al, 1988). This pyrrolnitrin also showed an excellent activity against some human pathogenic fungi as well as bacteria, thus can be used as a clinical antifungal and antibacterial agent, but it warrants further investigation. Pyrrolnitrin from *A. haemolyticus* A19 also can be used in agriculture as an effective fungicide as well as a biocontrol agent.

4.6. Conclusion :

- Pyrrolnitrin produced by *A. haemolyticus* A19 has broad spectrum of antimicrobial activity.
- This is the first report on pyrrolnitrin production in the genus *Acinetobacter.*
- Pyrrolnitrin production in *Acinetobacter haemolyticus* A19 is encoded by plasmid pUPI126.

4.7. Significance of this work :

- It is very important to note that *Acinetobacter* spp. isolated form rhizosphere of wheat has ability to produce an antibiotic. This property is very important in view of its existence in the rhizosphere area. It is also very interesting to know that pyrrolnitrin production in *A. haemolyticus* A19 is plasmid pUPI126 encoded. This is the first report on plasmid pUPI126 encoded pyrrolnitrin production by the genus *Acinetobacter.* Thus this work cleared the role of *Acinetobacter* in wheat rhizosphere as a rhizobacteria and *A. haemolyticus* A19 may be used as a biocontrol agent for wheat and many other crop plants.

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CHAPTER 5

Production of Novel Bioemulsifier by *Acinetobacter baumannii* A25 Isolated from Rhizosphere of Wheat.

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5.1. Abstract :

Three genospecies of *Acinetobacter* consisting of *A. baumannii* A25, A13, A16 and *A. junii* A6 isolated from rhizosphere of wheat produced novel bioemulsifier and showed lipase activity. Different triglycerides such as groundnut oil, mustard oil, sunflower oil, sesame oil, petrol, kerosene and toluene were degraded by two *Acinetobacter* genospecies such as *A. baumannii* and *A. junii*. Bioemulsifier production was found to be constitutive in *A. baumannii* A25, A13, A16 and *A. junii* A6. Rhamnose (300 $EUml^{-1}$) and phenylalanine (310 $EUml^{-1}$) showed highest increase in bioemulsifier activity among root exudates. Temperature 30^{0}C, pH 7, aeration 150 rpm and 1% NaCl, 1.5 % $CaCl_2$, and 1 % $MgSO_4$ were found to be most suitable condition for bioemusifier production and activity. *A baumannii* A25 showed maximum bioemulsifier activity (1200 EU ml^{-1}) even at 40^{0}C and at pH 6,7, and 8. This bioemulsifier contains protein (85 %), polysaccharide (14 %) and reducing sugars (1%). 99% reconstitution was achieved by the combining bioemulsifier protein (200µg/ml), bioemulsifier polysaccharide (200µg/ml), extracellular protein (100µg/ml) and exopolysaccharide (100µg/ml) respectively. Yield of bioemulsifier was found to be 6.6 g/liter in a given set of conditions. It reduced viscosity of groundnut oil by 50 % and increased viscosity of water by 10 %. Esterase produced by *A. baumannii* A25 in cell free supernatant was 190 U $ml^{-1}$and cell associated esterase was found to be 245 U mg^{-1}. Cell surface hydrophobicity of *A. baumannii* A25 towards groundnut oil was maximum, for hexadecane lower than groundnut oil and lowest for xylene. Poor congo-red binding to the cells of *A baumannii* A25 showed capsulated nature of *A. baumannii* A25. Bioemulsifier produced by *A. baumannii* A25 also showed good cleaning property. This bioemulsifier was very stable at room temperature (28-30^{0}C) and less stable at 10 and 50^{0}C. Surface tension of partly purified bioemulsifier was found to be 63 dynes /cm. This is the first report of production of novel bioemulsifier by *A. baumannii* A25 isolated from rhizosphere of wheat.

Key words : *A. baumannii*, Rhizosphere of wheat, Bioemulsifier, Chopadesan, Root exudates, Reconstitution, Surface tension, Cleaning property, Viscosity, Hydrophobicity.

5.2. Introduction :

Bioemulsifiers are surface active agents synthesized by certain microorganisms during their growth. These compounds have been studied extensively, specially due to their chemical structures and potential applications to several industrial fields. They are highly specific, less toxic and biodegradable (Zajic & Panchal, 1976). They are effective at extreme conditions of pH, temperature and salinity. (Desai,1987). They are easy to synthesize from cheaper renewable feed stock (Desai & Patel, 1994). Bioemulsifers are amphipathic molecules playing a vital role in microbial growth on hydrophobic substances like hydrocarbons and fatty acids. Many amphipathic

molecules produced by living cells posses surface active characteristics observed in the form of lowering of the interfacial tension at the oil-water interface which leads to the formation of microemulsions (Gutnick & Minas, 1987). These bioemulsifers have potential applications in agriculture, food, pharmaceutical, oil industries and cosmetics (Desai & Patel, 1994; Rosenberg, et al, 1980). It has significant application in oil spills as it minimizes health hazards of oil spills by employing bioremediation of specific microorganism (Gutnick & Rosenbreg, 1977).

Variety of microorganisms has been studied for the production of bioemulsifiers. Bioemulsifiers are produced by various members of genus *Acinetobacter* (Robert, et al, 1989; Rosenberg, et al, 1979a; Zajic et al, 1977; Rosenberg, 1986; Zajic & Panchal, 1976; Patil & Chopade 2001; Fought, et al, 1989). Emulsan an extracellular polymeric bioemulsifier produced by *A. calcoaceticus* RAG-1 has studied in detail and has great application in oil industry. *P. aeruginosa* produce bioemulsifers which are rhamnolipids in nature (Fiechter, 1992; Guerra-Santos, et al, 1984; McDonald, et al, 1981;Deziel, et al, 1996). Many species of *Acinetobacter*, isolated from soil, water, mud, etc have been reported for the production of bioemulsifiers (Rubinovitz, et al, 1982; Fought, et al, 1989). In recent years some new genospecies of *Acinetobacter* have been investigated for bioemulsifier production such as *A. calcoaceticus* BD4 and BD413, ATCC 17294, ATCC 17906, *A. junii* SC14 (Kaplan & Rosenberg; 1982; Kaplan et al, 1987, Patil & Chopade,2001). However, there was no report on bioemulsifier production by *Acinetobacter* spp isolated from rhizosphere of any plant species.

In present study we have isolated *Acinetobacter* species from rhizosphere of wheat and their ability to produce bioemulsifier. It is interesting to know that *Acinetobacter* is present in wheat rhizosphere in significant amount. (Huddedar & Chopade, 2000). It is known that rhizosphere is highly specialized and competitive environment for growth of microorganisms. Rhizosphere soil is rich in nutrients. Soil also contains some hydrocarbons or fatty acids and bacteria acts on them and make the soil nutrient rich, the process called bioremedation (Desai & Patel, 1994). In some seeds triglycerides are stored in the form of oil. The predominant food reserved in the wheat grain is starch. The major lipid component is triglycerides and those are hydrolysed during germination by the enzyme lipase. The products of enzymatic hydrolysis are then utilized by the growing wheat shoot (Plummer, 1992). In view of this background we proposed that *Acinetobacter* species from rhizosphere of wheat may produce bioemulsifier and therefore this work was initiated.

The present chapter describes bioemulsifier production by *A. baumannii* A25 isolated from rhizosphere of wheat. In this chapter we report characterization, partial purification, effects of different physico-chemical factors on bioemulsifier production, reconstitution of bioemulsifier and effect of wheat root exudates on activity of bioemulsifier.

5.3. Materials and Methods:

5.3.1. Isolation and identification of *Acinetobacter* strains and their lipase activity :

Isolation and identification of *Acinetobacter* spp. was done as described in chapter 3 (in section 3.3.1 and 3.3.2). All 37 strains of *Acinetobacter* were tested for lipase activity. Plates of tributyrin agar were spot inoculated with overnight grown cultures with *Acinetobacter* spp. Plates were incubated at 28°C for 2-3 days. After incubation, plates were observed for clear zone around the colony. Negative test showed absence of zone around the colony (Gerhardt, et al, 1994). After testing the lipase activity, positive strains were employed for emulsification assay.

5.3.2. Screening of *Acinetobacter* spp. for bioemulsifier production by emulsification assay :

Acinetobacter spp. were grown up to 48-72 h in Luria Bertoni broth. The cells were separated by centrifugation at 10,000 rpm for 15 min at 30°C. Three ml of cell free culture broth was mixed with 0.5ml test oil, vortexed vigorously for 2 min and incubated at 30°C for 1 h for phase separation. Aqueous phase was removed carefully by pipette and absorbance of the aqueous phase was recorded at 400 nm. The absorbance maxima arrived after scanning the entire visible light spectrum (UV- 1601 Shimadzu Corporation, Japan). The blank was prepared with sterile medium. An absorbance of 0.010 units at 400 nm multiplied by dilution factor , if any, was considered as one unit of emulsification activity per ml.(EU ml^{-1}) (Patil & Chopade, 2001).

5.3.3. Oils and hydrocarbons used for emulsification :

Edible oils such as groundnut oil, (Dhara Mills, Mumbai, India), sunflower oil,(Gemini Mills, Mumbai, India), sesame oil (Ahmad Mills, Mumbai, India), mustard oil, (Ahamad Mills, Mumbai, India) and hydrocarbons such as toluene (Qualigens, India), petrol and kerosene (Hindustan Petroleum, Ltd, India.) were used. All oils and hydrocarbons used were of analytical grade.

5.3.4. Effect of oils and hydrocarbons used for emulsification:

All above oils and hydrocarbons were used for emulsification. Emulsification was carried out as per emulsification assay (Patil & Chopade, 2001) and an absorbance of aqueous phase was measured at 400 nm.

5.3.5. Induction of bioemulsifier by groundnut oil :

Groundnut oil was used as an inducer for emulsion production in different concentrations such as 0.1, 0.2, 0.5 & 1% to see its effect on bioemulsifier production. At the same time bioemulsifier production without oil was also tested by growing the cells in LB broth without oil and tested for emulsification assay as above.

5.3.6. Bioemulsifier production :

Luria Bertoni broth (LB) was used as a production media. *Acinetobacter* strains were not grown properly in BNP medium by Fought et al, (1989). LB broth medium was selected because of

excellent growth of *Acinetobacter* in this medium and it is known that emulsion production is directly proportional to cell density. Bioemulsifier production was carried out in 200 ml of LB broth in a 500 ml conical flask at room temperature (30°C) with shaking at 150 rpm. Bacterial inoculum was grown in the same medium and 12 h culture (OD 0.1 at 660 nm) (100 µl) was inoculated into production media. Samples were tested for bioemulsifier production at each 5 h interval by employing emulsification assay and growth was detected by measuring absorbance at 660 nm (Patil & Choapde, 2001).

5.3.7. Effect of environmental factors on bioemulsifier production :

Bioemulsifier production of *Acinetobacter* spp., *A. baumannii* A 25, A13 and A16 , and *A. junii* A6 were tested for effect of different physico-chemical factors.

- ***For the effect of pH*** on production of bioemulsifier and activity, LB was adjusted to different pH ranges such as pH 5, 6, 7, 8 and 9 and inoculated with *Acinetobacter genospecies*. After each 10 h emulsification assay was carried out and bioemulsion was measured at 400 nm.
- ***To observer effect of different temperatures*** on production and activity of bioemulsifier, LB broth was inoculated with *Acinetobacter* genospecies and incubated at different temperatures such as 30, 33 37 and 40°C. Bioemulsifier was measured after each 10 h by emulsification assay and absorbance was measured at 400 nm.
- ***Effect of aeration*** on production of bioemulsifier was detected by incubating inoculated fermentation medium with *Acinetobacter* at different aeration conditions such as from 50, 100,120 and 150 rpm. Bioemulsifier was measured after each 10 h by emulsification assay and absorbance was measured at 400 nm.
- ***Effect of salt concentration*** was carried out by using different salts such as NaCl, $CaCl_2$ and $MgCl_2$ at concentration such as 1,2,4 and 6%. Bioemulsifier production and activity was measured after each 10 h by emulsification assay and absorbance was measured at 400 nm.

5.3.8. Effects of different oils and hydrocarbons on activity of bioemulsifier :

In the emulsification assay, after centrifugation, different oils such as groundnut oil, sesame oil, sunflower oil as well as mustard oil and different hydrocarbons such as kerosene, petrol and toluene were added separately in 0.5ml amount and emulsification assay was carried out. Emulsification was measured at 400 nm.

5.3.9. Stability of bioemulsifier :

For the stability of emulsion, emulsification assay was carried out and vortexed water and oil emulsion was measured at 400 nm. One set was incubated at room temperature (30°C) and other set at 10°C for 7 days. One set was kept at 50°C. Stability was also checked for different pH from 6 to 9. Absorbance of aqueous layer at 400 nm was noted after every 12 h. Stability was also checked for partial purified bioemulsifier as described above (Zosim, et al, 1982).

5.3.10. Effect of root exudates on activity of bioemulsifier :

Wheat root exudates were tested for emulsification activity. Wheat root exudates such as sugars, amino acids and organic acids were tested. We tested some of sugars such as lactose, mannitol, rhamnose, sucrose, innositol, glucose, galactose and maltose. Some amino acids like β-alanine, phenyl alanine, aspartic acid, tyrosine, histidine, arginine and ornithine and organic acids, such as propionic acid, malic acid and formic acid, for bioemulsification by adding each one separately and in different concentrations such as 20, 50, 100 µg/ml and combinations in emulsification assay and measuring the bioemulsifier activity at 400 nm.

5.3.11. Partial purification of bioemulsifier from *Acinetobacter baumannii* A25 :

A. baumannii A25 was selected for partial purification as it showed excellent bioemulsifier activity. Partial purification was carried out by taking 300 ml of 72 h grown Luria broth culture at 30°C and centrifuged at 10,000 rpm for 20 min at room temperature. After centrifugation, three volumes of chilled acetone was added in cell free broth, mixed thoroughly and incubated at 4°C for 15 h. The mixture was centrifuged at 10,000 rpm for 30 min at 10°C and brown precipitate was collected. This precipitate was dissolved in 3 ml of sterile distilled water and dialyzed extensively against sterile distilled water at 10°C for 48 h (seamless cellulose tubing , width 40mm, diameter 25 mm, retaining most proteins of molecular weight 12,000 or more, Sigma Aldrich Chemie , Gmbh, Steinheim, Germany). Distilled water was changed after each 12 h. The dialysate was then frozen at –20°C and lyophilized. The lyophilized powder was stored at room temperature (30°C) in airtight glass vials (Patil & Chopade, 2001).

5.3.12. Chemical analysis of bioemulsifier :

The partially purified bioemulsifier was chemically analyzed. Protein content was measured by method of method Lowry et al (1951), with bovine serum albumin (BSA) as a standard. Polysaccharide was assayed by method of Dubois et al (1956) using glucose as a standard. Reducing sugars were estimated by using *p*- dinitrosalicyclic acid method of Monreal & Resse (1969) with glucose as a standard. Extraction and quantification of lipids were done by two methods described by Reddy et al (1983) .

5.3.13. Reconstitution of emulsification activity of bioemulsifier :

The components of partly purified bioemulsifier were identified by chemical composition and this composition was considered for reconstitution. Protein from bioemulsifier powder was extracted by hot phenol method (Kaplan, et al, 1987). Capsular polysaccharide was obtained by homogenized culture supernatant and extracellular protein was extracted by 60% ammonium sulphate precipitation of cell free broth. The polysaccharide fraction of purified bioemulsifier was obtained by water extraction of phenol phase (Kaplan, et al, 1987; Patil & Chopade, 2001). Each fraction was again checked for its respective contents by the methods described as above and all the

fractions separately and in combinations were tested for reconstitution activity with groundnut oil as a substrate for emulsification assay.

5.3.14. Effect of proteases on partial purified bioemulsifier produced by *A. baumannii* A25 :

Different proteases such as trypsin, pepsine, chymotrypsine and protease k (Hi Media, Mumbai) were tested for their effect on partially purified bioemulsifier. Each (50µg/ml) proteases were mixed with bioemulsifier in increasing concentrations such as 10µg/ml, 20µg/ml and 50µg/ml. After mixing these enzymes, the mixtures were kept for 30 min.at room temperature. After this groundnut oil (0.5 ml) was added in 3 ml mixture (enzyme and supernatant) and emulsification assay was carried out. After assay, to see the effect of enzyme on bioemulsifiers activity, absorbance was taken at 400nm. Emulsifying units were measured and compared with control without enzyme.

5.3.15. Determination of viscosity :

Viscosity of the purified bioemulsifier was tested by two methods. In first method, different aliquots of purified bioemulsifier powder (1mg /ml up to 3 mg/ml) were used to emulsify a fixed 10 ml amount of groundnut oil. Viscosity was recorded by Ostwaldt's standard viscometer (Martin, et al, 1969) at room temperature (30^0C). Un-emulsified groundnut oil was used as a control. In second method purified bioemulsifier powder (10 mg /ml up to 30 mg/ml) were used to emulsify fixed volume (10ml) of water. Distilled water was used as a control.

5.3.16 Determination of esterase activity in *A. baumannii* A25:

Esterase activity was detected by a colorimetric assay described by Shabtai & Gutnick (1985). In this assay p- nitrophenol acetate was used as a substrate which is hydrolyzed by esterase enzyme and produce yellow-green colour. Cell pellet as well as cell supernatant was assayed for esterase activity. Activity was detected after every 12 h interval in 72 h fermentation. 0.2 ml washed cell suspension in saline or cell- free supernatant was mixed with 1.7 ml of phosphate buffer (75mM,pH7.0) containing 10mM MgSO4, and then reaction was started by addition of 0.1 ml of 100mM PNP-Ac in absolute ethanol. The reaction was run at 30^0C and was followed by recording the continuous change in absorbance for 5 min. at 405 nm. The activity was expressed in nanomoles of PNP released (the extinction coefficient of PNP at 405 nm is 9.940 litters per mol per cm). One unit of esterase activity is 1 nmol of PNP per min.

5.3.17. Cell surface hydrophobicity of *A. baumannii* A25 :

Cell surface hydrophobicity of *A. baumannii* A25 was determined by using n-hexadecane, xylene and groundnut oil (Rosenberg, et al, 1980). 1.2 ml of cell suspension made in PUM buffer (K_2HPO_4. $3H_2O$, 22.2 g; KH_2PO_4, 7.26 g; Urea, 1.8 g; $MgSO_4.7H_2O$, 0.2g; distilled water to 1 liter [pH 7.1]) and aliquots of each test hydrocarbon was added and incubated at 30^0C for 10 min. The mixture was vigorously agitated and allowed to stand for 20-30 min. Absorbance was measured at

400 nm using spectrophotometer (UV-1601 Shimadzu Corporation, Japan.). The volume of test hydrocarbon used varied from 0.1 to 0.6 ml. *P. aeruginosa* NCIM 1226 was used as a control.

5.3.17.1. Bath test for cell surface hydrophobicity :

This test was carried out, same as above method but in addition the cells treated with hydrocarbons were grown on Luria agar plates and incubated at 30°C for 24 h. The colony counts were revealed to see the relationship between colony count and optical density (Vanhaecke & Pijick, 1988).

5.3.17.2. Congo red (CR) binding assay to whole-cells :

A. baumannii A25 cells were harvested, resuspended in LB (OD 650 nm) in 1.5 ml ependoff tube. Congo red (21-25 nM) was added and allowed to bind for 10 min at room temperature (28°C). Cells were pulled on a centrifuge for 1 min. Congo red binding to cells was determined spectrophotometrically at 480nm as the residual dye in the supernatant (Camprubi, et al, 1992).

5.3.18 Cleaning property of bioemulsifier produced by *A. baumannii* A25 :

To check cleaning property of bioemulsifier, 10µg of purified powder of bioemulsifier was dissolved in 5 ml of distilled water. Clean glass tubes (16 x125 mm) were taken and about 7 ml glycerol, groundnut oil, mustard oil, sesame oil and sunflower oil were added separately. Then the tubes were inverted and oils were removed in such a way that the portion of oil should stick to the walls of the tubes. After this, 1ml of bioemulsifier solution (10 µg/ml) was added drop by drop, very slowly, in each tube by holding the tube in the horizontal position, and the inner surface of each glass tube was observed carefully for cleanness (Karanth et al, 1999).

5.3.19. Determination of surface tension of bioemulsifier :

To detect the surface tension of bioemulsifier, partly purified bioemulsifier was mixed in different concentrations with water to see its effect on water. Surface tension of bioemulsifier was measured by standard spinning drop tensiometer (Model 500, devised by Department of Physics, University of Pune).

5.4. Results :

5.4.1. Characterization, biotyping and lipase activity of *Acinetobacter* spp. :

All thirty seven bacterial strains showed Gram negative, non motile, oxidase negative, catalase positive and capsulated characters which are the diagnostic characteristics of genus *Acinetobacter*. Chromosomal DNA transformation assay of these strains confirmed that these strains belong to the genus *Acinetobacter*. The species level identification of these strains done as per Bouvet & Grimont (1986 & 1987), showed that there were five species of *Acinetobacter* viz. *A. calcoaceticus, A. baumannii, A. junii, A. haemolyticus* and *A*. genospecies 3. Out of thirty-seven, 15 *Acinetobacter* strains showed lipase activity. From these 15 strains, 4 *Acinetobacter* species were

selected for further studies. Among four *Acinetobacter* strains, three were identified as *A. baumannii* A25, A13 and A16, and one as *A. junii* A6.

5.4.2. Bioemulsifier production by *Acinetobacter* spp. isolated from rhizosphere of wheat :

All the four *Acinetobacter* strains showed good bioemulsification activity. The activity checked against four oils and three hydrocarbons. Activity was maximum with edible oils such as groundnut oil, sunflower oil, sesame oil and mustard oil as compared to kerosene, petrol and toluene. *A. baumannii* A25 showed maximum activity among four *Acinetobacter* strains to four oils and three hydrocarbons (Fig.1 a & b).

5.4.3. Time course of bioemulsifier production of *A. baumannii* A25:

It is very important to know that, at which growth phase of bacteria produce maximum bioemulsifier. Fig. 2 shows growth curve of *A. baumannii* A25 and bioemulsifier production in emulsifying units (EU). From Fig 2 it is clear that maximum bioemulsifier was produced in the late stationary phase at 72 h after which the production as well as growth of bacteria decreased.

5.4.4. Effect of oils and hydrocarbons on bioemulsifier activity :

Fig. 1 a & b. shows effect of plant oils on bioemulsifier produced by four *Acinetobacter* spp. Among four plant oils, groundnut oil showed maximum activity (1200 $EUml^{1}$) in four *Acinetobacter* species. All other oils showed little lower (1000 $EUml^{-1}$) activity than groundnut oil. Hydrocarbons used such as petrol, kerosene and toluene showed lower activity (250, 200 and 170 EU ml^{-1} respectively) of bioemulsifier. Among them petrol (200 EU ml^{-1}) showed more activity than toluene (200 EU ml^{-1}) and kerosene (170 EU ml^{-1}) .

5.4.5. Effect of oil on bioemulsifier production :

Two sets of bacterial growth, in one the fermentation were carried out with addition of different concentrations of groundnut oil and the second without oil. Fig.3 a & b shows effect of different concentrations of oils in comparison with and without oil. It was concluded from the Fig.3 that bioemulsifier production with oil and without oil is almost same. However, difference observed only in the stability of bioemulsifier produced. Bioemulsifier production decreases fast in LB without oil as compare to LB broth with oil. In LB broth with oil decrease in bioemulsifier production takes place slowly. It means bioemulsifier production is more stable in oil than without oil, but the maximum bioemulsifier activity exhibited in both conditions was same (1200 $EUml^{1}$).

Fig.1a. Effect of different plant oils on the activity and production of bioemulsifier produced by four different *Acinetobacter* genospecies

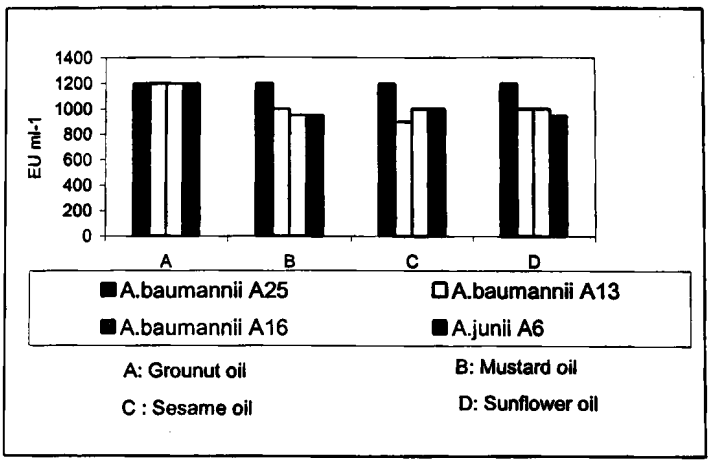
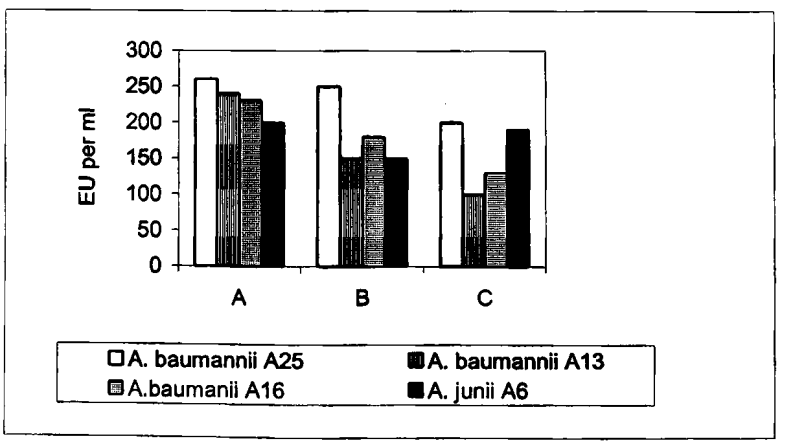


Fig.1.b. Effect of hydrocarbons on bioemulsifier activity produced by four different *Acinetobacter* genospecies.



A: Petrol; B: Kerosene; C: Toulene

Fig.2.Time course for production and activity of bioemulsifier produced by *baumannii* A25.

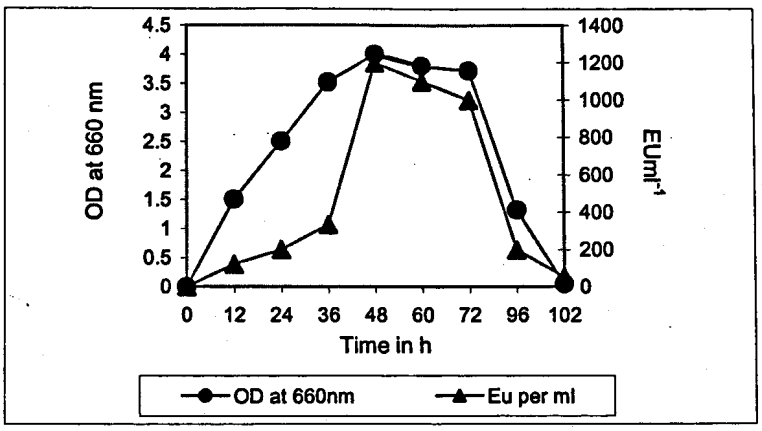
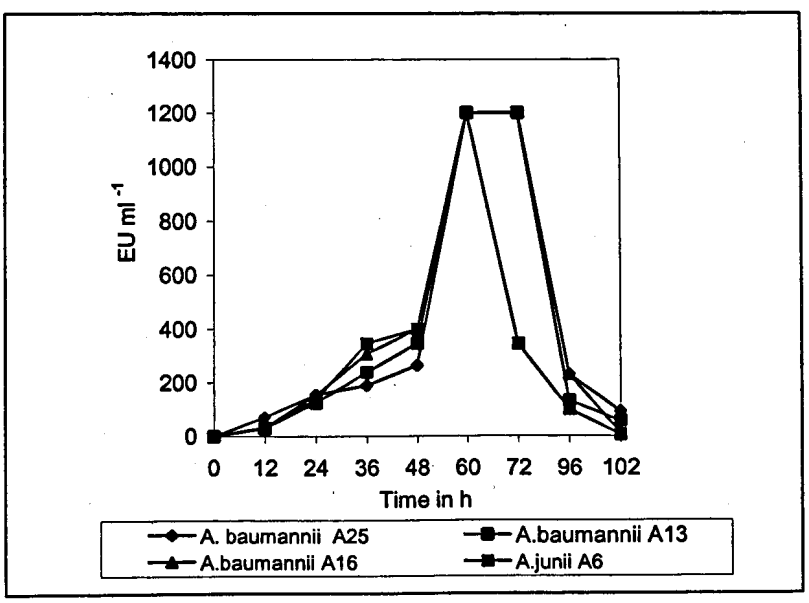


Fig.3a. Bioemulsion activity showed by four different *Acinetobacter* genospecies without oil.



5.4.6. Effect of different environmental factors on bioemulsifier production :

5.4.6.1.1. ***Effect of pH :*** It was found from Fig. 4a that pH affects the production of bioemulsifier produced by four *Acinetobacter* species and pH 7 was most effective for the production and activity for all four strains. *A. baumannii* A25 showed excellent activity even at pH 8 and pH 9 also. While remaining three *Acinetobacter* spp. showed decreased activity at pH 8 and pH 9.

5.4.6.1.2. ***Effect of temperature*** : It was observed that temperature plays an important role in bioemulsifier production. It was found that 30^{0}C and 37^0 C temperature was optimum for bioemulsifier production as their activity was same at these temperatures (1200 EUml^{-1}). Three *Acinetobacter* strains, *A. junii* A6, and *A. baumannii* A13 and A16 and their production and activity decreased as temperature increased, showing that they are not thermo-stable (Fig. 4b). While *A baumannii* A25 showed maximum activity even at 40^{0}C. Its activity decreased at 50^{0}C. It exhibited better thermo-stable activity as compare to other *Acinetobacter* spp.

5.4.6.3.1. ***Effect of aeration :*** Aeration showed direct effect on growth of *Acinetobacter* spp. and ultimately on the production and activity of bioemulsifier. 150 rpm was found to be most suitable aeration condition for all four *Acinetobacter* species (Fig. 4c). While 50 rpm was most poor aeration condition for bioemulsifier production and activity as there was poor growth of *Acinetobacter* spp.

5.4.6.4. ***Effect of salt concentration :*** Salts such as NaCl, $CaCl_2$ and $MgSO_4$ were found to be very effective in very less amount for all the four *Acinetobacter* species. NaCl in 1 %, $CaCl_2$ in 1.5 % and $MgSO_4$ in 1 % concentrations were most effective for all the four *Acinetobacter* species (Fig 5a, 5b and 5c).

5.4.7. Stability of Bioemulsifier :

Bioemulsifier from whole cells as well as partially purified powder was found to be extremely stable at room temperature (30^{0}C). It was found that after 7 days 92 % activity was maintained but at the same time it was found that bioemulsifier (cell as well as partially purified powder) was not much stable at low temperature (10^{0}C) and also high temperature (50^{0}C). It was observed that only 40 % activity retained at 10^{0}C and only 20 % activity retained at 50^{0}C. It was found that bioemulsifier from cell as well as partially purified powder was very stable at neutral pH (7) and 91% activity was maintained but at pH 6, 8 and 9 only 30 %, 40% and 50% activity was maintained respectively.

5.4.8. Effects of root exudates on bioemulsifier activity :

Amino acids when mixed with each other in different concentrations showed that increase in emulsification activity similarly more increase in emulsification activity was observed when amino acids and sugars were mixed. Among amino acids L-ornithine(232 EUml^{-1}) , L-arginine (262 EUml^{-1}), L-tyrosine (248 EUml^{-1}) and L-phenyl alanine (231 EUml^{-1}) exhibited increased bioemulsifier activity individually and in combinations. Among sugars rhamnose (300 EUml^{-1}),

maltose (250 $EUml^{-1}$), innositol (270 $EUml^{-1}$), and sucrose showed increased activity individually (270 $EUml^{-1}$) and in combinations with sugars such as lactose and glucose (290 (270 $EUml^{-1}$) and amino acids such as phenylalanine and tyrosine and histidine (310 (270 $EUml^{-1}$). No significant increase in activity was observed by all of the organic acids.

5.4.9. Partial purification and Chemical analysis of bioemulsifier from *A. baumannii* A25 :

A. baumannii A25 showed maximum bioemulsifier production as well as activity as compare to other three *Acinetobacter* species. Hence used further for purification. Partial purified bioemulsifier was obtained by acetone precipitation yielded a brownish-gummy powder after lyophilization. Activity of partially purified bioemulsifier was found to be 2287 $EUml^{1}$. The yield of bioemulsifier was found to be 6.6 g/liter. This is the highest yield of bioemulsifier without addition of any inducer, so far reported in the literature. The chemical composition and other properties of this bioemulsifier are given in Table1. It consists of 85% Protein, 14 % polysaccharide and 1 % reducing sugar. Lipid was not present in this bioemulsifier. This bioemulsifier is sparingly soluble in water. Its activity retained as it is at room temperature (30^0C) for several months (8 months).

Table 1. Properties of bioemulsifier produced by *A. baumannii* A25.

Color	:	Dark brown.
Yield	:	6.6 g/liter
Nature	:	Brown gummy powder and hygroscopic.
Chemical composition	:	Protein, polysaccharide and reducing sugars,
	:	Protein : 85%
	:	Polysaccharide : 14 %
	:	Reducing sugar : 1%.
Solubility	:	Completely soluble in cold water.
Stability	:	Room temperature (30^0C) for 8 months.
Storage at	:	Room temperature (30^0C) in tight glass vials for 8 months.

* This data is the mean of three experiments.

5.4.10. Reconstitution of emulsification activity of bioemulsifier :

Reconstitution of bioemulsifier was done by mixing the separated fractions of bioemulsifier individually and in combinations at different concentrations. Table 2 summarizes the percentage activity of reconstitution by each fraction individually and in combinations. The fractions individually showed less activity while the combination with all fractions showed enhanced activity. It was noted that protein is one of the major component of this bioemulsifier and showed more activity even individually. But for

Fig.3b. Bioemulsion activity showed by four different *Acinetobacter* genospecies

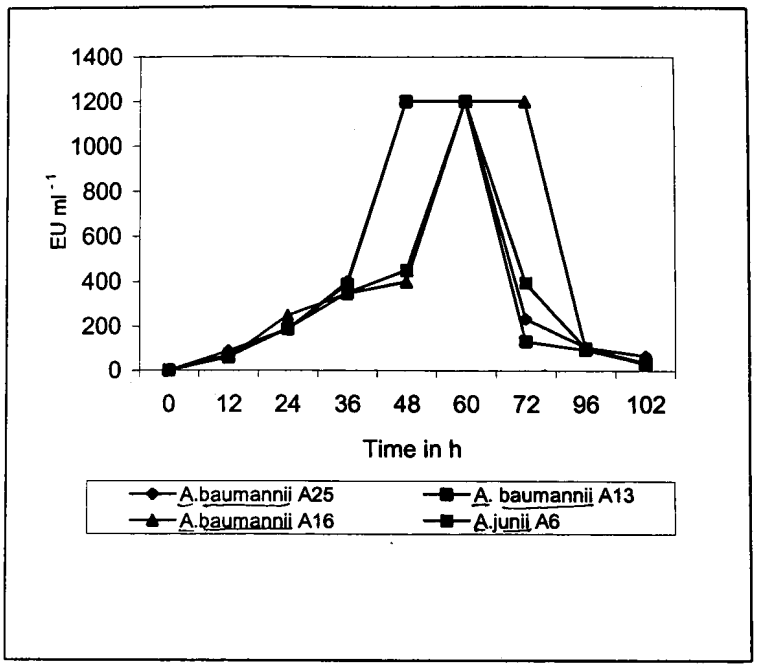


Fig.4.a. Effect of pH on bioemulsifier produced by four different *Acinetobacter* genospecies.

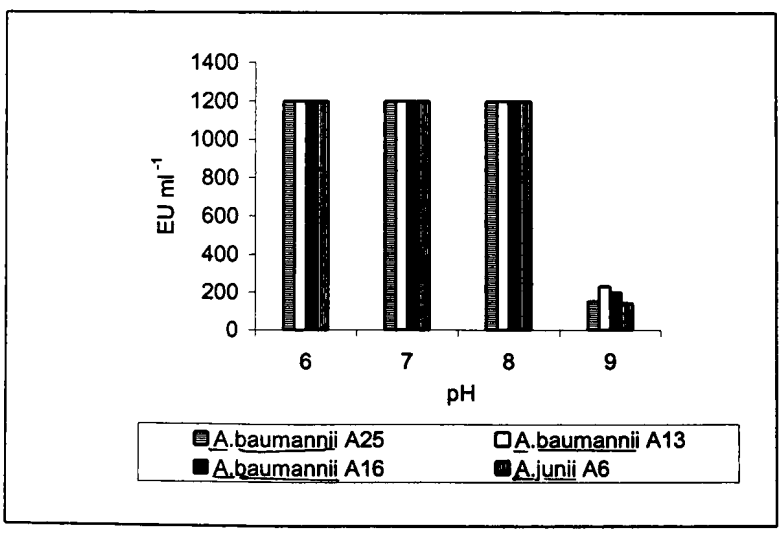


Fig.4.b. Effect of temperatures on bioemulsifier activity of four different *Acinetobacter* genospecies.

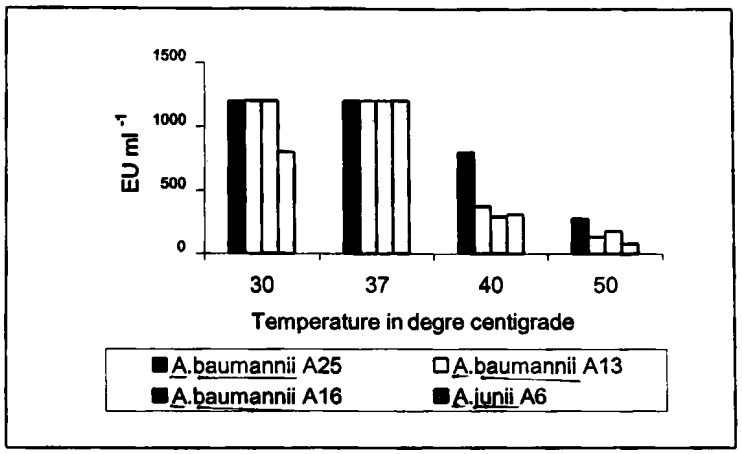


Fig.4c. Effect of aeration on bioemulsion activity of four different*Acinetobacter* genospecies.

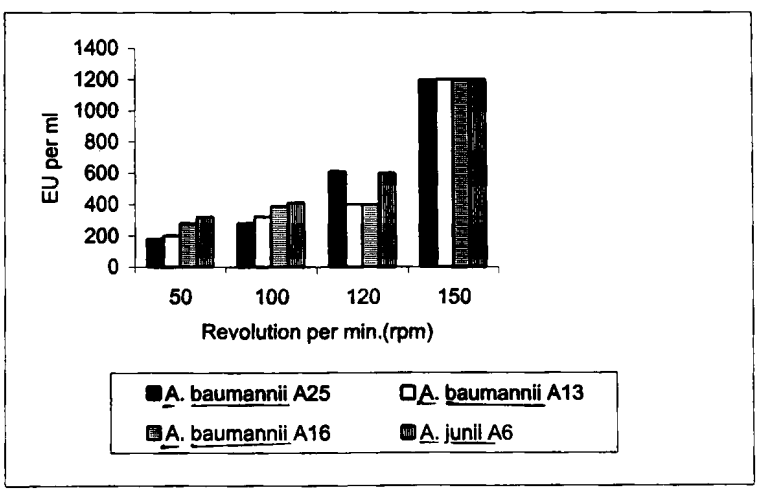


Fig.5a.Effcet of NaCl salt on activity of bioemulsifier produced by four different *Acinetobacter* species.

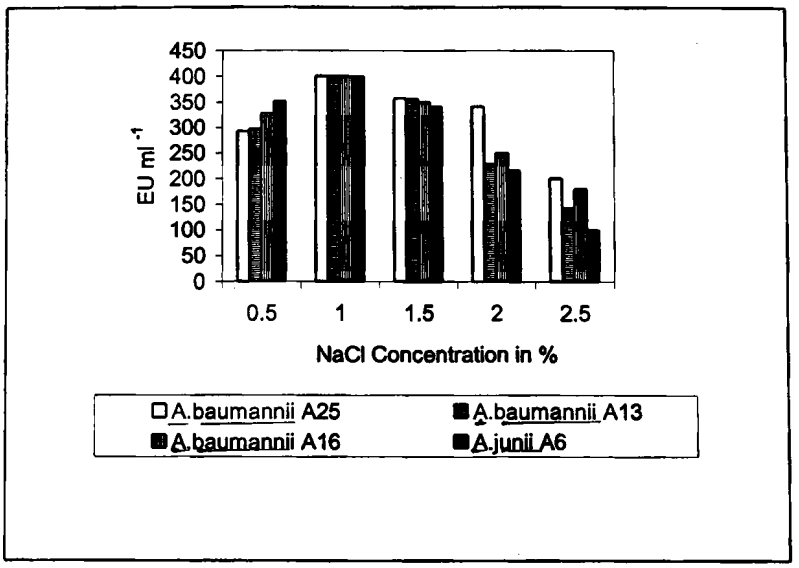


Fig.5b. Effect of $CaCl_2$ salt on bioemulsifier activity showed by four different *Acinetobacter* species.

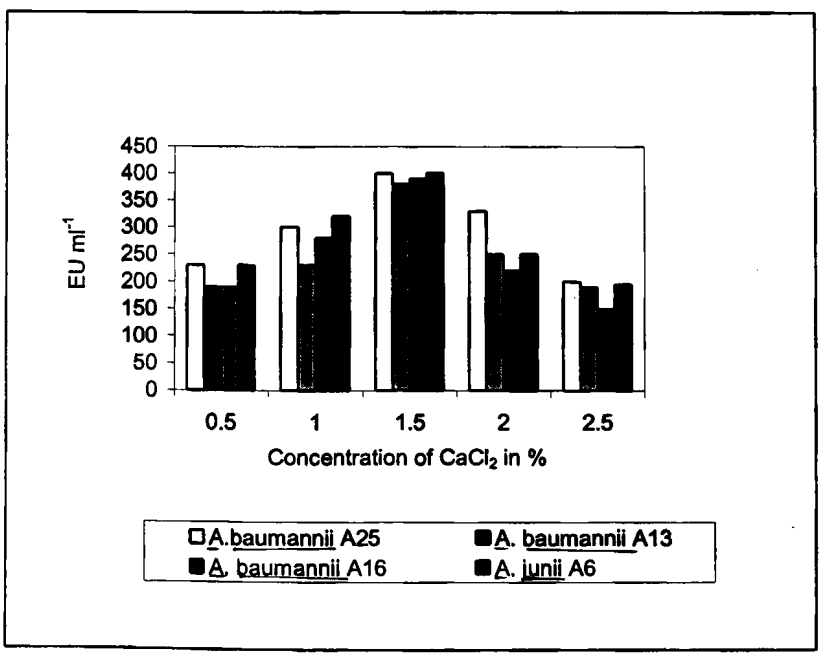


Fig.5c. Effect of $MgSo_4$ salt on bioemulsifier activity by four different *Acinetobacter* species.

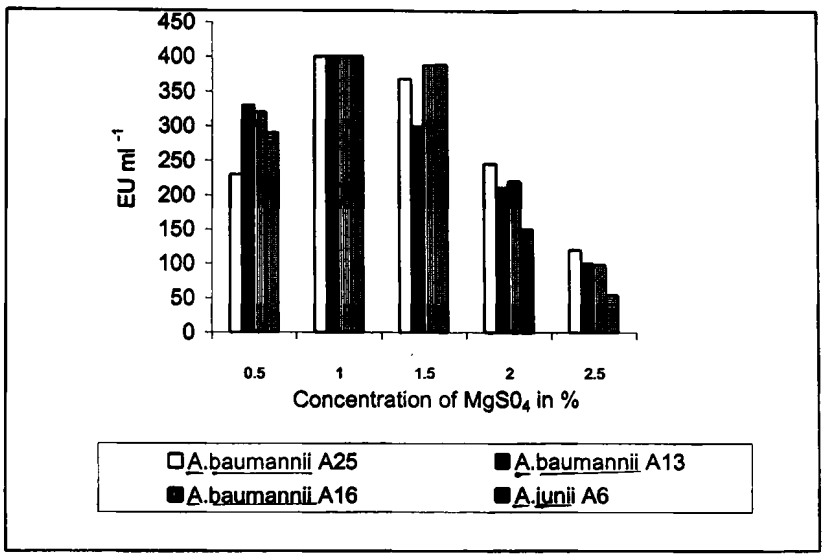


Fig .6 Cell free and cell associated esterase production by *A baumannii* A25.

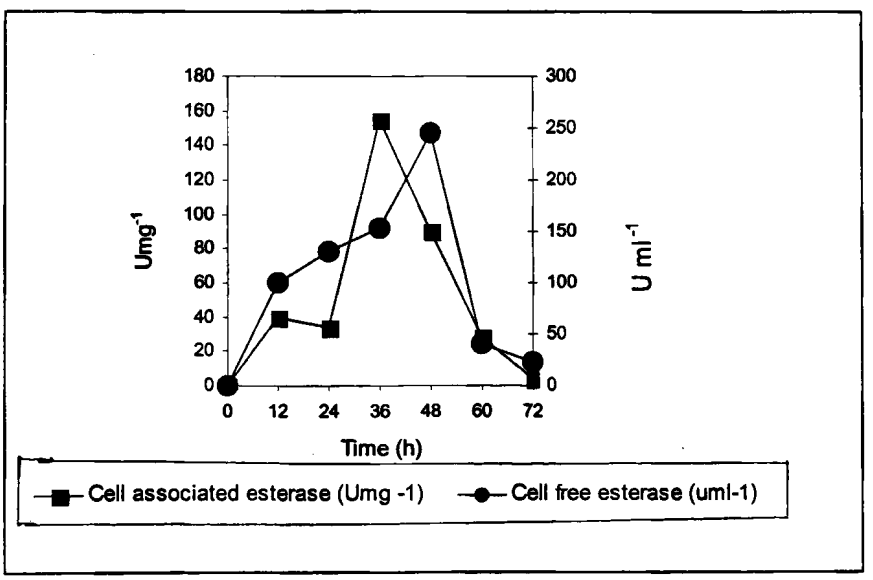


Table 2.Reconstitution activity of isolated fractions of bioemulsifier produced by *A. baumannii* A25.

Fractions of bioemulsifier		EU ml^{-1}	% activity
Purified bioemulsifier Factions(μg/ml)		2287	100
BEP	: 200	1055	46.1
EP	: 200	1566	68.4
BEPS	: 200	200	450
EPS	: 200	190	8.3
BEP+ EP	: 200+ 200	2222	97
BE+BEPS	: 200+200	2102	91.9
BE+EPS	: 200+200	2167	94.7
EP+BEPS	: 100+200	2159	94.4
EP+EPS	: 200+200	1710	74.7
BEPS+EPS	: 200+200	244	10.6
BE+EP	: 100+50	1126	49.2
BEP + BEP	: 100+50	2258	98.7
BEP+EPS	: 100+50	2260	98.8
EP+BEPS	: 100+50	2146	93.8
EP+EPS	: 100+50	1668	72.9
EP+BEP	: 100+50	2122	92.7
BEPS+EPS	: 100+50	1735	75.8
EPS+BEPS	: 100+50	1507	65.8
BEPS+BEP	: 100+50	600	26.2
EPS+BEP	: 100+50	1183	51.7
BEPS+EP ;	: 100+50	2101	91.8
EPS+EP :	: 100+50	821	35.8
BEP+BEPS+EP+EPS	: 200+200+200+200	2087	91.2
BEP+BEPS+EP+EPS	: 200+200+100+100	2265	99
BEPS+BPS+EPS+EP	: 200+200+100+100	2135	93.3
BEP+EP+EPS+BEPS	: 200+200+200+100	2243	98

BEP : bioemulsifier protein; EP : extracellular protein; BEPS : bioemulsifier polysaccharide; EPS : exopolysaccharide. Protein fraction of purified bioemulsifier was isolated by hot phenol treatment. Extracellular protein was obtained by 60 % ammonium sulphate precipitation of cell free broth. Polysaccharide fraction of purified bioemulsifier were prepared from water extraction of phenol phase. Capsular polysaccharide was isolated by acetone precipitation of homogenized culture supernatant.

complete activity, polysaccharide was necessary. Polysaccharide and reducing sugars showed less activity as compare to protein. Different combinations with different concentrations are showed in Table 2. Overall 99 % reconstitution of this bioemulsifier was achieved successfully.

5.4.12. Effect of proteases on bioemulsifier activity :

Bioemulsifier produced by *A. baumannii* A25 showed maximum amount of protein in its composition. Proteases are the enzymes, which acts on specific protein and reduces their activity. To see whether these proteases can act on this bioemulsifier and reduces its emulsification activity this experiment was performed. However, it is observed that addition of all four proteases was found to increase the activity of the bioemulsifier. In fact as the concentration of enzyme increased the corresponding increase in the activity of emulsion was observed.

5.4.12. Determination of viscosity :

It was noted that increase in bioemulsifier concentration from 0.5 to 3 ml against a fixed volume of 10 ml groundnut oil resulted in a decrease in viscosity of groundnut oil by 60 %. Different concentrations of bioemulsifier from 10 mg/ml to 30 mg/ml in water resulted in increase in viscosity of water by 20 %.

5.4.13. Esterase activity of *A. baumannii* A25 :

Esterase production by *A. baumannii* A25 is shown in Fig.6. The highest esterase activity was 190 Uml^{-1} showed by cell associated esterase at 48 h and in the cell free supernatant the activity noted was 245 Uml^{-1} at 48 h in the 72 h incubation.

5.4.14. Cell surface hydrophobicity of *A. baumannii* A25 :

Fig.7a, 7b & 7c shows cell surface hydrophobicity of *A. baumannii* A25 towards hexadecane, xylene and groundnut oil. It was noted that *A. baumannii* A25 was having affinity towards hexadecane and groundnut oil but it had lower affinity towards xylene. It can utilize hexadecane and groundnut oil more easily than xylene. Standard *P. aeruginosa* NCIM 1226 showed affinity towards hexadecane, xylene and groundnut oil. Thus the cell surface of the *A. baumannii* A25 appeared to be hydrophobic but relatively less than control. Bath test also showed similar result as above method and it proved that there is no correlation between OD and colony count of bacteria because it gives the count of only visible cells. This mainly showed wrong colony count in case of xylene, which may be toxic for the cells and the colony count came less as compare to OD. On results are similar to the results of Vanhaecke & Pijck (1988).

Fig. 7a. Cell surface hydrophobicity of*P. aeruginosa* NCIM1226 and *A. baumannii* A25 for xylene.

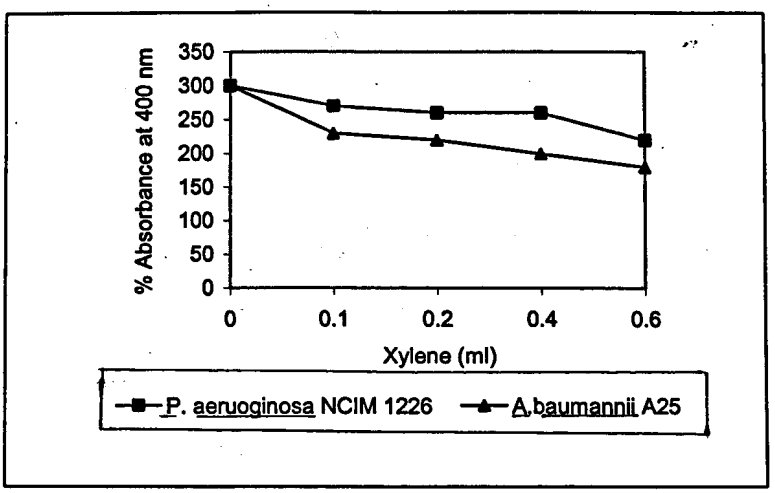


Fig.7b.Cell surface hydrophobicity of A*baumannii*A25 and *P. aeruginosa* NCIM1226 for hexadecane

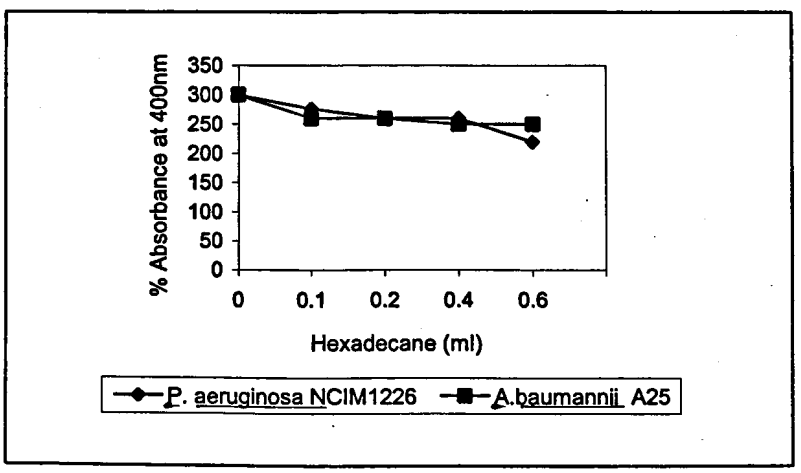


Fig.7 c. Cell surface hydrophobicity of *A. baumannii* A25 and *P. aeruginosa* NCIM 1226 for groundnut oil.

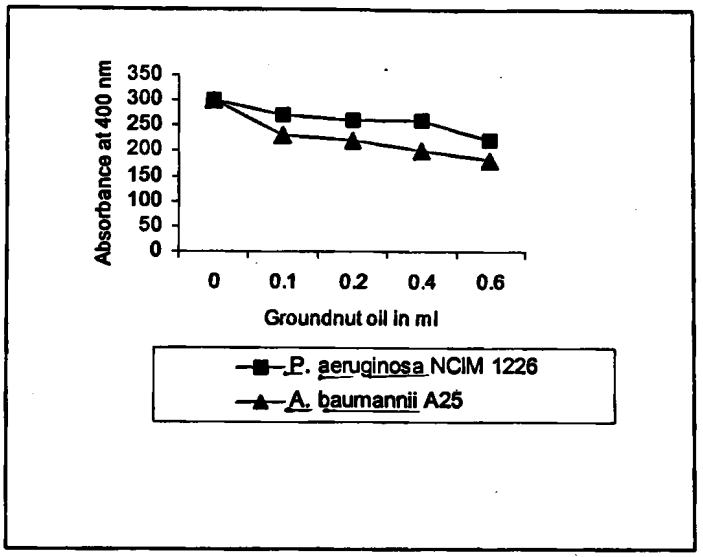
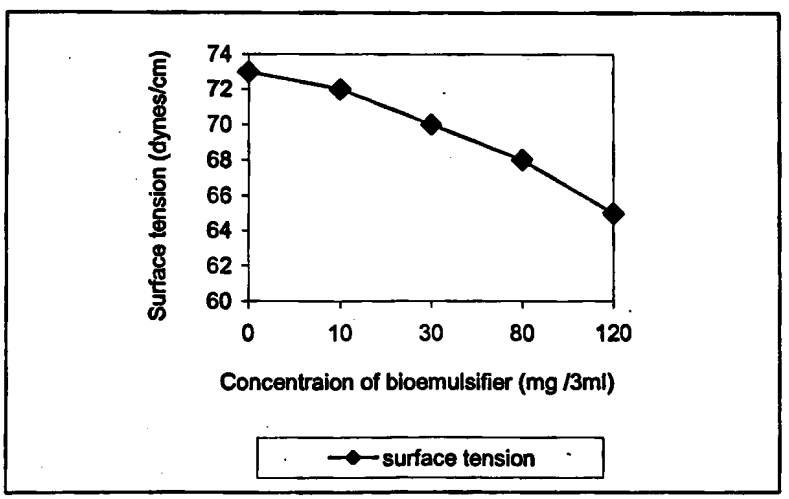


Fig 8. Surface tension of bioemulsifier produced by *A. baumannii* A25.



5.4.14.1. Congo red (CR) binding to whole cells :

CR binding to whole cells of four *Acinetobacter* species indicated very less percentage of CR binding. The percent activity for CR binding was noted as follows. *A. junii* A6 , 0.025 % ; *A. baumannii* A13, 0.015 % ; *A. baumannii* A16, 0.020 % and *A. baumannii* A25 , 0.049 %. The above result showed that the percentage of all bacteria was very low. Among them *A. baumannii* A25 showed little more percentage however, it did not indicate any significant binding of CR to whole cells.

5.4.15. Determination of Surface tension of bioemulsifier :

Fig.8 showed surface tension of partly purified bioemulsifier. It was observed that when this bioemulsifier was mixed with water it decreased the surface tension of water but the degree of decreasing the surface tension was very low. It required 40 mg/ml concentration of biosurfactant to decrease the surface tension of water up to 63 dynes/cm.

5.4.16. Cleaning property of bioemulsifier produced by *A. baumannii* A25 :

It was satisfactory to know that this bioemulsifier is having very good cleaning property even in very small amounts. It showed cleaning property against glycerol, groundnut oil, mustard oil, sunflower oil and sesame oil. It is very surprising that its activity is very fine against glycerol which is very viscous, oily and generally difficult to clean.

5.5. Discussion :

Acinetobacter is ubiquitous in nature and present in soil, water, food and on human skin (Baumann, 1968; Huddedar & Chopade, 2001; Chopade et al, 1994; Patil & Chopade, 2001; Saha & Chopade, 2001). *Acinetobacter* is well known for the production of bioemulsifier and a bioemulsifier produced by *A. calcoaceticus* known, as Emulsan is very effective agent for hydrocarbon in oil system (Desai, 1987). Very recently, *Acinetobacter* from human skin is also reported to produce a bioemulsifier, which is protein, polysaccharide and lipid in nature (Patil & Chopade, 2001). In this study we have isolated *Acinetobacter* from rhizosphere of wheat. It should be noted that rhizosphere is very specific and competitive area because of limited nutrients as compare to microbial population (Subba Rao, 1986). We have isolated 37 *Acinetobacter* among which 15 showed lipase activity. Lipase is enzyme acts on water-oil surfaces (Kloosterman, et al, 1988). From this result it is concluded that *Acinetobacter* from rhizosphere of wheat showed presence of lipases and may be able to produce bioemulsifier and it may have some role in the rhizosphere of wheat.

We found that all lipase positive *Acinetobacter* spp. showed emulsification activity. Among which 4 *Acinetobacter* strains were selected in which three were IAA producer and one is antibiotic producer. It was observed that among four *Acinetobacter* strains *A. baumannii* A25

showed maximum bioemulsifier producing activity (1200 EU ml^{-1}) hence studied in detailed. Different workers have utilized different media for the production of bioemulsifier, but mainly BNP medium was used (Fought, et al, 1989). In case of *Acinetobacter* spp. isolated from rhizosphere of wheat we found that cell count of*Acinetobacter* spp. was very less in BNP medium and therefore production of the bioemulsifier. To overcome this problem we used Luria Bertani broth (LB), was supplemented with 0.25 % peptone was used as production medium. Maximum bioemulsifier production was observed at 72 h of growth phase of*A. baumannii* A25. After 72 h there was decrease in the bioemulsifier production and activity (Fig.1). Rosenberg et al (1989) reported that bioemulsifier of *A. calcoaceticus* RAG-1 was produced during stationary growth phase and it has been previously demonstrated that cell mass and bioemulsifier production are directly proportional to each other (Sar & Rosenberg, 1983). *Acinetobacter* strains isolated from healthy human skin (Patil & Chopade, 2001) as well as a biosurfactant produced by*B. subtilis* (Cooper, et al, 1981) were reported to produce bioemulsifier during stationary growth phase.

The optimum pH was found to be 7 for the activity as well as production. However, this bioemulsifier could also show activity at pH 6 and pH 8. These results showed that bioemulsifier production and activity was good at alkaline pH as compared to acidic pH. Generally pH optimum for growth of organism is also optimum for bioemulsifier production (NavonVenezia, 1995). It was determined that activity and production of bioemulsifier was maximum in acidic pH, which is observed in case of alasan produced by*A. radioresistens* KA53 (Navon-Venezia, et al, 1995). The activity showed over a wide pH range of 3.3 to 9.2. But this didn't found true in case of rhizosphere *Acinetobacter* spp. however, at pH 5, rhizosphere*Acinetobacter* spp. were unable to grow. At pH 6 to 8 it showed good activity andactivity decreased to 80 % at pH 9. It was found that pH of the rhizosphere soil is between 7 to 8.2. Therefore, it is obvious that these rhizosphere strains showed higher activity in the corresponding pH. Similar results were observed by *Pseudomonas* spp., and *Acinetobacter junii* SC14, isolated from healthy human skin as production of bioemulsifier was not affected in the pH range 6.5-8.0 (Powalla et al, 1989; Chopade & Patil, 2001).

28^{0}C temperature, was found to be optimum for the production and activty of bioemulsifier. Production was also detected at 30^{0}C and 37^{0}C. At 40 and 50^{0}C the production as well as activity decreased near about 70 % for*A. baumannii* A13 and 16 and*A. junii* A6. But the activity of the bioemulsifier of*A. baumannii* A25 decreased only near about to 25 %. This is because of the temperature of the soil in winter wheat is relatively less and becomes maximum in start of summer (March-April) it becomes 30^{0}C to 40^{0}C. This may be the reason that as temperature increases there was 70% loss in bioemulsifier production by *Acinetobacter* species from rhizosphere of wheat. Aeration of 150 rpm was found to be good for production and activity of this bioemulsifier. This is quite obvious as Fig. 6 showed that as aeration increased, production

and activity of bioemulsifier also increased up to 150 rpm. $MgSO_4$ increased emulsifying activity than $CaCl_2$ and NaCl. This increase in activity of emulsifier by $MgSO_4$ was same as detected before in case of protein-polysaccharide complex produced by *P. fluorescens* (Desai,1987). But more addition of $MgSO_4$ will be found inhibitory for bioemulsifier production. Similar results have been reported for surfactin produced by *B. subtilis*, where iron and manganese caused significant enhancement of surfactin production. In case of *A. junii* SC14, 1 % concentration of NaCl caused 31.3 % inhibition of the emulsification activity.

It has been determined previously that production of bioemulsifier was induced by addition of hydrocarbons or oils or ethanol (Patel & Desai, 1997;Pines & Gutnick, 1986). Production of bioemulsifier takes place by degrading hydrocarbon and utilizing them as a nutrient by bacteria in a limited nutrient conditions. However, we observed that bioemulsifier production in case of *Acinetobacter* species isolated from rhizosphere of wheat was constitutive and there was decrease in bioemulsifier production due to addition of oil in the media. There were no study was done in this respect. Similar results were obtained from *B. subtilis*, produces surfactin constitutively and bioemulsifier production was completely inhibited by hydrocarbon addition in the medium (Cooper, et al, 1981).

The plant oils showed very good activity than the hydrocarbons such as petrol, kerosene and toluene. This indicates that this bacteria can utilizes and acts very easily on triglycerides than hydrocarbons. The choice of these edible plant oils was done according to the past record of crops of the agriculture field from past 10 years. Crop record of agriculture field indicated that inthe past years some vegetables and oily plants such as groundnut, mustard, sunflower and sesame were grown in agricultural college experimental field in Pune. So we decided to test these oils for bioemulsification. One can expect probability for the presence of traces of these oils in this soil through these plants. It should be noted that seeds are always in contact with soil and while harvesting some part of plant remains there in the soil, which is subsequently used as a nutrient by soil microorganisms. The lipid part of seed (specially above referred plants) remain undegradable and here is the chance for these bioemulsifier producing bacteria to degrade them in nutrient limited condition and it is important to note that rhizosphere is always a very nutrient limited area as compare to microbial population since there is always competition for the nutrients. If we observe the compositions of these oils, it is revealed that these are triglycerides, fatty acids and mainly composed of saturated, monosaturates and polysaturates (Plummer, et al, 1985). There are also reports that bacteria produce bioemulsifier only in nutrient limited or starvation conditions (Singh, et al, 1990). This may be one of the reasons for the production of bioemulsifier by *Acinetobacter*. It is also known that triglycerides in the small amounts stimulate the growth of*A. lwoffii* on hexadecane (Breuil & Kushner, 1979).

Root exudates of wheat plant such as amino acids, sugars and organic acids (Subba Rao, 1986; van Elssas, 1992) affect on bioemulsifier activity and showed that protein, sugars and protein plus sugar combination increased the activity of bioemulsifier. This provided the clue that bioemulsifier production may be enhanced or stimulated by root exudates of wheat plant. Some sugars such as rhamnose, maltose, innositol showed increase in activity, also in amino acids L- ornithine, L-arginine, L-tyrosine and L-phenyl alanine enhanced the activity of bioemulsifier. Combinations of these sugars and amino acids showed more increased activity. Interestingly, rhamnose and phenylalanine showed highest emulsification activity. It concludes that rhamnose and phenylalanine may be present in the structure of this bioemulsifier due to which addition of these two compounds increased activity. However, at the same time, addition of organic acid did not show much increase in the activity of bioemulsifier. So far to the best of our knowledge there is no report on effect of root exudates on bioemulsifier production. This constitutes the first report.

Stability of the bioemulsifier formed with groundnut oil remained stable at room temperature for more then seven days as its 92% activity was retained. This is very important characteristic of this bioemulsifier as it benefits in increasing self life of he product as well as easier storage of the bioemulsifier (Patil & Chopade,2001). Similar results were obtained in case bioemulsifier produced from *P. fluorescens* (Powalla, et al, 1989) and *B. subtilis* (Cooper et al, 1981).This emulsifier is highly soluble in water, which is very important in the view of application as it can be easily applicable in all the fields such as in formulation of pesticides, pharmacy, food and medicine. It showed considerable decrease in the viscosity of groundnut oil and at thesame time it showed increase in the viscosity of water which is an important property of a good bioemulsifier.

This bioemulsifier can also act very effective cleaning agent, and dissolves in cold water immediately, shows property of a true surfactant. Itconcludes that it can act as a good surfactant. The surface tension of water was reduced by 10 dynes/cm by this bioemulsifier. If we compare surface tension with other bioemulsifier such as bioemulsifier of glycolipids produced by *P. aeruginosa* which showed surface tension, 29 dynes /cm, bioemulsifier of fatty acids / neutral lipids produced by *N. erythropolis* had surface tension 32 dynes/cm. Surface tension of lipopeptides/lilpoproteins in nature produced by *P. fluorescens* was 26.5 dynes/cm, and polymeric surfactant produced by *P. fluorescens* had surface tension 27 dynes/cm (Robert et, al, 1989; Martin, et al. 1969; Desai & Patel, 1994). Emulsan, a bioemulsifier produced by *A. calcoaceticus* RAG-1 also showed decrease in surface tension of water by 10 dynes/cm (Gutnick & Shabtai, 1987). Similar results were obtained with bioemulsifier produced by *A. baumannii* A25.

The bioemulsifier produced by *A. baumannii* A25 was a protein-polysaccharide complex which may be made up of long chains of protein polymer. Thisbioemulsifier is rich in protein 85%, and polysaccharide 15%. This chemical composition appears to be new if compared with

previous structures of bioemulsifiers. There was no detail study of this type of bioemulsifier where protein is more than polysaccharide. However, there are reports of bioemulsifier where polysaccharide is more than protein such as bioemulsifier of *A. calcoaceticus* BD4 (Gutnick & Shabtai, 1987; Kaplan et al, 1987) . Also Emulsan of *A. calcoaceticus* RAG-1 had a major part of polysaccharide (Zuckerberg, et al, 1979). Alasan is also one of the bioemulsifiers, rich in polysaccharides (80 %) (Navon-Venezia, et al, 1995). If we observe carefully, we find that these all bioemulsifiers had a little part of lipid and major part of polysaccharide and then protein. In view of this background, bioemulsifier of *A. baumannii* A25, has major difference in that there is no lipid in this bioemulsifier. To the best of our knowledge there is no bioemulsifier which is rich in protein up to 85%. Therefore, this definitely appears to be a new bioemulsifier which gives very high yield of 6.6 g/liter and activity as compare to other bioemulsifier. Therefore we propose the name of this bioemulsifier as " Chopadesan".

Reconstitution of *A. baumannii* A25 reveled that its protein was the main fraction and alone exhibited the high emulsification activity. From Table 2, it is clear that both protein (Bioemulsifier Protein + Extracellular Protein) in different combination showed highest activity at the same time polysaccharide alone and in combinations (Exopolysaccharide + Bioemulsifer polysaccharide) exhibited low activity as compared to protein. Intrestingly we achieved reconstitution up to 99% (2280 $EUml^{-1}$) which requires all the fractions, only protein gave activity up to 98 % but for maximum reconstitution, polysaccharisde were also required. Protein fraction in this bioemulsifier played a crucial role as in case of *A. calcoaceticus* BD4. *A. calcoaceticus* BD4 had also a macrocapsule, responsible for high emulsification activity (Kaplan & Rosenberg, 1982) and the capsule of RAG-1 has been demonstrated as cell-bound, emulsan (Sar & Rosenberg, 1983). *A baumannii* A25 have capsule, like *A. junii* SC14 from human skin (Patil & Chopade, 2001). Disappearance of cellular capsule during stationary growth phase and its subsequent release into the fermentation broth are attributable to the simultaneous maximum production of the bioemulsifier (Patil & Chopade, 2001).

In case of emulsan, bioemulsifier accumulates on the cell surface during logarithmic growth phase and subsequently releases into the medium in the stationary phase (Sar & Rosenberg, 1983). *A. baumannii* A25 from rhizosphere of wheat, showed same pattern for esterase. At 48 h stationary phase 245 U ml^{-1} was reported as well as 90 Umg^{-1} was reported from cell associated esterase at 36 h. In case of *A. junii* SC14, isolated from healthy human skin, significant esterase activity was observed to be associated with the cells while only a minor fraction was in the fermentation broth (Patil & Chopade, 2001). Role of esterase is to release the bioemulsifier from the cell. This bioemulsifier showed an increase in cell free esterase at 48 h and at the same time cell associated esterase was decreased. This suggests that like emulsan, this bioemulsifier also released from cells into the medium and increased production of bioemulsifier.

Cell surface hydrophobicity assay of *A. baumannii* A25 for xylene compared with *P. aeruginosa* NCIM 1226 showed that *A baumannii* A25 had slightly less affinity towards xylene than *P. aeruginosa* NCIM 1226. Emulsification activity of *P. aeruginosa* (300 EU ml^{-1}) is more for xylene as compare to *A. baumannii* A25 (200 EUml^{-1}). In case of hexadecane, *A. baumannii* A25 showed lower affinity towards hexadecane than *P. aeruginosa* NCIM 1226 but its emulsification activity is more (350 EU ml^{-1}) than *P. aeruginosa* NCIM 1226 (210 EU ml^{-1}). Cell hydrophobicity for groundnut oil is greater for *A. baumannii* A25 than *P. aeruginosa* NCIM 1226 and it also showed very high emulsification activity (1200 EU ml^{-1}) than *P. aeruginosa* NCIM 1226 (250 EU ml^{-1}). In earlier report *P. aeruginosa* PAS 279 has been reported for the same results (Rosenberg, et al, 1980). Thus it was observed that xylene is less utilized by *A. baumannii* A25 and it also showed lower affinity towards xylene as compare to *P. aeruginosa* NCIM 1226. *A. baumannii* A25 showed lower affinity than *P. aeruginosa* for hexadecane but it utilizes hexadecane more efficiently than *P. aeruginosa* NCIM 1226. In case of groundnut oil, *A. baumannii* A25 showed greater affinity than *P. aeruginosa* NCIM 1226 and also utilize it more efficiently than *P. aeruginosa* NCIM 1226.

It is interesting to know that rhizobacteria are also able to produce emulsifiers. It is already known that *Bacillus* spp. present is soil can produce bioemulsifiers which have antimicrobial activities (Fiechter, 1992), thus these bacteria produce bioemulsifers to protect themselves from pathogens. It is also known that these bacteria can degrade hydrocarbons which cannot degrade easily by other bacteria, hence used effectively in bioremedation (Desai & Patel, 1994). To summerize, present work has demonstrated excellent emulsifying activity by *A. baumannii* A25, isolated from rhizosphere of wheat. On the basis of the results obtained from present work, it appears that rhizosphere of wheat is indeed excellent source of novel bioemulsifier producing bacteria. It can be successfully used in the bioremedation of heavily contaminated soils. This is the foundation for search of novel biopolymer like bioemulsifiers from rhizosphere. This is also a study on *Acinetobacter* and plant interactions on which the future investigation can be done.

5.6. Conclusion :

- *Acinetobacter* spp. isolated from rhizosphere of wheat is able to producebioemulsifier.
- Bioemulsifier produced by *A. baumannii* A25 is protein and polysaccharide complex.
- This bioemulsifier is named as "Chopadesan" has interesting properties such as high yield, good cleaning property and high emulsification activity.

5.7. Significance of this work :

- This is the first report on production of bioemulsifier by *Acinetobacter* spp. isolated from rhizosphere of wheat. On the basis of these interesting results during the present study, rhizosphere of wheat appears to be good source of bacteria capable of bioemulsifier production. Bioemulsifier

produced by rhizosphere *Acinetobacter* may play an important role for existence in rhizosphere by colonization and adhesion of roots of crop plants. In addition it can degrade hydrocarbons from soil and hence can be used in bioremediation.

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CHAPTER 6

Isolation, Characterization and Plasmid pUPI126 Mediated Indole - 3- Acetic Acid (IAA) Production in *Acinetobacter* Strains from Rhizosphere of Wheat.

Chapter 6

Isolation, Characterization and Plasmid pUPI126 Mediated Indole 3 Acetic Acid (IAA) Production in *Acinetobacter* Strains from Rhizosphere of Wheat.

6.1. Abstract

37 strains of *Acinetobacter* isolated and characterized from rhizosphere of wheat were screened for indole acetic acid (IAA) production. Only eight *Acinetobacter* strains showed indole acetic acid production. The genus *Acinetobacter* was confirmed by chromosomal DNA transformation assay. Biotyping of eight strains was carried out and were found to be genospecies of *A. junii*, *A. baumannii* , *A.* genospecies 3 and *A. haemolyticus*. Five strains, out of eight produced IAA at the early stationary phase : *A. haemolyticus* A19, *A. baumannii* A18, A16, A13 and *A.* genospecies 3 A15. *A. junii* A6 showed maximum IAA production at log phase and *A.* genospecies 3 A28 and *A. baumannii* A30 showed maximum IAA production at late stationary phase. IAA was extracted by ethyl acetate and purified by preparative TLC. Purified IAA was confirmed by ^{1}H-nuclear magnetic resonance and infrared spectrum analysis. Pot experiments showed significant increase in plant growth inoculated with eight *Acinetobacter* strains such as *A. haemolyticus* A19, *A. baumannii* A18, A16, A13, A30, *A.* genospecies 3 A15, A28 and *A. junii* A6, as compared to control plants. It was also observed that development of inflorescence stage (45 days) and fruiting stage (60 days) were observed 10 days earlier in inoculated plants as compared to control. IAA production was encoded by plasmid pUPI126. All eight strains of *Acinetobacter* contain a plasmid pUPI126 with molecular weight of 40 kb. Plasmid pUPI126 was transformed into *E. coli* HB101 at a frequency of 5 x10^5 per μg of DNA and *E. coli* HB101 (pUPI126) transformants also showed IAA activity. pUPI126 also encoded resistance to selenium, tellurium and lead. This is the first report of plasmid encoded IAA production in the genus *Acinetobacter*.

Key words : *Acinetobacter* spp, Wheat rhizosphere, Tryptophan, IAA production, PlasmidpUPI126, Transformation, IR analysis, ^{1}H-NMR, Plant growth promotion.

6.2. Introduction :

Acinetobacter species are ubiquitous in nature (Baumann, 1968; Juni, 1972,). *Acinetobacter* is commonly found in soil, water, food and also present on healthy human skin (Saha & Chopade, 2001; Patil & Chopade 2001; Dhakephalkar and Chopade, 1994). *Acinetobacter* is one of the known opportunistic human pathogens (Dhakephalkar & Chopade 1994; Chopade et al, 1994a & 1994b). It also possesses number of naturally occurring plasmids exhibiting resistance to antibiotics and heavy metals (Deshpande et al 1994; Shakibaie et al, 1999). There are few reports on presence of *Acinetobacter* in soil, however, detail studies regarding its occurrence, distribution, growth pattern, physiology and its interactions with other soil microorganisms are not known. There is only one statement on presence of *Acinetobacter* in wheat rhizosphere (Kleeberger et al, 1983). However, detail information about role of *Acinetobacter* in rhizosphere is not known.

Soil is a rich environment for growth of microorganisms and specifically rhizosphere is highly specialized environment in soil for growth of microorganisms. Since rhizosphere contains large number of microorganisms one would expect plasmid transfer and dynamics of plasmid transfer from *Acinetobacter* to other microorganisms and vice versa in the rhizosphere environment. The rhizosphere of each and every plant is very specific with respect to the root exudates as it is the main source of nutrients for rhizosphere microorganisms (Subba Rao, 1986).

Until now there is no report on involvement of plasmid/s in the production of IAA from genus *Acinetobacter*. IAA is one of the major plant growth promoting hormones produced by plants as well as some bacteria and fungi (Arshad & Frankenberger,1991). Many species of bacteria produce IAA, especially when growth media are supplemented with tryptophan, a precursor of IAA. Number of microorganisms like *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Pseudomonas savastanoi*, *Pseudomonas* spp.,(Leinhos & Vocek, 1984), *Rhizobium* spp., (Baldi et al, 1991), *Bradyrhizobium* spp. and *Azospirillum* spp (Bashan et al, 1989) present in the rhizosphere of plants are known to produce IAA (Costacurta & Vanderleyden,1995). To some extent the biosynthesis of plant growth promoting substances like auxin/s from phosphate solubilizing rhizobacteria from rhizosphere of wheat and rye is reported (Leinhos & Vocek, 1984). Aim of this work was to isolate and characterize *Acinetobacter* from rhizosphere of wheat and to find out the role of *Acinetobacter* in plant growth promotion in general and involvement of plasmid/s in production of plant growth promoting substance like indole acetic acid (IAA).

6.3 Materials and Methods :

6.3.1. Sample Collection :

Sample collection was done as described in Chapter 2 (section 2.3.1 and 2.3.2).

6.3.2. Culture Media :

Culture media used are described in Chapter 2 (section 2.3.3).

6.3.3. Isolation of *Acinetobacter* from rhizosphere :

Isolation of *Acinetobacter* spp. from rhizosphere of wheat was done as described in Chapter 2 (section 2.3.4).

6.3.4. Chromosomal DNA transformation assay :

Chromosomal DNA transformation assay was done as described in Chapter2 (in section 2.3.5).

6.3.5. Identification of *Acinetobacter* strains up to species level :

Identification of *Acinetobacter* strains up to species level by employing different biochemical tests as described in Chapter3 (section 3.4).

6.3.6. Detection of IAA production in *Acinetobacter* spp. :

IAA production was detected by two methods as described below :

6.3.6.1. Nitrocellulose paper assay :

All 37 *Acinetobacter* strains were tested by nitrocellulose paper assay for the production of IAA (Bric et al 1991). 37 *Acinetobacter* strains were spot inoculated on LB medium supplemented with 5mM tryptophan (LBT). The spot inoculated agar surface was overlaid with a nitrocellulose membrane filter and incubated at 28°C for 48 h. The membrane filter (Sartorious, Germany, 0.45 mm) was aseptically removed from the plate, after 48 h and transferred to Whatman filter paper No. 2 and 500µl of Salkowaski reagent (2% of 0.5M $FeCl_3$ in 35 % perchloric acid or $FeCl_3$ 2.025 g in 300 ml of conc. H_2SO_4 and 500 ml of distilled water) was added on the nitrocellulose paper and kept for 1-2 min. at room temperature. IAA production was indicated by red ring around the colony on LB agar plates.

6.3.6.2. Salkowaski method :

37 *Acinetobacter* spp were grown up to 48 h at 28°C in the LB broth supplemented with 1mg/ml of tryptophan. After 48 h of incubation cells were harvested by centrifugation at 10000 rpm for 15 min. at room temperature and 1 ml of (supernatant) sample and 4 ml of Salkowaski reagent were mixed and allowed to react in dark at room temperature for 30 min. 1ml of uninoculated LBT and 4 ml of Salkowaski reagent was treated as blank. Optical density (O.D) was checked at 540 nm. Red color formation was considered as positive evidence for IAA production (Gordons & Weber, 1951).

6.3.7. Time course of IAA production in *Acinetobacter* spp. :

IAA production by *Acinetobacter* strains at different growth phases was also studied. *Acinetobacter* strains were inoculated in (100µl) LBT medium, incubated at 28°C at 120 rpm and production of IAA was checked after every 2 h up to 108 h by Salkowaski method.

6.3.8. Extraction and purification of IAA by preparative thin layer chromatography (TLC) :

IAA produced by *Acinetobacter* genospecies was purified by method described by Koga, et al (1991). In brief : all strains were grown in (150 ml) LBT medium, at 200rpm and 28°C, till it shows maximum IAA production. 150 ml culture broth was centrifuged at 8000 rpm (Remi, RMI2C, India) for 20 min at room temperature. pH of the supernatant was adjusted to 7 . This was treated as neutral extract and it was extracted with 1:1 volume of ethyl acetate. The aqueous phase was carefully separated and pH was adjusted to 2.8 with HCl. This was treated as acid extract. This acid extract was again extracted with 1:1 volume of ethyl acetate. Organic phases from both extractions were mixed together and evaporated on rota-evaporator at 60°C (Buchi, Switzerland) to get powdered IAA. At each phase of extraction, Salkowaski test was done for organic as well as aqueous phase. The preparative TLC was run for the extracted samples with the standard indole 3 acetic acid (Sigma,USA) using methanol : chloroform (10: 90) as a solvent system. The TLC was carried out on polygram G / UV 254 precoated aluminium sheet of 20 X 20 and 60 mm (Merck ,

Germany). The TLC spots were observed under UV (245 nm) and compared with standard IAA sample (Sigma, USA). IAA spots were scratched with a fine spatula and sample with silica gel was collected in a clean glass bottle. The sample was dissolved in 2 ml of chloroform or ethyl acetate and filtered through cotton to remove silica. TLC of the sample was done to check purity of the sample.

6.3.9. Identification of IAA by infra red (IR) spectrum and melting point :

IR spectrum of extracted IAA sample was taken using Perkin Elmer 1600 FTIR Spectrophotometer and the spectra were recorded in nujol mull or in KBr pellets and expressed in wave number (cm^{-1}). The melting point of extracted IAA was tested on Thomos Hoover melting point apparatus in the Degree Celsius.

6.3.10. Analysis of IAA by ^{1}H-NMR :

The purified sample of IAA was analyzed by Mercury ^{1}H-NMR (300 MHZ, Vavion , USA) and the peaks were identified for IAA. The ^{1}H-NMR of standard IAA was also checked. The two ^{1}H-NMR were compared. The purified IAA was dissolved in 25 µl of DMSO.

6.3.11. Effect of pH on IAA production :

To study effect of pH on the production of IAA, buffered LBT broth (100 ml) was prepared in the standard buffers such as acetate, phosphate and Tris –HCl (Gerhardt, et al, 1994). pH in the range of 4 to 9 was checked. The IAA production at different pH was checked by Salkowaski test.

6.3.12. Effect of IAA production by *Acinetobacter* spp. on growth of wheat plant :

The effect of IAA production on growth of wheat plant was tested by employing pot experiments. All eight *Acinetobacter* strains were grown separately in following four media i) LB, ii) LBT: containing 1mg /ml of tryptophan iii) AMM, iv) AMMT: containing 1mg /ml of tryptophan. LB and AMM were used as a control media and LBT and AMMT were used for IAA production. Eight *Acinetobacter* genospecies were inoculated in all 4 media and incubated at 120 rpm at 28^{0}C up to 48h. The wheat seeds were surface sterilized by 2% $HgCl_2$ and washed with sterile distilled water for 6 to7 times to remove $HgCl_2$ completely (Subba Rao, 1988). After washing, seeds were added in above-mentioned culture and kept on shaker at 120 rpm for 2 h at 28^{0}C. After 2 h, wheat seeds were aseptically collected and inoculated in pots containing sterile soil. Wheat seeds mixed with uninoculated media as well as with distilled water were treated as control. The pots were kept in sunlight and raised under close supervision. The growth of plants was observed everyday for 21 days. After 21 days plants were carefully uprooted and root and shoot lengths were measured. Same experiment was done simultaneously using large size pots up to 4 months covering complete life cycle of wheat plant.

6.3.13. Statistical analysis:

Root length (RL) and shoot length (SL) were considered as main parameters to find out effect of IAA on wheat plant. Statistical analysis was done with the help of mean, standard deviation and analysis of variance (ANOVA) (Kulkarni et al, 1999).

6.3.14. Plasmid isolation :

Eight strains were checked for presence of plasmid/s by three different methods described by Kado & Liu (1981), Sambrook et al (1989) and Birnboim & Doly (1979). The presence of plasmid/s was tested by 0.7 % agarose gel electrophoresis in TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8), at 52 V for 6 to 8 h. Ethidium bromide (0.05 lg/ml) stained gels were observed under gel documentation system (Alpha Imager™ 2200 Documentation and Analysis System, Alpha Innotech Corporation, California, USA) and photographed. Molecular weight of plasmid was determined by comparing with 1 kb DNA ladder.

6.3.15. Plasmid curing :

Plasmid curing was done for all eight strains of *Acinetobacter* genospecies using ethidium bromide (1024 μg/ml) and heat (52^0C) as described by Deshpande & Chopade (1994).

6.3.16. Transformation :.

Plasmid DNA isolated from *A. haemolyticus* A19 was used for transformation of DNA as this strain showed good IAA production and also exhibited other interesting characteristics such as resistance to selenium, lead and tellurium, which were used as genetic markers for plasmid transformation. Chitinase production and antimicrobial activity against plant as well as human pathogenic fungi and bacteria (Huddedar & Chopade, 2000). Transformation was carried out by preparing *E. coli* HB101 rif^r mutant cells competent by $CaCl_2$ method (Sambrook et al, 1989) and competent *E. coli* cells were mixed with plasmid pUPI126 DNA (10 μg/ml). The transformants were selected and checked for IAA production by Salkowaski test and parent recipient *E. coli* HB101 rif^r mutant was used as control.

6.4. Results :

6.4.1. Isolation, identification and confirmation of *Acinetobacter* genospecies :

Colonies showing mucoid character were selected and tested for Gram character and morphology, motility, presence of capsule, oxidase and catalase production. Gram negative, coccobacilli, non motile, oxidase negative, catalase positive and capsulated strains were considered as tentative *Acinetobacter* spp. and confirmed by chromosomal DNA transformation assay. 37 *Acinetobacter* strains isolated from five life stages of wheat plant were confirmed as genuine *Acinetobacter* spp. *Acinetobacter* strains were identified to species level on the basis of biochemical tests. These *Acinetobacter* spp. were not biotypable by API 20NE system. Eight IAA producing strains were

identified as *A. baumannii* A18, A16, A13 and A30, *A. haemolyticus* A19, *A. junii* A6 and *A.* genospecies 3, A15 and A28 (Table 1).

6.4.2. Production of IAA by *Acinetobacter* spp. :

All 37 *Acinetobacter* strains were tested for IAA production by nitrocellulose paper assay. It was observed that among 37 *Acinetobacter* strains, only 8 *Acinetobacter* strains showed red ring around growth of bacterial colony within 1 min on addition of Salkowaski reagent. IAA production for these eight *Acinetobacter* strains was also detected by Salkowaski method and development of red color indicated presence of IAA. 1 ml distilled water and 4 ml of Salkowaski reagent was taken as negative control.

6.4.3. Time course of IAA production in *Acinetobacter* spp. :

It was found that *A. haemolyticus* A19, *A. baumannii* A18, A16, A13 and *A.* genospecies 3 A15, showed maximum IAA production in early stationary phase (48 h). *A. junii* A6 showed maximum IAA production in log phase (24 h) and *A.* genospecies 3 A28 and *A. baumannii* A30 showed maximum IAA production in late stationary phase (60 and 72 h). Fig. 1. A and B, represent the growth phase with maximum IAA production by each *Acinetobacter* spp.

6.4.4. Extraction and purification of IAA by TLC :

Extraction of IAA was done by ethyl acetate. The Salkowaski test was done at each step of the extraction showed that only the organic phase contained IAA. The dry powder obtained after evaporation of ethyl acetate showed presence of IAA which was further fractionated by preparative TLC. The band pattern of purified IAA was comparable with standard IAA. (Fig 2). It was observed that when these bands from TLC were scratched and again tested by TLC, showed a single band as that of standard IAA with R_f value 0.5. The amount of auxin found in the culture filtrate was 4 mg/liter.

6.4.5. Identification of IAA by IR spectrum and by melting point :

The IR spectrum of purified IAA showed OH frequency at 3384.9 cm^{-1} and C = O frequency at 1698.4 cm^{-1} (Fig 3). IR spectrum of standard IAA also showed same results. The melting point of purified IAA was found to be 168^0C , which is also same as that of standard IAA (Sigma, USA).

6.4.6. Analysis of IAA by ^{1}H-NMR :

The ^{1}H-NMR of purified eight IAA samples was found to be same as that of ^{1}H-NMR of standard IAA (Fig. 4). The first peak from right is of acid, of value 9.0 δ bs (-OH), lateral peaks are of protons having values 7.8 δ d 1H (C_8H) , 7.5 δ d 1H (C_5H), 7.31δ d 1H (C_2H) and 7.07 δ m 2H (C_6 & C_7 H). Middle big peak is of DMSO having value of 3.04 δ S (d^6) and moisture. The next peak is of carbon and hydrogen, having value of 2.59 δ S 2H ($-CH_2-$) and last peak is of internal standard, tetra-methyl-silnate (TMS) of value 0.08.

6.4.7. Effect of pH on IAA production :

It was observed that at acidic pH (pH 4 & 5) *Acinetobacter* genospecies could not grow. The growth and IAA production was observed from pH 6 to pH 9 and found that pH 7 was optimum for IAA production by *A. baumannii* A16, A18, A30, *A.* genospecies 3 A15, *A. haemolyticus* A19, and *A. junii* A6. Two strains viz. *A. baumannii* A13 and *A.* genospecies 3 A28 showed optimum IAA production at pH 9 (Table 2).

6.4.8. Effect of IAA produced by *Acinetobacter* spp. on growth of wheat plant :.

Statistical analysis showed significant difference in root and shoot length of wheat plants tested (21 days) as compared to control plants (Table 3). Similar difference was observed when the same experiment was done using large size pots (Table 4). It was observed that there was a difference in color of leaves of control and inoculated plants. Color of leaves of plants inoculated with *Acinetobacter* spp. was dark green as compared to pale green of control. Interestingly width of shoots of inoculated plants was found to be almost double as compared to control. It was also observed that development of inflorescence stage (flowering stage, 45 days) and fruiting stages were observed 10 days earlier in inoculated plants as that of control. (Fig. 5).

6.4.9. Plasmid isolation :

All eight *Acinetobacter* strains contained one plasmid of the molecular weight 40 kb. All three methods of plasmid DNA isolation showed the presence of only one plasmid in each eight strains. The plasmid was designated as pUPI126.

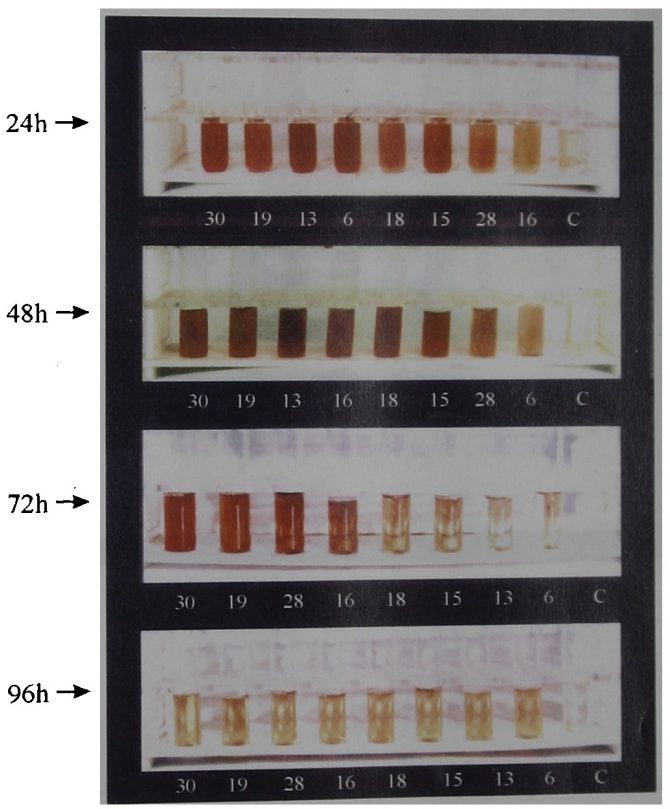
6.4.10. Plasmid curing :

Plasmid pUPI126 was not cured by ethidium bromide even at concentration of 1024 µg/ml and heat (52^0C). It is sensitive to 2048 µg/ml concentration of ethidium bromide.

6.4.11. Transformation of plasmid pUPI126 :

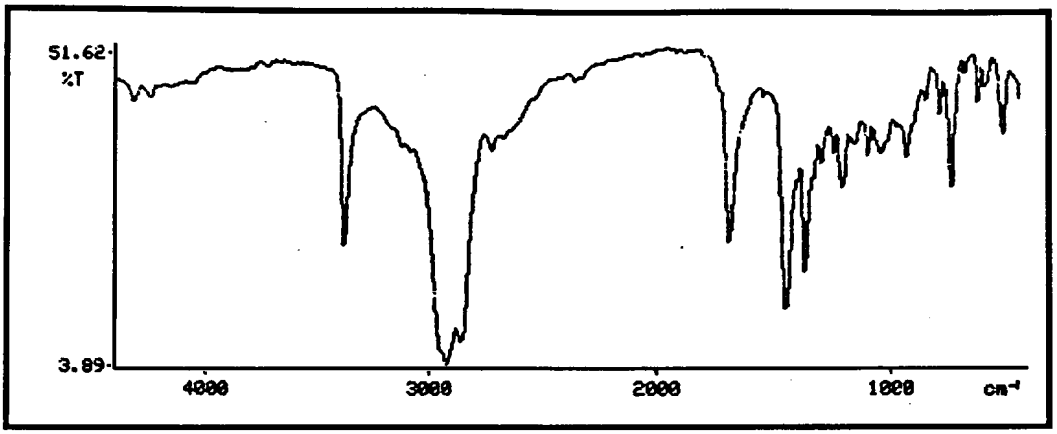
Plasmid pUPI126 was transformed to *E. coli* HB101 rif^r mutant at the frequency of 5×10^5 per µg of DNA. Along with IAA, selenium, tellurium and lead were also co-transferred almost at the same frequency. Transformants showed IAA production as checked by Salkowaski test. The color developed was pink as compared to red, developed by original host *A. haemolyticus* A19. *E. coli* HB101(rif^r) mutant did not show any color formation with Salkowaski reagent. Plasmid pUPI126 was isolated from transformants and observed by 0.7 % agarose gel electrophoresis.

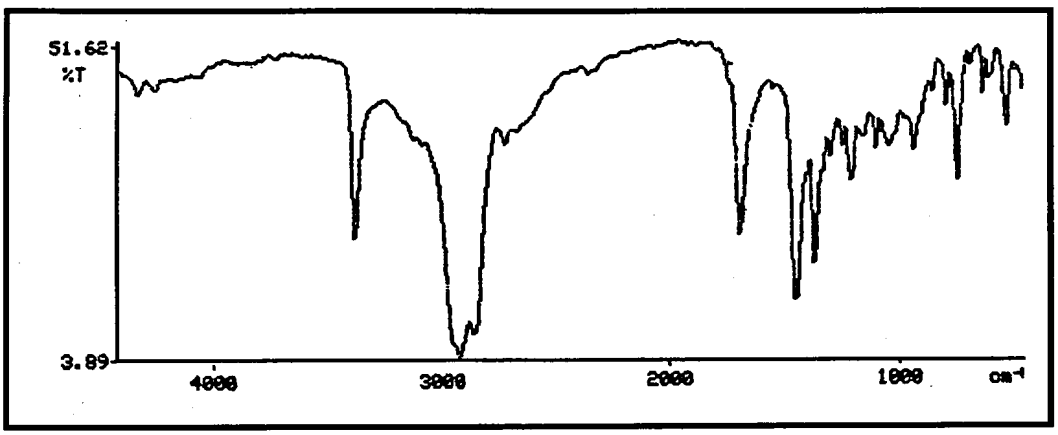
Fig.2A.IAA production at different growth phases of *Acinetobacter* spp. by Salkowaski method .



30: *A. baumannii* A30;
19: *A. haemolyticus* A19;
16:*A. baumannii* A16;
13:*A. baumannii* A13;
28: *A.* genospecies 3 A28;
18 :*A. baumannii* A18;
15: *A.* genospecies 3 A15;
6: *A. junii* A6; C: Control.

Fig.2B. TLC of purified and extracted IAA produced from *Acinetobacter* genospecies
S: purified IAA; 30: *A. baumannii* A30; 28: *A.* genospecies 3 A28;
19: *A. haemolyticus* A19;18 :*A. baumannii* A18; 16:*A. baumannii* A16;
15: *A.* genospecies 3 A15; 13:*A. baumannii* A13;
6: *A. junii* A6 and I: standard IAA (Sigma,USA).





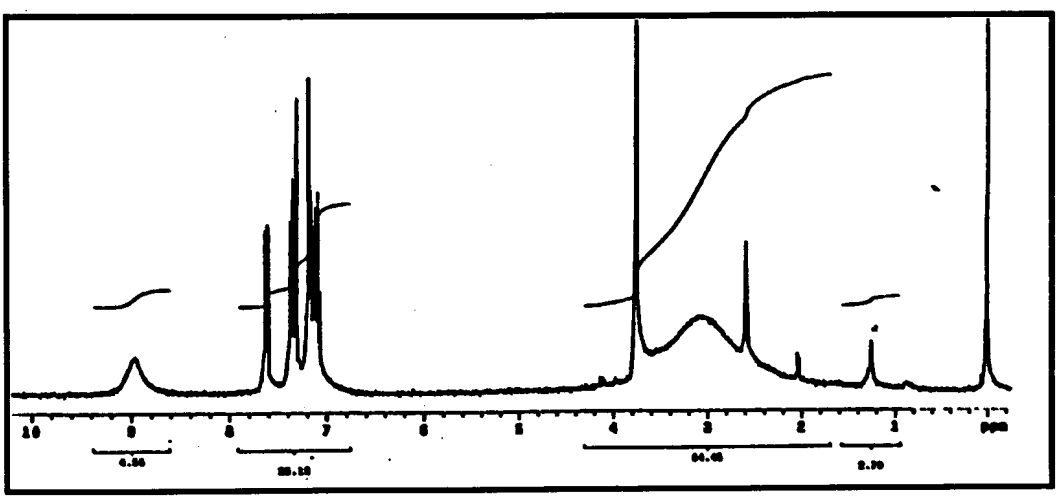


Fig.3. IR analysis of purified IAA from *Acinetobacter* genospecies.
A: The Infra Red (IR) spectrum of purified IAA from *Acinetobacter* genospecies.
% T: percent transmision, cm^{-1} : wave length in cm.
B: The Infra Red (IR) spectrum of standard IAA from sigma.
% T: percent transmision, cm^{-1} : wave length in cm.
Fig.4. ^{1}H-NMR analysis of purified IAA from *Acinetobacter* genospecies A28.*
*Similar pattern was observed in other 7 IAA producing *Acinetobacter* genospecies.

Table .1. Identification of IAA producing *Acinetobacter* spp. isolated from rhizosphere of wheat.

Characteristics	*A* .genospecies 3 (A15, A28)	*A .baumannii* (A13, A16, A18, A30)	*A. haemolyticus* (A19)	*A .junii* (A6)
Gram nature	Gram-ve	Gram-ve	Gram-ve	Gram-ve
Morphology	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli
Motility	-	-	-	-
Oxidase	-	-	-	-
Catalase	+	+	+	+
Capsule	+	+	+	+
Growth at				
44^{0}C	-	+	+	+
41^{0}C	+	+	+	+
37 ^{0}C	+	+	+	+
28 ^{0}C	+	+	+	+
Gelatin hydrolysis	-	-	+	+
Haemolysis	+	-	+	-
Citrate	+	+	+	+
Acid from glucose	+	+	+	+
Utilization of :				
Glutarate	+	+	+	+
L-phenylalanine	+	+	+	-
Phenylacetae	-	-	+	-
Malonate	+	+	+	+
L-histidine	+	+	+	+
D-malate	+	+	+	+
L-aspartate	+	+	+	+
L-leucine	+	+	-	+
L-tyrosine	+	+	-	+
β-alanine	+	+	+	+
Ethanol	+	+	+	+
Trans aconitate	+	+	+	-
L-arginine	+	+	+	+
DL-4-amino butyrate	+	+	+	-
DL-lactate	+	+	+	+

* - : Negative, + : Positive.

Table 2. Effect of pH on IAA production by *Acinetobacter* genospecies*.

pH	*A.* genospecies 3		*A. baumannii*				*A. junii*	*A. haemolyticus*
	A15	A28	A16	A18	A30	A13	A6	A19
6	0.02	0.08	0.07	0.81	1.23	0.06	1.46	1.22
7	0.13	0.14	0.32	1.10	1.90	1.45	1.43	1.95
8	0.11	0.23	0.21	0.92	1.12	1.46	1.35	1.01
9	0.10	0.35	0.29	0.58	0.88	1.52	1.28	0.55

* OD measured at 540 nm.

* At pH 4 and 5 there was no growth of *Acinetobacter* genospecies hence no IAA production.

Table. 3. Effect of IAA producing *A.* genospecies on root length and shoot length of 21 days wheat plant by ANOVA test.

Source	Root Length (cm)				Shoot Length (cm)			
	df	SS	MS	F	df	SS	MS	F
Medium	4	2193.7	548.4	33.2	4	3407.8	851.9	42.5
Bacteria	8	3900.4	487.5	29.5	8	1770.0	221.2	11.0
Interaction	24	3814.7	158.9	9.6	24	2669.7	111.2	5.5
Error	1069	17622.6	16.4	-	648	154726.4	20.0	-
Total	1105	27,531.4	-	-	684	-	-	-

df : degree of freedom, SS : sum of squares, MS : mean squares, F : F-test., Medium : AMM, LB, AMMT and LBT, - : not necessary, Bacteria : *Acinetobacter* genospecies, Interaction : Interactions takes place between bacteria-bacteria and media-bacteria.

Table 4. Effect of IAA produced by *Acinetobacter* genospecies on root and shoot length of wheat plant at ripened fruiting stage (75days)[a].

	Root length (cm)		Shoot length (cm)	
A. genospecies	Mean	SD	Mean	SD
A. *baumannii*				
A18	36.7	5.02	40.1	0.1
A13	32.3	2.5	36.9	5.7
A30	32.4	4.8	35.1	4.8
A16	37.2	2.3	33.5	5.5
A. haemolyticus				
A19	35.3	3.5	41.7	2.8
A. junii				
A6	35.6	2.5	40.1	0.1
A. genospecies 3				
A15	37.2	4.3	40.3	0.3
A28	41.4	2.1	42.6	2.08

[a] Values are based on three sets of experiments.

SD : Standard Deviation.

Fig. 1.A. IAA production by four *Acinetobacter* genospecies - *A. baumannii* A13, *A.* genospecies 3 A15 & A28, *A. junii* A6 isolated from rhizosphere of wheat.

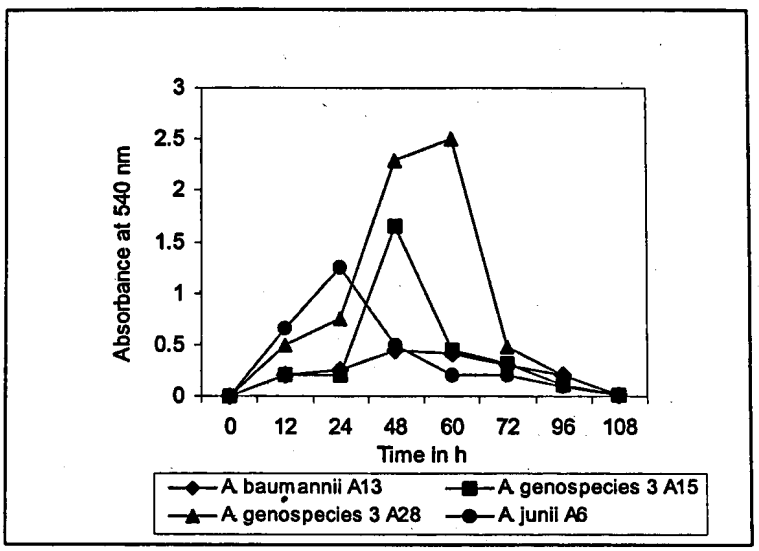


Fig. 1.B. IAA production by four *Acinetobacter* genospecies, *A. haemolyticus* A19 and *A. baumannii* A18, A16 and A30 isolated from rhizosphere of wheat

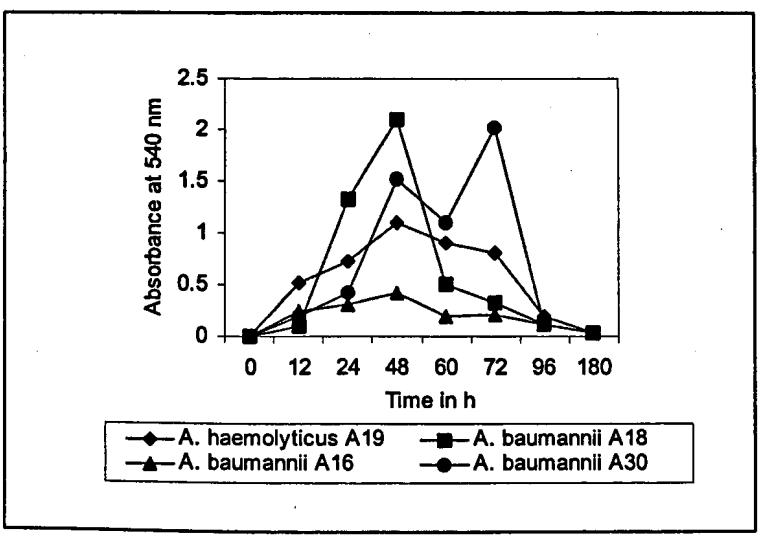


Fig.5

Fig.6

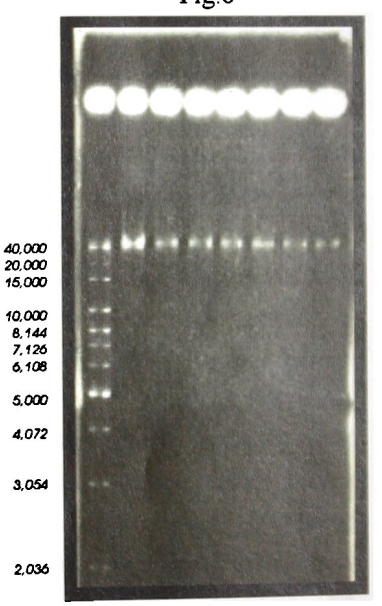


Fig.5. Effect of IAA produced by *Acinetobacter* genospecie 3 A28 on growth of wheat plant.*
A : Root and shoot length of 21-d wheat plant; B : Shoot width of 60-d wheat plant.
C : Leaf width of 60-d wheat plant; D: fruiting size and number of grains of 75-d wheat plant.
C : Control; S : Sample; d : Days

* Note : Similar growth pattern was observed by other 7 IAA producing *Acinetobacter* genospecies.

Fig.6. Plasmid pUPI126 in IAA producing *Acinetobacter* genospecies.
Lane 1 : 1 Kb DNA ladder; Lane 2 : *A. baumannii* A13; Lane 3 : *A. baumannii* A16
Lane 4 : *A.* genospecies 3. A15; Lane : 5 *A. baumannii* A18; Lane 6 : *A. junii*;
Lane 7 : *A. haemolyticus* A19 and Lane 8 : *A.* genospecies 3 A28.

6.5. Discussion :

Acinetobacter is commonly found in soil (Baumann, 1968; Dhakephalkar & Chopade 1994). Therefore it was logical to believe that it may be present in rhizosphere. However there is no report on the presence of *Acinetobacter* in the rhizosphere of plants including wheat plant, which is an economically important plant. There is only a passing statement on the presence of *Acinetobacter* in the wheat rhizosphere (Kleeberger et al, 1983). *Acinetobacter* is relatively recently studied group of microorganisms. This is because previously this bacteria was known by 40 different names and hence there has been a lot of confusion about the systematics of *Acinetobacter* spp. (Henriksen, 1973). With the development of chromosomal DNA transformation assay by Juni (1972 & 1978), a genuine method of confirmation of *Acinetobacter* was developed. This assay is specific only for *Acinetobacter* genospecies and based on this assay the authenticity of genus *Acinetobacter* was established (Juni, 1972 & 1978). At present the systematics of *Acinetobacter* is well defined and it consists of 21 genospecies (John, et al 1994; Bouvet & Grimont 1986 & 1987). Aim of this study was to find out the effect of IAA production by *Acinetobacter* strains on the overall life cycle of wheat plant.

Our work in this area was done systematically because of use of chromosomal DNA transformation assay for confirmation of genus *Acinetobacter* (Juni, 1972; Deshpande & Chopade, 1994). Interestingly all 37 isolates of *Acinetobacter* were confirmed by this assay. It is important to note that, out of 21 genospecies, only five *Acinetobacter* genospecies were detected from rhizosphere of wheat. Our findings have revealed that *Acinetobacter* is present in the rhizosphere of wheat in significant number (Huddedar & Chopade, 2001). This finding gave us the most valuable clue that *Acinetobacter* may have some role in the rhizosphere of wheat. It should be noted that *Acinetobacter* is not a plant pathogen. To find out its role in the plant growth promotion, we did screening of *Acinetobacter* spp for IAA production. It is important to note that until now there is no report on the production of IAA in the genus *Acinetobacter* from wheat rhizosphere. The biology of *Acinetobacter* is very similar to that of *Pseudomonas*, which is also found in the wheat rhizosphere. Moreover, *Pseudomonas* spp. from wheat rhizosphere also produced IAA (Kleeberger, et al 1983, Costacurta & Vanderleyden, 1995). Like *Pseudomonas savastanoi*, *Acinetobacter* genospecies also produced IAA by using tryptophan as a precursor. Determination of IAA producing capacity of microorganism is useful in its identification and provided a valuable marker when examining physiological role or ecological significance of IAA in establishment and persistence of organism in the rhizosphere (Bric et al, 1991). As compared to other IAA producing bacteria, production of IAA with respect to growth phase of *Acinetobacter* is similar that it produced IAA in stationary phase, but *A. junii* (A6) produced IAA in log phase. Interestingly the

IAA production by *Acinetobacter* is qualitatively strong as it takes dark red color within one min. when it reacts with Salkowaski reagent on the nitrocellulose paper as described by Bric et al, (1991).

The TLC of extracts clearly showed presence of IAA in all eight *Acinetobacter* genospecies (Fig.2). The ^{1}H-NMR, IR and melting point of extracted samples matched with the standard IAA. We found that pH also affected the IAA production and neutral pH (7) was found to be best for production of IAA for four *Acinetobacter* genospecies which were, *A.* genospecies 3, *A. baumannii*, *A. junii* and *A. haemolyticus* (A15, A16, A18, A13, A6 and A19). Out of eight, in two *Acinetobacter* genospecies, *A. baumannii* A13 and *A.* genospecies 3 A28, IAA was produced in maximum amount at alkaline pH as compared to acidic pH. This fact has ecological significance as, pH of clay soil used for cultivation of wheat in Maharashtra state in India, is alkaline (pH 8 to 10). The effect of IAA on plant root, shoot length and width, fruiting capacity and health of the plants as compared with control plant clearly indicated that IAA is produced by *Acinetobacter* and it is directly involved in plant growth promotion. The *Acinetobacter* genospecies grown in AMMT or LBT promoted maximum growth of wheat plants in pots since the growth media was supplemented with tryptophan, the precursor for IAA production. While the *Acinetobacter* strains grown in AMM and LB promoted less growth of plants, as there was lack of tryptophan. This observation indicates that plant growth promotion was definitely by IAA produced from *Acinetobacter* genospecies. Similarly the effect of an inoculation with IAA producing 3 *Pseudomonas* and 1 *Acinetobacter* on root growth resulted in increased shoot growth of maize plant has been demonstrated (Lippmann, et al, 1995).

Plasmid isolation and transformation of plasmid pUPI126 to *E. coli* HB101 rif^{r} mutant provided evidence that production of IAA and resistance to selenium , tellurium and lead genes are encoded on the plasmid pUPI126 in *Acinetobacter haemolyticus* A19 and *E. coli* HB101 transformants also produced IAA in stationary phase. Our findings are similar to previously published results of *Pseudomonas savastanoi* in which IAA producing genes are also encoded on plasmid pIAA1 (Comai & Kosuge,1982; Costacurta & Vanderleyden ,1995). To the best of our knowledge this is the first report of the IAA production in the genus *Acinetobacter*. It is observed that plasmid pUPI126 was not cured either by ethidium bromide or heat which suggests that this plasmid is very stable in its original host *Acinetobacter*. Plasmid transfer and behavior is well established in *Acinetobacter* (Chopade et al, 1985; Deshpande & Chopade 1994; Naik et al, 1994). It would be worth to investigate the behavior of plasmid pUPI126 in the rhizosphere microorganisms.

Besides indole acetic acid (IAA) encoded by plasmid pUPI126, other characteristics such as production of hormone/s like cytokinin as it has been reported in plasmid pP4TH in *Erwinia herbicola pv. phypsophiloe* (Clark, et al, 1993) are encoded by plasmid and this warrants further

investigation. This work has indeed established the role of *Acinetobacter* in wheat rhizosphere. It is expected that this work will provide stimulus to the work on *Acinetobacter* plant interactions in variety of economically important plants. Present study has successfully shown the effect of IAA on the growth of wheat plant. Large size pot experiments have confirmed this observation. Besides enhancement of growth of wheat plant, the flowering (inflorescence) and fruiting stages of life cycle were reached about 10 days earlier and persisted longer than the control. Overall, health of wheat plant was very much improved as compared to control. This indicates the potential of *Acinetobacter* as a novel bioinoculant for wheat plant. Further studies on cloning of IAA genes and its regulation, pathway of IAA biosynthesis and field studies on effect of IAA produced by different *Acinetobacter* genospecies on growth and yield of wheat plant and development of bioinoculant are in progress.

6.6. Conclusions :

- *Acinetobacter* spp. isolated from rhizosphere of wheat produced indole acetic acid (IAA).
- This is the first report on plasmid pUPI126 encoded IAA production in genus *Acinetobacter.*
- Plasmid pUPI126 is self transferable to *E. coli* HB101 by conjugation and transformation.
- IAA produced by *Acinetobacter* spp. increased shoot and root length of wheat plant and hence promotes plant growth.

6.7. Significance of this work :

- *Acinetobacter* is able to produce IAA and hence able to promote plant growth. It indicates that *Acinetobacter* has positive interaction with wheat and other crop plants and stimulates plant growth as other rhizobacteria. Thus *Acinetobacter* could be further used as plant growth promoting rhizobacteria .

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CHAPTER 7

Studies on Isolation of Rifampicin Resistant Mutants of *Acinetobacter* spp. and their Survival in a Lignite Carrier

Chapter 7

Studies on Isolation of Rifampicin Resistant Mutants of *Acinetobacter* spp. and their Survival in a Lignite Carrier.

7.1. Abstract :

Rifampicin resistant mutants from eight strains of *Acinetobacter* spp. producing indole acetic acid (IAA) were isolated. Frequency of spontaneous mutation of rifampicin resistant mutants were as follows : for genospecies *A. baumannii* A13.1 : 5 x 10^6, for *A baumannii* A16.1: 3 x 10^6, for *A. baumannii* A18.1: 6 x10^6 and for *A. baumannii* A30.1 : 4 x 106 cfu/ml, for *A.* genospecies 3 A15.1: 4 x 10^3, and for A28.1 : 2 x 10^4 cfu/ml. For *A. haemolyticus* A19.1: 8 x 10^5 and for *A. junii* A6 : 1 : 6 x 10^5 cfu/ml. All mutants were found to be 95 % stable. Biochemical tests carried out for mutants did not shown any significant differences in tests except some substrate utilization tests such as D-malate, phenyl acetate, L-leucine, tyrosine and 4-aminobutyric acid. Plasmid isolation from rif ^{r} mutants also showed presence of plasmid pUPI126 with same molecular weight 40 Kb as wild *Acinetobacter* spp. IAA was also produced by all eight rif ^{r} mutants with change in the growth phases of IAA production. *A. haemolyticus* A19.1, *A. baumannii* A18.1, A16.1, A13.1 and *A.* genospecies 3 A15.1, showed maximum IAA production in mid-stationary phase (60 h). *A. junii* A6.1 in early stationary phase (36 h) and *A.* genospecies 3 A28.1 and *A. baumannii* A30.1 in late stationary phase (60 and 72 h) same as parent. Wild as well as rif ^{r} mutants of all *Acinetobacter* spp. survived 95 % in a lignite carrier for 4 months at room temperature (28^{0}C). After 4 to 6 months 80 % decrease in the survival of *Acinetobacter* spp. was observed.

Key Words : *Acinetobacter* spp., IAA production, rif ^{r} mutants, Lignite.

7.2 Introduction :

Acinetobacter spp. isolated from rhizosphere of wheat were found to produce indole 3 acetic acid (IAA). IAA has a direct role on wheat plant growth promotion. (Huddedar et al, 2002). *Pseudomonas, Azotobacter, Azospirillum* are some of the bacterial species known for production of IAA and their effect on plant growth promotion. There is only one passing report (Leinhos et al, 1984) on production of IAA by *Acinetobacter* spp. However, no detailed study was carried out on its role in rhizosphere. Most of the study was carried out on *Pseudomonas* species and its colonization to wheat roots. For colonization study it is important to introduce the test bacteria in the actual field and observe the response of introduced bacteria on plants in the field. In field experiments, when bacteria are introduced in the field, it is very difficult to isolate and identify the introduced bacteria. For this purpose mutants of interested bacteria, having some special selective markers, differentiating from other bacteria present in rhizosphere and easy for detection were introduced. Spontaneous mutant isolation with the help of specific antibiotics is one of the widely used methods (Obaton 1971; Brockwell et al, 1977). Mainly rifampicin antibiotic is used for this purpose, as most of the soil bacteria are sensitive to rifampicin (Obaton 1971; Brockwell et al, 1977).

Different carriers can be used for the bioinoculum preparations. Peat has been widely used as a carrier for legume inoculants (Swtrijdom & Deschodt, 1976). In many areas of the world, however, peat is not readily available and is expensive. The alternative carriers can be used those include filter mud (Philpotts, 1976), lignite (Kandasamy & Prasad, 1971), coal (Crawford & Berryhill, 1983), a coal-bentonite mixture (Deschodt & Swtrijdom, 1976), cellulose (Pugashetti, et al, 1971), bagasse (Liderman, 1971), wheat straw (Schiel & Dieguez, 1970), a compost of core dust and soil (John, 1966) and charcoal, manure, compost, powdered coconut shells, ground teak leaves, and combinations of these substances (Tilak, et al, 1978). Carrier selection is very important, as bacteria have to survive in carrier with deficient nutrients. For bioinoculum preparation, survival of interested bacteria in a carrier, is one of the important parameters, on which further field experiments are dependent. Survival of bacteria in these carriers depends on the moisture and nutrient availability (Chao & Alexander, 1982). Survival of bacteria also depends on bacterial type (Date, 1976; Mary et al, 1985; Calcott & MacLeod, 1974). Survival at room temperature is also important parameter in storage of bioinoculum.

In the present study, we have selected lignite as a carrier having maximum moisture holding capacity and protection of bacteria from desiccation. Bacteria can also use it as a source of carbon. We have isolated rifampicin resistant mutants of *Acinetobacter*, and studied their biochemical characterization, plasmid isolation and IAA production. Survival of rif^r mutants in lignite carrier was also studied in comparison to wild strains of *Acinetobacter* spp.

7.3. Materials and Methods :

7.3.1. *Acinetobacter* spp. used for mutant isolation :

Eight wild *Acinetobacter* strains, producing IAA were used for isolation of rifampicin resistant mutants. These include : *A. baumannii* A18, A16, A13 and A30, *A. haemolyticus* A19, *A. junii* A6, and *A.* genospecies 3 A15 and A28.

7.3.2. Isolation of rifampicin resistant mutants of *Acinetobacter* spp.:

For the isolation of mutants, spontaneous method was used. 100 ml of Luria broth (pH 7-7.2), was inoculated with fresh cultures of each eight IAA producing *Acinetobacter* spp. separately, and flasks were incubated at 28^0C for 12-18 h at 120 rpm. Luria agar plates with 100µg/ml of rifampicin were prepared. 100µl of 18 h grown culture of each eight *Acinetobacter* spp. was spread on Luria agar containing 100µg/ml of rifampicin in each plate and plates were incubated at 28^0C for 5-6 days. After 6 days colony characters were observed and colonies of mutants developed on plates were isolated and purified. Purified colonies of resistant mutants were preserved on Luria agar slants and further biochemical characterization was carried out.

7.3.3. Stability of rifampicin resistant mutants of *Acinetobacter* :

Purified mutants were checked for stability. For stability checking, each rifampicin resistant mutant was grown in 100 ml of Luria broth with 100µg/ml of rifampicin and Luria broth

without rifampicin separately and flasks were incubated at 28°C for 18 h at 120 rpm. After 18 h, 100 µl of each grown culture was spread on Luria agar plates without rifampicin as well as Luria agar with 100 µg/ml of rifampicin, and plates were incubated for 24-48 h at 28°C and colony count was taken from all the plates. Stability of mutants was calculated as: Stability = Number of colonies on LA+ Rifampicin / Number of colonies on LA without rifampicin x 100.

7.3.4. Biochemical characterization of rifampicin resistant mutants of *Acinetobacter* spp. :

Biochemical characterization of rifampicin resistant mutants was carried out as per Bouvet & Grimont (1986 & 1987) and compared with respective wild *Acinetobacter* spp.

7.3.5. IAA production by rifampicin resistant mutants of *Acinetobacter* spp. :

Rif ^{r} mutants, isolated from IAA producing *Acinetobacter* spp. were tested for their IAA production employing Salkowaski method. In brief : In this method, eight *Acinetobacter* rif ^{r} mutants were grown at 28°C in the LB broth supplemented with 1mg/ml of tryptophan separately. After 48 h of incubation, cells were harvested by centrifugation at 10,000 rpm for 15 min. at room temperature (28-30°C) and 1 ml of supernatant and 4 ml of Salkowaski reagent were mixed and allowed to react in dark at room temperature for 30 min. (Gordons & Weber, 1951). 1ml of uninoculated LBT and 4 ml of Salkowaski reagent was treated as blank. Optical density (O.D) was checked at 540 nm. Red color formation was considered as positive evidence for IAA production (Bric, et al, 1991).

7.3.6. Time course of IAA production of rifampicin resistant mutants of *Acinetobacter* :

IAA production by each rifampicin resistant mutants of *Acinetobacter* spp. at different growth phases was also studied. *Acinetobacter* mutants were inoculated in 150ml LBT medium, incubated at 28°C at 120 rpm and production of IAA was checked after every 5 h up to 108 h by Salkovaski method.

7.3.7. Plasmid isolation from rifampicin resistant mutants of *Acinetobacter* spp. :

Plasmid isolation of rifampicin resistant mutants was also carried out by employing two methods. i.) Kado & Liu, (1981), and ii.) Sambrook, et al. (1989). Isolated plasmids were detected by agarose gel electrophoresis and molecular weight of plasmid /s was determined by comparing it with 1 kb DNA ladder.

7.3.8. Survival of *Acinetobacter* spp. and its mutant in a lignite carrier :

Survival of these 8 *Acinetobacter* wild strains and their respective mutants was observed in a lignite carrier. All these strains were grown in Luria broth, separately, for 24 h at 28°C at 150 rpm and 250 ml of growth (3 x 10^{8} *Acinetobacter* per g of lignite) was mixed with 500g of lignite in sterile conditions. Before mixing these cultures with lignite, pH of lignite (4.2) was adjusted near to neutral by adding 3-5 g of $CaCO_3$. In the process of mixing, the broth is spread to powdered lignite and left in trays of 15 x 15 cm for1-2 days, at room temperature (28°C). 50g mixture was then packed into sterile polythene bags (30 x 17 cm, 0.038-0.051mm of high density sheets) and was incubated at room temperature (28-30°C) for 6 months. After each

month the survival of wild as well as mutants was observed by spread plate method and number of colonies were counted.

7.4. Results :

7.4.1. Isolation of rifampicin resistant mutants of *Acinetobacter* spp. :

Mutants of *A. baumannii* A13, A16, A18 & A30, *A.* genospecies 3 A15 & A28, *A. junii* A6 and *A. haemolyticus* A19 were obtained. Colonies were raised on the medium after 48 h. Colony size observed was bigger than wild (Fig.1A & B). The morphological change observed in the mutants with comparison to wild is shown in Table 1. Frequency of mutation for each mutant was calculated (Table 2), as follows : for *A. baumannii* A13.1 : 5 x 10^6, for *A. baumannii* A16.1 : 3 x 10^6, for *A. baumannii* A18.1 : 6 x 10^6 & for *A. baumannii* A30.1 : 4 x 10^6 cfu/ml for *A.* genospecies 3 A15.1 : 4 x 10^3 & for *A.* genospecies A28.1 : 2 x 10^4, for *A. junii* A6.1 : 6 x 10^5 cfu/ml and for *A. haemolyticus* A19.1 : 8 x 10^5 cfu/ml.

7.4.2. Stability of rifampicin resistant mutants of *Acinetobacter* spp. :

Stability of each rifampicin resistant mutant such as of *A. baumannii* A13.1, A16.1, A18.1 and A30.1; *A.* genospecies 3 A15.1 and A28.1; *A. junii* A6.1 and *A. haemolyticus* A19.1 was checked and it was found that almost all rif^r mutants showed 95 % stability. Mutants from each strain was purified by isolating single colony and preserved on Luria agar slants at 4^0C.

7.4.3. Biochemical characteristics of rifampicin resistant mutants of *Acinetobacter* spp. :

Biochemical tests were carried out as per Bouvet & Grimont (1986 & 1987). Comparison between biochemical tests of wild and mutant are shown in Table 3. It was observed that there were no significant differences between biochemical characteristics of mutants as compared to wild except utilization of D-malate, phenylalanine, 4-aminobutyric acid, L-leucine, tyrosine, phenylacetate, glutarate, transaconitate, aspartic acid and β-alanine.

7.4.4. IAA production by rifampicin resistant mutants of *Acinetobacter* spp. :

Indole acetic acid production was also tested for all the mutants and observed that mutants were able to produce IAA as wild. Fig.2 A & B, shows IAA production by rifampicin resistant mutants of *Acinetobacter.*

7.4.5. Time course of the IAA production by rifampicin resistant mutants of *Acinetobacter* spp. :

Time course of each rifampicin resistant mutant was studied and compared with wild strain of *Acinetobacter.* Fig.2A & B, shows time course for rifampicin resistant mutants. It was observed that growth of mutants was slow (20h) as compared to wild (18h) and growth phase for IAA production was changed such as *A. haemolyticus* A19.1, *A. baumannii* A18.1, A16.1, A13.1 and *A.* genospecies 3 A15.1, showed maximum IAA production in mid-stationary phase (60 h). *A. junii* A6.1 showed maximum IAA production in early stationary phase (36 h) and *A.* genospecies 3 A28.1 and *A. baumannii* A30.1 showed maximum IAA production in late stationary phase (60 and 72 h) same as wild. Fig. 2 A & B represent the growth phase with maximum IAA production by each rifampicin resistant mutant of *Acinetobacter* spp.

Fig 1 A

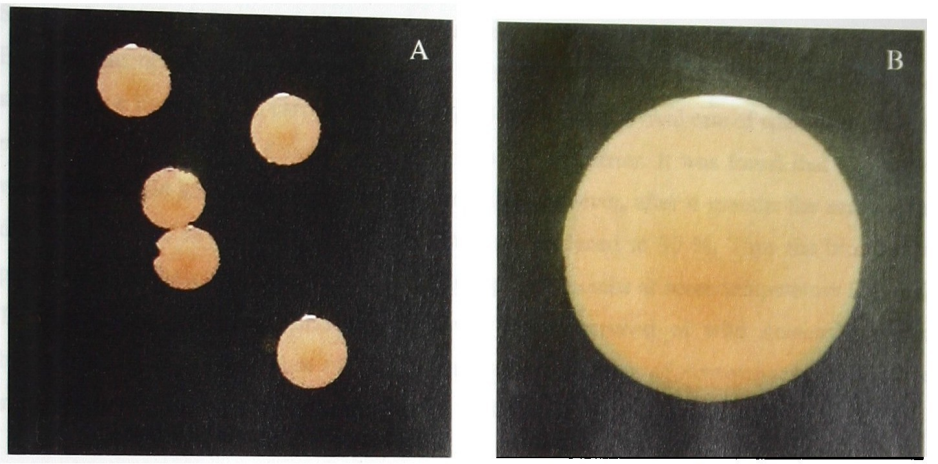


Fig 1 B

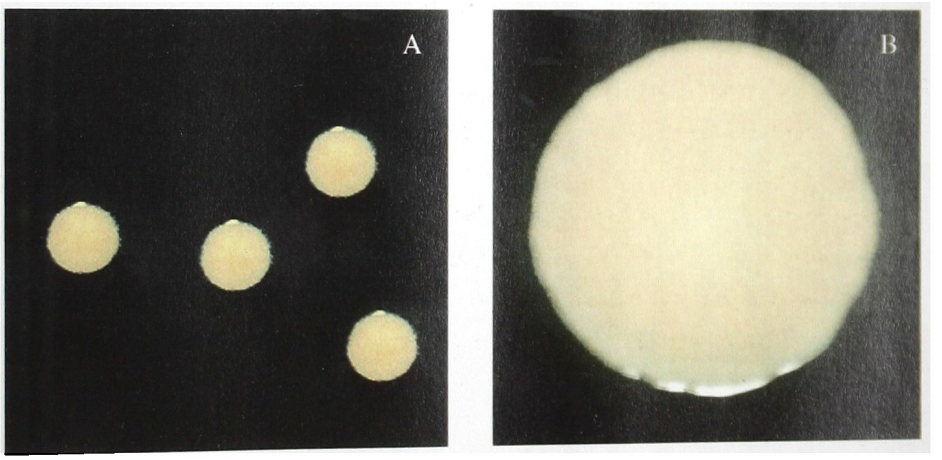


Fig.1A. Colony morphology of parent and rifampicin resistant mutant of *A.* genospecies 3 A28
A: *A.* genospecies 3 A28 ; B: *A.*genospecies 3 A28.1.
Fig.1B. Colony morphology of parent and rifampicin resistant mutant of *A. haemolyticus.*
A: *A. haemolyticus* A19 ; B: *A. haemolyticus* A19.1.

7.4.6. Plasmid isolation from rifampicin resistant mutants of *Acinetobacter* spp. :

Plasmid isolation was carried out by employing methods such as Kado & Liu (1981), and Sambrook et al (1989), showed presence of one plasmid in each mutant as wild. Molecular weight of each plasmid was 40Kb.

7.4.7. Survival of *Acinetobacter* and its rifampicin resistant mutants in a lignite carrier :

Eight strains of wild *Acinetobacter* spp. and their mutants producing IAA were tested for their survival in a lignite based carrier. Table 4 A & B shows the survival rate of rifampicin resistant mutants as well as wild *Acinetobacter* spp. in a lignite carrier. It was found that mutant and wild, both could survive in lignite up to 4 months. However, after 4 months the survival rate decreased by 20 % and by 6th month, the rate was reduced to 90 %. Thus the bioinoculum prepared with these strains would remain best up to 4 months at room temperature (28-30^{0}C). There was no significant difference observed in the survival of wild *Acinetobacter* spp. bioinoculum and bioinoculum of rifampicin resistant mutants of *Acinetobacter* spp. Percent survival rate for both is very similar (Table 4 A & B).

Table1: Characteristics of rifampicin resistant mutants of *Acinetobacter* spp on Luria agar containing rifampicin.

Characteristics	*A baumannii*				*A.*genospecies3		*A junii*	*A .haemolyticus*
	A13.1	A16.1	A18.1	A30.1	A15.1	A28.1	A6.1	A19.1
Colony size	round	round	round	round	round	round	round	round
Margin	entire	entire	entire	entire	entire	entire	entire	entire
Opacity	translucent	translucent	translucent	translucent	translucent	translucent	translucent	translucent
Consistency	sticky	sticky	sticky	sticky	sticky	sticky	sticky	sticky
Surface	smooth	smooth	Smooth	smooth	smooth	smooth	smooth	smooth
Elevation	flat	convex	convex	flat	flat	flat	flat	flat
Gram staining	Gram-ve	Gram-ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve
Morphology	coccobacilli	coccobacilli	coccobacilli	coccobacilli	coccobacilli	coccobacilli	coccobacilli	coccobacilli
Motility	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Capsule	+	+	+	+	+	+	+	+

This is the mean of three experiments. + : Positive; -: Negative.

Table 2 : Frequencies of rifampicin resistant mutants of *Acinetobacter* strains isolated by spontaneous mutation.

Name of the strains	Frequency of spontaneous mutation (cfu per ml)
Acinetobacter baumannii A13.1	5×10^6
Acinetobacter baumannii A16.1	3×10^6
Acinetobacter baumannii A18.1	6×10^6
Acinetobacter baumannii A30.1	4×10^6
Acinetobacter genospecies 3 A15.1	4×10^3
Acinetobacter genospecies 3 A28.1	2×10^4
Acinetobacter haemolyticus A19.1	8×10^5
Acinetobacter junii A6.1	6×10^5

Table.3. Biochemical characteristics of rif^r mutants of *Acinetobacter* species.

Tests	*A. baumannii*				*A.* genospecies 3		*A. junii*	*A. haemolyticus*
	A13.1	A16.1	A18.1	A30.1	A15.1	A28.1	A.6.1	A19.1
Growth at :								
28^{0}C	++	++	++	++	++	++	++	++
37^{0}C	++	++	++	++	++	++	++	++
41^{0}C	++	++	++	++	++	++	++	++
44^{0}C	++	++	++	++	--	--	--	++
Acid from Glucose	++	++	++	--	++	++	--	++
Gelatin hydrolysis	--	--	--	++	--	--	++	++
Haemolysis	--	--	--	++	--	--	--	++
Citrate	++	++	++	++	++	++	++	++
Utilization of :								
Malate	++	++	++	++	++	++	++	++
Phenylalanine	++	--	++	++	++	++	++	++
4 Aminobutyric acid	--	--	--	--	--	++	--	--
Histidine	++	++	++	++	++	++	++	++
L- Leucine	++	++	++	++	++	++	++	++
L- Ornithine	++	--	++	++	++	++	++	++
Ethanol	++	++	++	++	++	++	++	++
Tyrosine	++	++	++	++	++	++	++	++
Phenylacetate	++	++	++	++	++	++	++	++
L- Arginine	++	++	++	++	++	++	++	++
Glutarate	++	++	++	++	++	++	++	++
Trans- aconitate	++	++	++	++	++	++	++	++
Malonate	++	++	++	++	++	++	++	++
Lactate	++	++	++	++	++	++	++	++
Aspartic acid	++	++	++	++	++	++	++	++
β- Alanine	++	++	++	++	--	++	++	++

++ : Positive ; -- : Negative.

Table.4. A. Survival of wild *Acinetobacter* genospecies in a lignite carrier.

Time in days	% survival of *Acinetobacter* genospecies wild*							
	A15	A28	A13	A16	A18	A30	A6	A19
0	100	100	100	100	100	100	100	100
30	98	98	98	98	97	98	95	97
60	98	98	98	98	97	98	95	97
90	98	98	98	98	97	98	95	97
120	98	98	98	98	97	98	94	97
150	50	50	50	50	54	55	49	55
180	20	30	35	25	23	41	13	22

*A 15 & A28 : *A.* genospecies 3; A13, A16, A18 & A30 : *A. baumannii*; *A6: *A. Junii*; A19 : *A. haemolyticus*.

Table.4. B. Survival of rif^r mutants of *Acinetobacter* genospecies in a lignite carrier* :

Time in days	% survival of *Acinetobacter* rif^r mutants*							
	A15.1	A28.1	A13.1	A16.1	A18.1	A30.1	A6.1	A19.1
0	100	100	100	100	100	100	100	100
30	95	98	96	98	96	98	95	97
60	98	98	98	98	97	98	95	97
90	98	98	98	98	97	97	95	97
120	98	98	96	98	97	97	94	96
150	52	53	50	51	48	53	34	52
180	20	30	35	25	23	41	13	22

A. genospecies 3 : A15.1 & A28.1; *A. baumannii :* A13.1, A16.1, A18.1 & A30.1; A. *junii* : A6.1 : *A. haemolyticus :* A19.1*.

Fig.2A.Time course for IAA production by four rif^r mutants of *A. baumannii* strains.

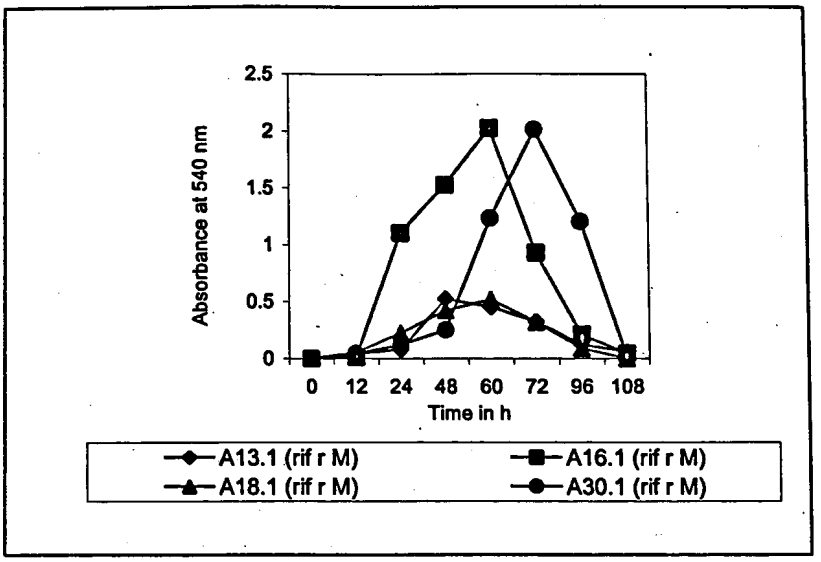
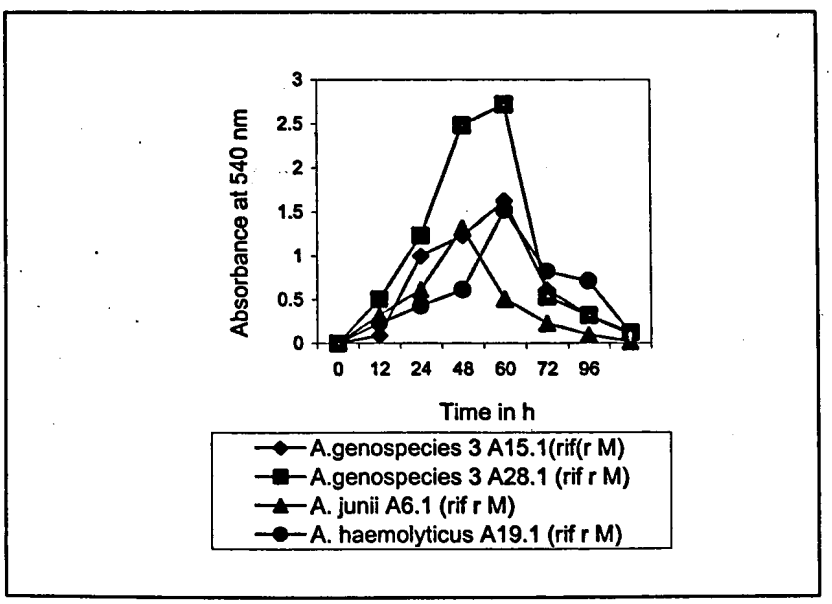


Fig.2.B. Time course for IAA production of rif^r mutants of four *Acinetobacter* spp.



7.5. Discussion :

Rifampicin resistant mutants of *Acinetobacter* spp. were isolated by spontaneous mutation. Mutants are important to study the colonization of bacteria in the rhizosphere (Brockwell, et al, 1977). Different types of mutants were isolated and used in biocontrol studies (Loon, et al, 2000; Lindow, 1985). These include different antibiotics as well as heavy metals. Antibiotics mainly used for spontaneous mutation are rifampicin and streptomycin (Obaton, 1971; Brockwell et al, 1977). Heavy metals used for isolation of resistant mutants were cadmium, nickel and chromium (Gupta et al, 2002). These mutants are important in the field identification and root colonization studies because of their antibiotic or metal resistant characteristics (Suslow, 1982; Weller & Thomashow, 1993).

In this study we used eight rifampicin resistant mutants of *Acinetobacter* spp. to observe colonization of *Acinetobacter* spp. to wheat root. These mutants were also studied for their biochemical characterization in comparison with wild. We found that these mutants can utilize some amino acids, sugars and organic acids which were not utilized by wild *Acinetobacter* spp. such as *Acinetobacter baumannii* A18 were not able to utilize D-malate, phenylalanine, 4 amino butyric acid, glutarate and transaconitate but *A. baumannii* A18.1 can utilize D-malate, phenylalanine, 4 amino butyric acid, glutarate and transaconitate. *A. baumannii* A13 was not able to utilize phenylalanine and 4 aminobutyric acid while *A. baumannii* A13.1 is able to utilize both. *A. baumannii* A16 was not able to utilize 4 aminobutyric acid and transaconitate but mutants were able to utilized both. *A. baumannii* A30 was unable to utilize phenylalanine, 4 aminobutyric acid, L-leucine, tyrosine, phenylacetate and aspartic acid but *A. baumannii* A30.1 was able to utilize all. Phenylalanine, phenylacetate and transaconitate was not utilized by *A. junii* A6 but utilized by *A. junii* A6.1. *A.* genospecies 3 A15 was not able to utilize 4 aminobutyric acid, phenylacetate and β-alanine but *A.* genospecies 3 A15.1 utilized all. Tyrosine and phenylacetate were not utilized by *A.* genospecies 3 A28, while utilized by *A.* genospecies A28.1. *A. haemolyticus* A19 was not able to utilize L-leucine, tyrosine and β-alanine but mutant of *A. haemolyticus* A19.1 was able to utilize all.

Overall, rifampicin resistant mutants isolated from *Acinetobacter* spp. were able to utilize more substrates than wild. Thus the mutants isolated from the wild may prove more beneficial to survive in rhizosphere as they have more capacity to utilize maximum substances than wild. This change in utilization pattern between wild and mutants is because of spontaneous mutation. Josey et al, (1979), reported similar results for antibiotic resistance of rifampicin resistant mutants, it was observed that rifampicin resistant mutants of *Rhizobium* obtained by spontaneous mutation had altered pattern of resistance that appeared to be unrelated to the known mutation. The rifampicin resistant mutants of *Acinetobacter* also showed increased resistance to rifampicin (increased by 8μg/ml to 100μg/ml) and nalidixic

acid (increased by 8μg/ml to 16μg/ml). Rifampicin resistant mutants obtained by *Acinetobacter* spp. did not exhibit physiological or enzyme tests different than wild. However, except above utilization tests, all other tests gave similar results as wild. Thus from the results obtained, we were able to use these mutants for further studies.

IAA production by eight rif^r mutants of *Acinetobacter* spp. was carried out and we observed that there was no significant difference in the IAA production by wild and rif^r mutants of *Acinetobacter* spp. The difference was found in the production phases of IAA. In rif^r mutants of *Acinetobacter* strains of *A. baumannii* A18.1, A16.1, A13.1, *A.* genospecies 3 A15.1 and *A. haemolyticus* A19.1, showed maximum IAA production in mid-stationary phase (60 h), while in wild it was observed at early stationary phase. *A. junii* A6.1 rif^r mutant showed maximum IAA production in early stationary phase (36 h), while wild *A. junii* A6 showed maximum IAA production in log phase (48 h) and rif^r mutants of *A.* genospecies 3 A28.1 and *A. baumannii* A30.1 showed maximum IAA production in late stationary phase (60 and 72 h) same as wild. One reason for this may be the slow growth pattern of rif^r mutants than wild.

Plasmid isolation carried out for rif^r mutants exhibited presence of plasmid of same molecular weight 40 kb in each strain and also produced IAA as wild. This result support to IAA production by mutants as IAA production is encoded by plasmid and plasmid isolation from rif^r mutants, proved that there was no effect on the plasmid by spontaneous mutation.

Survival of rif^r mutants as well as wild *Acinetobacter* spp. in a lignite carrier was observed. Most of the studies on the preparation of bioinoculum and carriers were done on *Rhizobium* spp.(Chao & Alexander, 1984 & 1982; Kremer & Peterson, 1983; Crawford & Berryhill, 1982; Mary et al, 1985, Osa-Afiana & Alexander, 1981& 1979; Danso & Alexander, 1974). It was observed that desiccation affects the survival of bacteria, as in case of *Rhizobium*, it can not survive in dry environment for long time (Marshall, 1964; Pena-Carbriales & Alexander, 1979). Mineral soil is particularly attractive carrier because it is available in all farming regions and cheap. A problem of its use is due to the presence of soil plant pathogens and weed seeds, but this can be eliminated or reduced in numbers by heating (Pena-Carbriales & Alexander, 1979). A second potential problem is marked decline of many rhizobia in mineral soils undergoing drying (Pena-Carbriales & Alexander, 1979). The effect of drying rates on the survival of microorganisms during storage at 31 % relative humidity (RH) is well documented (Antheunisse & Arkesteijn-Dijksman, 1979). Thus drying is one of the important parameter, which affects the survival of bacteria in particular carrier.

We used lignite as a carrier for bioinoculum preparation as it has capacity to hold 60 % moisture and can act as a carbon source (Kandasamy & Prasad, 1971; Saha, et al, 2001). It was observed that *Acinetobacter* as well as rif^r mutants of *Acinetobacter* can survive properly in the lignite. The rate of their survival was more than 95 % for 4 months and after that it was decreased. One more advantage of lignite is, that being a very fine powder can

bind to the seeds properly. We also observed that the survival rate at room temperature (28-30^{0}C) being important for the bacteria as the inoculum is applied to the seeds have to survive at room temperature. The method carried out for the survival of bacteria is most probable number or spread plate method. (Alexander, 1965). We also observed the effect of freeze storage of this bioinoculum which showed slight increase in shelf life of bacteria (by 8 days), these results are very similar to results obtained by Berton, (1967) and Vincent (1958 & 1965). They observed similar results for *R. japonicum* peat based inoculants packed in polythene and incubated at room temperature and lyophilized inoculants. They found that, lyophilized inoculants have a slightly longer shelf life period than do peat-based cultures at 28-30^{0}C, but their death rate after application to seed is much higher. In case of rhizobia survival rate of bioinoculum increased more from 3 months to 12 months when stored in fridge. Similar results were observed in case of *Azospirillum* spp. and *Azotobacter* spp. bioinoculants. Shelf life of both the inoculants were increased by 3 months when stored at low temperature (Subba Rao, 1988).

Thus in the present study we have isolated rif ^{r} mutants and studied their other characteristics, which proved that these mutants as well as wild strains of *Acinetobacter* can be further used in field experiments and can survive successfully at room temperature for 4 months. Thus the bioinoculum prepared by these *Acinetobacter* strains can be easily stored at room temperature. Storage at room temperature makes its use easier for the agriculture applications.

7.5. Conclusions :

- Rifampicin resistant mutants isolated from wild IAA producing *Acinetobacter* spp. were able to produce significant IAA as wild.
- Rifampicin resistant mutants are 95 % stable. They also contain plasmid of molecular weight of 40 kb as wild, which is responsible for IAA production same as parent.
- Survival of wild as well as rif ^{r} mutants of IAA producing *Acinetobacter* spp. in a lignite carrier at room temperature was found excellent up to 4 months.

7.6. Significance of this work :

- Overall, from above study, it is clear that rifampicin resistant mutants of *Acinetobacter* spp. were able to produce IAA same as wild and also contain plasmid responsible for IAA production as parent. Thus these mutants can be successfully used in the field experiments. Lignite was found to be best carrier for *Acinetobacter* bioinoculum as *Acinetobacter* spp. and its mutants were survived in a lignite carrier at room temperature for four months.

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CHAPTER 8

Preparation of Novel *Acinetobacter* Bioinoculum and its Use in Plant Growth Promotion.

Chapter 8

Preparation of Novel *Acinetobacter* Bioinoculum and its Use in Plant Growth Promotion.

8.1. Abstract :

Lignite based *Acinetobacter* bioinoculum was prepared and field trials were carried out in two different sites of fields at Pune. Effects of *Acinetobacter* bioinoculum with respect to growth and yield proved best for wheat plant. Yield of wheat grains was found to increase 60 % by *Acinetobacter* bioinoculum and increased to 75 % when applied with half dose of fertilizer, as compared to control and standard bioinoculums of *Azotobacter* and *Azospirillum*. These standard bioinoculums showed 10-20 % increase in yield of wheat grains. *Acinetobacter* was found to colonize wheat roots successfully at each life stage of wheat plant in both the fields. Soil analysis, showed little difference between the soils of two fields, with respect to soil texture, pH, macro and micronutrients. Yield obtained from both the fields was similar. In treated wheat plants, increase in number of tillers and height of the plant was observed as comparedto controls and standards. Dry weight of wheat plants treated with *Acinetobacter* bioinoculum was 26.1g, which was found to be more as compared to controls and standards. 1000 grain weight was measured as 62.66 g and found to be maximum for the treated plants with *Acinetobacter* bioinoculum than controls and standards. Chlorophyll content of *Acinetobacter* bioinoculum treated plants was observed more (7.74 mg/g) than standard (3.30 mg/g) and controls (0.8 mg/g). IAA oxidase enzyme activity was also recorded more (0.16) in wheat plants treated with *Acinetobacter* bioinoculum. *Acinetobacter* bioinoculum was found suitable to 12 different crop plants and showed similar results. These plants included i.) *Phaseolus vulgaris* (Rajma), ii.) *Sorghum bicolor* (Sorghum), iii.) *Pennisetum americanum* (Black millet), iv.) Zea maize (Maize), v.) *Arachis hypogea* (Groundnut), vi.) *Vigna radiata* (Mung), vii.) *Cicer arietinum* (Gram), viii. *Coriander cilantro* (Coriander), ix) *Spinacia olerecea* (Spinach), x). *Lycopersicon esculentum* (Tomato), xi.) *Glycine max* (Soybean), and xii.) *Phaseolus vulgaris* (Black bean). Thus, *Acinetobacter* bioinoculum can be used effectively as a biofertilizer for these crop plants.

Key Words: *Acinetobacter*, IAA production, *Acinetobacter* bioinoculum, *A.* genospecies 3 A28.1, *A.* genospecies 3 A28 , *A. haemolyticus* A19 .1, *A. haemolyticus* A19, Root colonization, Biofertilizer.

8.2. Introduction:

Among the world crops, wheat is pre-eminent both in regards to its antiquity and its importance as a food of mankind (Rangaswami, 1996). Although rice is the principal food of a large portion of the human race, a greater amount of wheat is grown and consumed in the form of bread, which constitute the chief food of the most highly civilized races (Rangaswami,1996). On account of particularly, physical and chemical quantities of the gluten in its grains, wheat makes more potable and better bread than any other cereals. Thus wheat is the most important staple food of the world,

covering about 200 million hectors. In India it is grown in about 23 million hectares, which is about 20 % of the area under cultivation for cereals. The annual production is about 36 million tonnes of grains (Rangaswami,1996; Zehnder et al, 2001) Byproducts of wheat obtained while manufacturing are most valuable foods for all kinds of stocks. In addition to its agricultural value, wheat straw is utilized in the manufacture of mattresses, straw hats and paper (Rangaswami,1996).

India is known as one of the highest population country, which need more yields in less engagement of land. To achieve the desired increase in productivity, fertilizers were added in large amounts, which results in the production of unfertile soil. In agriculture, soil texture and fertility is very important on which the production rate of crops depends. In soil, rhizobacteria play very important role and ecology of rhizosphere is maintained by soil microflora, which keeps the soil fertility intact (Subba Rao, 1986; Zehnder et al, 2001). Excess use of fertilizers affect soil texture and after some years soil becomes unfertile. To avoid use of such chemical fertilizers and to get more productivity biofertilizers are being used.

Biofertilizers are the living fertilizer compounds of microbial inoculants or group of microorganisms, which are able to fix atmospheric nitrogen or solubilize phosphorus, decompose organic material or oxidize sulphur in the soil. It produces plant growth promoting substances. On application, it increases plant yield and also improves soil fertility and reduce pollution (Brown, 1968; Brakel & Hilger, 1965; Denarie & Blachere, 1966; Rovira, 1965; Schank & Smith, 1984; Tien, et al, 1979). Biofertilizers have definite advantages over chemical fertilizers. Chemical fertilizers supply over nitrogen whereas, biofertilizers in addition to nitrogen provide, certain growth promoting substances like, hormones, vitamins, amino acids, and plant protection by producing antibiotics etc (Gaskin et al,1985; Ramamoorthy et al, 2001). Crops have to be provided with chemical fertilizers repeatedly to replenish the loss of nitrogen utilized for crop growth. On the other hand, biofertilizers supply the nitrogen continuously through out the entire period of crop growth in the field under favorable conditions. Continuous use of chemical fertilizers adversely affects the soil structure whereas biofertilizers when applied to soil improve the soil structure. The deleterious effects of chemical fertilizers are that, they are toxic at higher doses. In contrast biofertilizers have no toxic effects (Backman et al, 1997; Tien, et al, 1979).

Until now five types of biofertilizers have been developed and used successfully, these are: *Rhizobium* biofertilizers, *Azotobacter* biofertilizers, *Azospirillum* biofertilizers, *Pseudomonas* biofertilizer, Cyanobacteria known as blue-green algae biofertilizers and *Azolla* biofertilizers (Subba Rao, 1986, Baraibar, et al, 1999; Gaur & Algawadi, 1992; Berge, 1990; Glick, 1995; Zimmerman, et, al, 1980; Fay, 1983; Lehari & Malhotra, 1968; DeBrito, et al, 1995). These were mainly based on the property of bacteria to fix nitrogen and ability of phosphate solubilization.

In the present study, we have prepared bioinoculum of *Acinetobacter* spp., isolated from rhizosphere of wheat, having capacity to produce indole acetic acid and pyrrolnitrin. Field

experiments with lignite based *Acinetobacter* bioinoculum was carried out with different treatments and results were noted.

8.3. Materials and Methods :

8.3.1. Preparation of *Acinetobacter* bioinoculum :

Acinetobacter bioinoculum was prepared by using lignite as a carrier. Lignite (500g) was sterilized at 121^{0}C for 1 h. after 1h autoclaving lignite was kept for cooling for 3h.*A.* genospecies 3 A28 wild and mutant (A28.1) and *A. haemolyticus* A19 wild and mutant (A19.1) were inoculated in 500 ml of Luria broth and flasks were incubated at 30^{0}C for 48 h at 120 rpm. After 48 h flasks were removed, sterile lignite and cultures of *A.* genospecies 3 A28 wild and mutant as well as *A. haemolyticus* A19 wild and mutant were mixed separately in the proportion of 500 g lignite and 250 ml of each culture, aseptically using sterile gloves. The mixture of each bioinoculum was labeled properly and incubated at 30^{0}C for 24 h in sterile plastic bags (25 cmx 27 cm) in two sets. These sets were then used in field treatments of bioinoculum for wheat plants.

8.3.2. Field trials of *Acinetobacter* bioinoculum for wheat plant :

Field trials of novel bioinoculum of *A.* genospecies 3 A28 wild and mutant (A28.1) as wel as *A. haemolyticus* A19 wild and mutant (A19.1) were taken, *Acinetobacter* bioinoculum was used for the field trial on the wheat plant. Variety of wheat plant used was HD 2189 and bioinoculum as well as chemical fertilizer treatments were given in two different fields. First was experimental field of Mahatma Phule Agriculture College, Shivajinagar, Pune. Second was a field of local farmer at Hadapsar, Pune. Following types of the treatments were given to the wheat seeds with and without application of bioinoculum and by using different doses of chemical fertilizers. Chemical fertilizers used were: Urea : 0.63 Kg/100 m^2 and Single Super Phosphate : 1.3 Kg/100 m^2 (values for half dose of chemical fertilizers).

(a) Control 1 : Without bioinoculum and chemical fertilizer.

(b) Control 2 : Without bioinoculum and with half dose of chemical fertilizer such as Urea 0.6 kg/100m^2, and single super phosphate 1.3 kg/100m^2.

(c) Treatment 1: Wheat seeds treated with only bioinoculum of *A.* genospecies 3 A28 wild.

(d) Treatment 2: Wheat seeds treated with only bioinoculum of *A. haemolyticus* A19 wild.

(e) Treatment 3: Wheat seeds treated with only bioinoculum of *A.* genospecies 3 A28.1(rif^r mutant).

(f) Treatment 4: Wheat seeds treated with only bioinoculum of *A. haemolyticus* A19.1 (rif^r mutant).

(g) Treatment 5 : Wheat seeds treated with bioinoculum of *A.* genospecies 3 A28 wild and half dose of fertilizer as in step (b) above.

(h) Treatment 6 : Wheat seeds treated with bioinoculum of *A. haemolyticus* A19 wild and half dose of fertilizer as in step (b) above.

(i) Treatment 7 : Wheat seeds treated with bioinoculum of *A.* genospecies 3 A28.1 (rif^r mutant) and half dose of fertilizer as in step (b) above.

(j) Treatment 8 : Wheat seeds treated with bioinoculum of *A. haemolyticus* A19.1 (rif^r mutant) and half dose of fertilizer as in step (b) above.

(k) Treatment 9 : Wheat seeds treated with standard *Azotobacter* bioinoculum*.

(l) Treatment 10 : Wheat seeds treated with standard *Azotobacter* bioinoculum* with half dose of fertilizer as in step (b) above.

(m) Treatment 11: Wheat seeds treated with standard *Azospirillum* bioinoculum*.

(n) Treatment 12: Wheat seeds treated with standard *Azospirillum* bioinoculum* with half dose of fertilizer as in step (b) above.

* : Standard bioinoculums of *Azotobacter* and *Azospirillum* spp. was provided by Mahatma Phule Agriculture College, Shivajinagar, Pune.

After these treatments entire life cycle (3 months) of wheat plant was observed for different growth parameters.

8.3.4. Colonization of wheat roots by *Acinetobacter* spp. in the field :

Rifampicin resistant mutants isolated from *A.* genospecies 3 A28.1 and *A. haemolyticus* A19.1 were mainly used for this purpose. Colonization by these mutants was observed at 4 major life stages of wheat plants, involving : i) 30 days, ii). 45 days, iii). 60 days and iv).75 daysAt all these 4 life stages of wheat plants treated with only mutants of *A.* genospecies 3 A28 and *A. haemolyticus* A19 were uprooted and the roots were washed thoroughly by sterile distilled water for 56 times. Roots were then cut into small pieces of 2-3 cm, weighed 1g and kept in sterile saline (0.85%) then kept on shaker for 30 min. After those serial dilutions were made in the sterile saline and 100µl of each dilution was plated out in duplicate on Luria agar plates containing 100µg/ml of rifampicin. Plates were incubated at 28°C for 48-60h. Colony count was taken after 60 h. The colonization of wheat root was also carried out by root mapping method (Brown, 1962). Wheat roots were mapped on Luria agar plates with 100µg/ml of rifampicin and incubated at 28°C for 24-48 h and observed for colonization.

8.3.5. Effect of *Acinetobacter* bioinoculum on growth of wheat plant :

Effect of *Acinetobacter* bioinoculum on wheat plant was observed considering following parameters.

a) Measurement of shoot length of wheat plants (total height of plant).

b) Measurement of number of tillers of wheat plants.

c) Measurements of width of leaves of wheat plants.

Significant difference between controls and different treatments was calculated by employing statistical methods such as, mean, standard deviation, ANOVA and T- test.

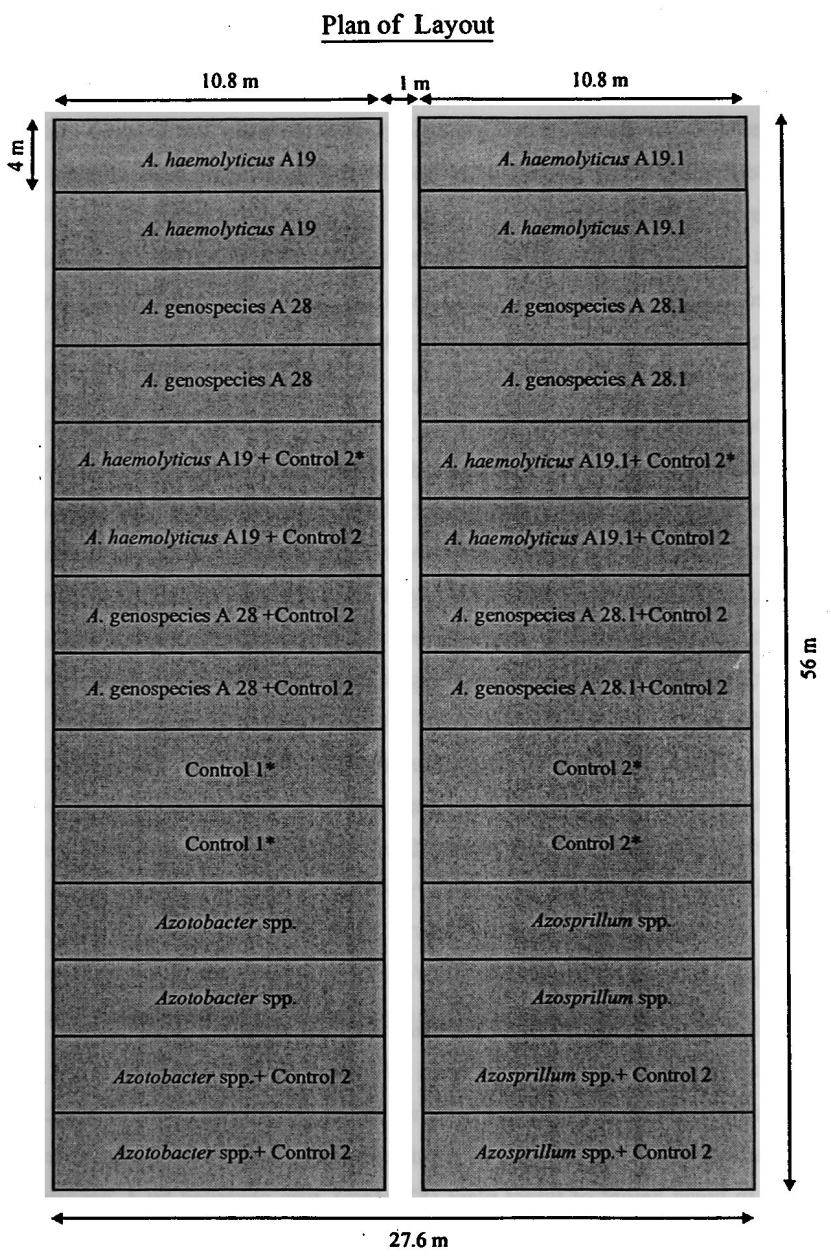

Fig.4 : Schematic Diagram of wheat field treated with different treatments.

Control 1*: wheat seeds without any fertilizer
Control 2*: wheat seeds with half dose of chemical fertilizer
Dose of chemical fertilizer:
i.) SSP: 1.3 Kg per 100 m^2 ii) Urea : 0.6 Kg per 100 m^2
Replications : 2 Varity of wheat plant : HD2189
No. of treatments : 14 Date of sowing : 17 Dec. 2001
Total area 0.12 hector

8.3.6. Biochemical analysis for detection of effect of bioinoculum on wheat plants.

To observe the effect of bioinoculum on wheat plant biochemical analysis was carried out. Total chlorophyll content was detected by the method, described by Whitman et al,(1971). It is also very important to know the effect of IAA produced on the plant. To observe this, indole acetic acid oxidase enzyme test was carried out as described by Sadashivam & Manickam, (1991).

8.3.7. Analysis of rhizosphere soil :

Analysis of rhizosphere soils was carried out for two experimental fields. The analysis was employed for pH, soil texture, macro nutrients such as nitrogen ,phosphorous and potassium, micronutrients such as Zn, Cu, Fe and Mg. These tests were carried out at laboratory of Zuari Agro Chemicals Ltd., Pune. Comparison between these two soils was done and effect of soil on the yield of wheat was observed.

8.3.8. Effect of *Acinetobacter* bioinoculum on different crop plants :

To observe the effect of *A.* genospecies 3 A28 bioinoculum on the other types of plants, 12 different plant seeds were treated with this novel bioinoculum. Soil was sterilized and seeds of the following 12 plants were treated with *A.* genospecies 3 A28 bioinoculum. Seeds selected were : Sorghum, (*Sorghum bicolor*), Pearl millet (*Pennisetum americanum*), Gram (*Cicer arietinum*) Rajma, (*Phaseolus vulgaris*), Mung,(*Vigna radiata*), Soybean (*Glycine max*), Maize (*Zea maize*), Black beans, (*Phaseolus vulgaris*), Groundnut (*Arachis hypogea*) , Spinach (*Spinacia olerecea*), Tomato (*Lycopersicon esculentum*) and Coriander (*Coriander cilantro*). All these plants were observed for 21 days and after 21 days root and shoot length was measured to see the effect of bioinoculum on these plants. Seeds without bioinoculum was kept as control. This experiment was carried out in triplicate.

8.3. Results :

8.4.1. Field trials of *Acinetobacter* bioinoculum for wheat plant :

Field trials of *Acinetobacter* bioinoculum was taken as described in section 8.3.2. The lay out of wheat field and different treatments is shown in Fig.4. Different parameters such as height of wheat plant, number of tillers and width of plant leaves were measured. It was observed that, maximum increase in mean height of wheat plant (87.84cm), was observed by *A.* genospecies 3 A28.1 with half dose of chemical fertilizer followed by *A.* genospecies 3 A28 with half dose of chemical fertilizer (84.96cm). Maximum increase in height of wheat plant without fertilizer was observed by *A.* genospecies 3 A28.1(66.92cm) followed by *A. haemolyticus* A19 (66.40cm). Number of tillers of wheat plants were increased maximum by *A.* genospecies 3 A28 with half dose of fertilizer (12.75) followed by *A. haemolyticus* A19.1 with half dose of chemical fertilizer (11.9). Number of tillers of wheat plants were increased maximum without chemical fertilizer by *A. haemolyticus*

A19.1 (9.68) followed by *A.* genospecies 3 A28.1(9.32). Maximum increase in width of wheat plant leaves was observed by *A.* genospecies 3 A28 with half dose of fertilizer (1.69cm), followed by *A.* genospecies 3 A28 without fertilizer (1.5cm). Table 1. shows details about the height, width and number of tillers of wheat plant. Overall, Table 1, indicates that treatment with biofertilizer with half dose of chemical fertilizer proved best as compared to other treatment such as treatments with standard bioinoculum as well as control (without bioinoculum). Treatment with only *Acinetobacter* biofertilizer was also proved best next to treatment withbiofertilizer and half dose of chemical fertilizer.

Total dry weight for wheat plants with all above treatments was calculated. It was found that total dry weight of wheat plant recorded maximum of the wheat plants with treatment of*A. haemolyticus* A19 with half dose of fertilizer (26.1g) followed by treatment with*A.* genospecies 3 A28.1 with half dose of fertilizer (25g). Treatments with only biofertilizer indicated maximum increase in dry weight of wheat plant by*A.* genospecies 3 A28 (20g) followed by*A.* genospecies 3 A28.1(19g). Table 2 indicates total dry weights of wheat plants with different treatments.

From Table 2, it is clear that total dry weight of wheat plant increased maximum by the treatments with *Acinetobacter* bioinoculum and half dose of chemical fertilizers than only bioinoculum. But it should be noted that wheat plants treated with only*Acinetobacter* bioinoculum (*A.* genospecies 3 A28 and *A. haemolyticus* A19) had more dry weight as compared to controls and standard bioinoculums. Total yield of wheat grains produced by different treatments applied to wheat plant was calculated. It was found that maximum increase in yield of wheat grains was observed by the plants treated with *A.* genospecies 3 A28 and half dose of chemical fertilizer i.e. 12.30Kg, followed by*A. haemolyticus* A19.1 with half dose of chemical fertilizer, i.e. 11.30 Kg. It was also found that yield of wheat grains was increased by treatments with only*Acinetobacter* bioinoculum (*A.* genospecies 3 A28, A28.1 and *A. haemolyticus* A19, A19.1) by 1.30 Kg than control as well as standard bioinoculums without any treatment of fertilizer.

Table 1.Effect of *Acinetobacter* bioinoculum and fertilizer (urea and ssp) treatments on wheat plants.

Name of Treatment	Number of Samples	Mean	Standard Deviation
I) Height of wheat plant (cm)			
Control without fertilizer	25	58.52	2.74
Control with fertilizer	25	74.12	10.59
A28 .1 with fertilizer	25	87.84	10.37
A 28.1 without fertilizer	25	66.92	6.16
A 28 W with fertilizer	25	84.96	10.54
A 28 W without fertilizer	25	65.48	4.70
A 19 .1 with fertilizer	25	76.20	10.7
A 19 .1 without fertilizer	25	65.68	5.8
A19 W with fertilizer	25	78.84	10.04
A19 W without fertilizer	25	66.40	4.5
Azotobacter with fertilizer	25	73.68	9.32
Azotobacter without fertilizer	25	59.44	2.50
Azospirillum with fertilizer	25	68.72	9.04
Azospirillum without fertilizer	25	55.4	3.78
II.) Number of tillers			
Control without fertilizer	25	3.8	1.04
Control with fertilizer	25	8.4	1.80
A28 .1 with fertilizer	25	11.88	1.61
A 28.1 without fertilizer	25	9.32	1.97
A 28 W with fertilizer	25	12.75	1.91
A 28 W without fertilizer	25	10.64	1.70
A 19 .1 with fertilizer	25	11.9	2.26
A 19 .1 without fertilizer	25	9.68	2.5
A19 W with fertilizer	25	11.0	2.7
A1 9 W without fertilizer	25	8.84	1.79
Azotobacter with fertilizer	25	9.16	1.57
Azotobacter without fertilizer	25	5.56	1.12
Azospirillum with fertilizer	25	8.72	1.48
Azospirillum without fertilizer	25	4.28	1.20
III.) Width of wheat plant leaves (cm)			
Control without fertilizer	25	1.15	0.18
Control with fertilizer	25	1.32	0.24
A28 .1 with fertilizer	25	1.46	0.25
A 28.1 without fertilizer	25	1.32	0.21
A 28 W with fertilizer	25	1.50	0.29
A 28 W without fertilizer	25	1.69	0.41
A 19 .1 with fertilizer	25	1.48	0.29
A19.1 without fertilizer	25	1.25	0.19
A 19 W with fertilizer	25	1.30	0.23
A19 W without fertilizer	25	1.20	0.18
Azotobacter with fertilizer	25	1.45	0.25
Azotobacter without fertilizer	25	1.21	0.19
Azospirillum with fertilizer	25	1.20	0.11
Azospirillum without fertilizer	25	1.16	0.18

*Fertilizer : Half dose of fertilizer : Urea 0.63 Kg/100 m^2 ; SSP: 1.3 Kg/100 m^2 .A 28 .1 : *A.* genospecies 3 A28 rif^r mutant, A 28 W : *A.* genospecies 3 A28 Wild, A 19.1 : *A haemolyticus* A19 rif^r mutant, A 19 W : *A. haemolyticus* A19 wild.

Table 2. Total dry weight of wheat plant produced by different treatments applied to wheat plant.

Treatment given to wheat seeds	Dry weight in g*	Treatment given to wheat seeds	Dry weight in g*
Control without fertilizer	11.7	A 19 .1 without F	16.6
Control with fertilizer	14	A19 W with F	26.1
A28 .1 with fertilizer	25	A19 W without F	18.5
A 28.1 without fertilizer	19	*Azotobacter* with F	15.4
A 28 W with fertilizer	19.5	*Azotobacter* without F	15
A 28 W without fertilizer	20	*Azospirillum* with F	17.1
A 19 .1 with fertilizer	19.2	*Azospirillum* without F	16

*Dry weight was measured after complete drying wheat plants. (after 6 months). Fertilizer : Half dose of fertilizer : Urea 0.63 Kg/100 m^2 ;Single Super Phosphate : 1.3 Kg/100 m^2 .A 28 .1 : *A.* genospecies 3 A28 rif^r mutant, A 28 W : *A.* genospecies 3 A28 Wild, A 19.1 : *A haemolyticus* A19 rif^r mutant, A 19 W : *A. haemolyticus* A19 wild.

Table 3. Total yield of wheat grains produced by different treatments applied to wheat plant.

Treatment given to wheat seeds	Weight of wheat grains in Kg	Treatment given to wheat seeds	Weight of wheat grains in Kg
Control without fertilizer*	8	A 19 .1 without fertilizer	9.30
Control with fertilizer	8.5	A19 W with fertilizer	11.00
A28 .1 with fertilizer	13	A19 W without fertilizer	10
A 28.1 without fertilizer	9.30	*Azotobacter* with fertilizer	8.8
A 28 W with fertilizer	12.30	*Azotobacter* without fertilizer	8.2
A 28 W without fertilizer	9.30	*Azospirillum* with fertilizer	8.7
A 19 .1with fertilizer	11.30	*Azospirillum* without fertilizer	9

*Fertilizer: Half dose of fertilizer ; Urea 0.63 Kg/100 m^2 ; Single Super Phosphate: 1.3 Kg/100 m^2 .
A 28 .1 : *A.* genospecies 3 A28 rif^r mutant, A 28 W : *A.* genospecies 3 A28 Wild,
A 19.1 : *A haemolyticus* A19 rif^r mutant, A 19 W : *A. haemolyticus* A19 wild.

Overall, from Table 3 it is clear that yield of wheat grains increased maximum with treatment of *Acinetobacter* bioinoculums and half dose of fertilizer followed by treatments with only *Acinetobacter* bioinoculums. 1000 grains weight was also calculated. Table 4, indicate the 1000 grain weight for different treatments applied to wheat plant, as follows:

Table 4. Thousand grain weight fordifferent treatments applied to wheat plant.

Treatment given to wheat seeds*	1000 grain weight (in g)	Treatment given to wheat seeds*	1000 grain weight (in g)
Control without fertilizer	30.8	A 19 .1 without fertilizer	46.2
Control with fertilizer	40.2	A19 W with fertilizer	54.40
A28 .1 with fertilizer	47.2	A19 W without fertilizer	44.04
A 28.1 without fertilizer	54.13	*Azotobacter* with fertilizer	47.6
A 28 W with fertilizer	62.66	*Azotobacter* without fertilizer	43.3
A 28 W without fertilizer	53.24	*Azospirillum* with fertilizer	49.2
A 19 .1 with fertilizer	56.78	*Azospirillum* without fertilizer	43.84

*Fertilizer : Half dose of fertilizer ; Urea 0.63 Kg/100 m^2 ; Single Super Phosphate: 1.3 Kg/100 m^2 .A 28 .1 : *A.* genospecies 3 A28 rif^r mutant, A 28 W : *A.* genospecies 3 A28 Wild, A 19.1 : *A haemolyticus* A19 rif^r mutant, A 19 W : *A. haemolyticus* A19 wild.

It was observed from table 4 that maximum thousand grain weight noted by wheat plants treated with *A.* genospecies 3 A28 with half dose of chemical fertilizer (62.66g) followed by *A. haemolyticus* A19 with half dose of chemical fertilizer (56.78). Thousand grain weight of wheat plants treated with only *Acinetobacter* bioinoculums *A.* genospecies 3 A28 (54.13) and *A haemolyticus* A19 (54.40) were found more as compared to controls as will as standard bioinoculum of *Azospirillum* and *Azotobacter* spp.

8.4.2. Colonization of wheat roots by *Acinetobacter* spp. in the field :

Colonization of wheat roots by *Acinetobacter* spp. in the field was studied by using mutants of *A.* genospecies 3 A28.1 and *A haemolyticus* A19.1. Colonization was calculated by taking colony count at different life stages of wheat plant. Table 5, indicates colonization of wheat roots by these two mutants in all major life stages of wheat plant as follows :

Table 5. Colonization of *A.* genospecies 3 A28.1 and *A. haemolyticus* A 19.1 to wheat roots in all major life stages of wheat plant. (cfu x 10^6)*

Life stage of wheat plant	*A.* genospecies 3 A28.1	*A haemolyticus* A19.1
30 days	202	296
45 days	403	445
60 days	510	540
75 days	173	195

*Colony count was taken per g of wheat root. cfu : Colony forming units are calculated in mean values. This data is the mean of three experiments.

From Table 5, it is clear that both *Acinetobacter* spp. inoculated with wheat seeds were able to colonize wheat plant successfully. Maximum colonization was observed by *A. haemolyticus* A19.1 (540 x 10^4) at 60 days life stage of wheat plant than *A.* genospecies 3 A28.1 (510 x 10^4).

8.4.3. Biochemical analysis for detection of effect of bioinoculum on wheat plants :

In biochemical analysis chlorophyll content of wheat plant and IAA oxidase enzyme test was done. Table 6, indicates the total chlorophyll content of wheat flag leaf as follows :

Table 6. Chlorophyll content of wheat plants leafs treated with different treatments.

Treatment given to wheat seeds*	Total Chlorophyll (a+b)* in mg/g	Treatment given to wheat seeds*	Total Chlorophyll (a+b)* in mg/g
Control without fertilizer	0.065	A 19 .1 without fertilizer	4.66
Control with fertilizer	0.80	A19 W with fertilizer	4.33
A28 .1 with fertilizer	4.29	A19 W without fertilizer	6.31
A 28.1 without fertilizer	4.42	*Azotobacter* with fertilizer	2.55
A 28 W with fertilizer	4.78	*Azotobacter* without fertilizer	1.32
A 28 W without fertilizer	7.74	*Azospirillum* with fertilizer	3.30
A 19.1 with fertilizer	3.31	*Azospirillum* without fertilizer	1.33

*Fertilizer : Half dose of fertilizer ; Urea 0.63 Kg/100 m^2 ; Single Super Phosphate: 1.3 Kg/100 m^2 .A 28 .1 : *A.* genospecies 3 A28 rif^r mutant, A 28 W : *A.* genospecies 3 A28 Wild, A 19.1 : *A haemolyticus* A19 rif^r mutant, A 19 W : *A. haemolyticus* A19 wild. * Total chlorophyll (a+b) = Chlorophyll a + chlorophyll b.

From Table 6, it is clear that total chlorophyll content of wheat leaves was increased by the wheat leaves treated with *Acinetobacter* bioinoculums without half dose of chemical fertilizer, such as by *A.* genospecies 3 A28 (4.42 mg/g) and A28. 1(7.74 mg/g) and *A. haemolyticus* A19 (6.31 mg/g) and A19.1(4.66 mg/g). Wheat plants treated with *A.* genospecies 3 A28.1 bioinoculum showed maximum increase in total chlorophyll content. Total chlorophyll content of all other treatments, such as control as well as standard bioinoculum was found less than *Acinetobacter* bioinoculum.

IAA oxidase enzyme activity was also observed and Table 7, shows IAA oxidase enzyme activity of wheat plant. From Table 7, it was observed that, maximum IAA oxidase enzyme activity was shown by wheat plants with *Acinetobacter* bioinoculum than standard and controls. Maximum IAA oxidase activity was shown by wheat plants treated with *A.* genospecies 3 A28 with half dose of fertilizer (0.16 µl/min/mg of protein) as well as without fertilizer (0.16µl/min/mg of protein), followed by *A. haemolyticus* A19 (15µl/min/mg of protein). The details about the IAA oxidase enzyme production is shown in Table 7.

Fig. 1

Fig. 2

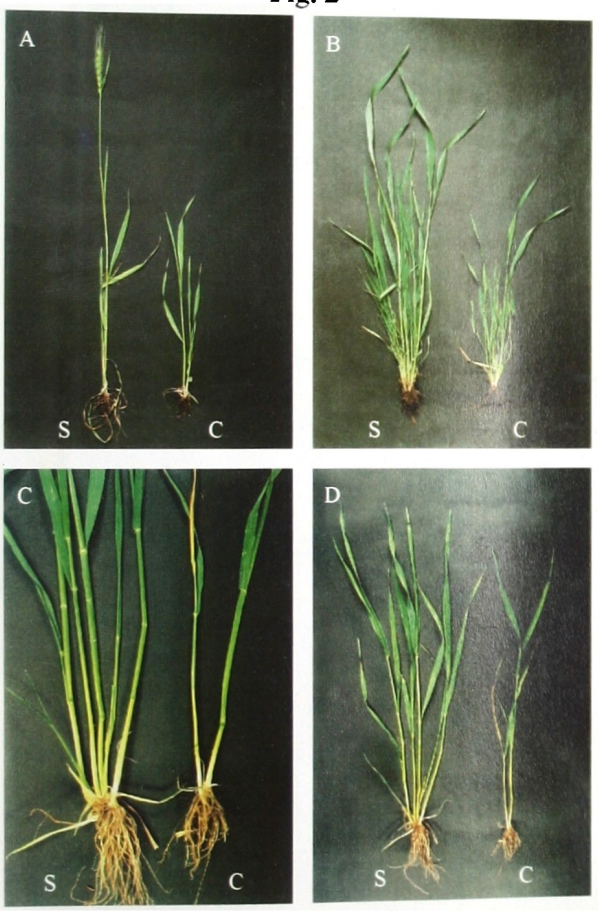

Fig. 3

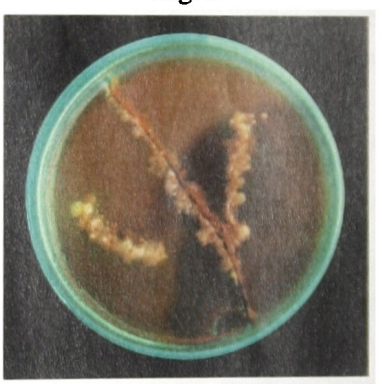

Fig.1. View of wheat fields treated with *Acinetobacter* bioinoculum A28 and A19.
1 : Experimental field of Mahatma Phule Agriculture College, Pune.
2 : Experimental field of Mr. Kodare, Hadapsar, Pune.

Fig.2. Effect of *Acinetobacter* genospecies 3 A28 bioinoculum on wheat plant.*
A: Increase in height of wheat plant compare to control.
B: Increase in overall growth of wheat plant compare to control.
C: Increase in number of tillers of wheat plant compare to control.
D: Increase in width of shoot of wheat plant compare to control.
Control I: Wheat plants without any fertilizer or bioinoculum treatment.
C: Control; S: Sample.
*Note : Similar results were observed by *Acinetobacter haemolyticus* A19 bioinoculum on wheat plant.
Fig.3. Wheat root colonization by *Acinetobacter* genospecies.

Table 7. IAA oxidase activity of wheat plant.

Treatment given to wheat seeds	IAA oxidase activity in µl/min/mg of protein	Treatment given to wheat seeds	IAA oxidase activity in µl/min/mg of protein
Control without fertilizer*	0.06	A 19 .1 without fertilizer	0.15
Control with fertilizer	0.07	A19 W with fertilizer	0.12
A28 .1 with fertilizer	0.11	A19 W without fertilizer	0.14
A 28.1 without fertilizer	0.12	*Azotobacter* with fertilizer	0.09
A 28 W with fertilizer	0.16	*Azotobacter* without fertilizer	0.10
A 28 W without fertilizer	0.163	*Azospirillum* with fertilizer	0.08
A 19.1 with fertilizer	0.14	*Azospirillum* without fertilizer	0.10

*Fertilizer : Half dose of fertilizer ; Urea 0.63 Kg/100 m^2 ; Single Super Phosphate: 1.3 Kg/100 m^2 .A 28 .1 : *A.* genospecies 3 A28 rif^r mutant, A 28 W : *A.* genospecies 3 A28 Wild, A 19.1 : *A haemolyticus* A19 rif^r mutant, A 19 W : *A. haemolyticus* A19 wild. IAA oxidase enzyme activity is measured perµl/min/mg of protein.

From Table 7, it is observed that, maximum IAA oxidase enzyme activity was shown by wheat plants with *Acinetobacter* bioinoculums than standard and controls. Maximum IAA oxidase activity was shown by wheat plants treated with *A.* genospecies 3 A28 with half dose of fertilizer (0.16 µl/min/mg of protein) as well as without fertilizer (0.16µl/min/mg of protein), followed by *A. haemolyticus* A19 (15µl/min/mg of protein).

8.4.4. Analysis of rhizosphere soil :

Table 8 shows, pH of soil was alkaline in bdh the fields. Both field soils were of medium lomy type. In case of Macro elements N was in same percent (1.20) in both the fields. Phosphate was little more (12 Kg/Acre) in Hadapsar field and Potash is also observed more amounts (134 Kg/Acre) in Hadapsar field. In case of micro elements such as zinc, copper and manganese, are present in more amounts in M.A.P.H. field and Iron is observed in more amounts in Hadapsar than M.A.P.H. field. It can be conclude that both the soils are having adequate macro and micro elements required for plant growth. Soil analysis of two experimental fields was done. Table, 8 indicates soil analysis as follows.

Table 8. Soil analysis of the two experimental fields.

Parameters	M.P. A. C., Pune	Hadapsar field , Pune.
Soil type	Medium loamy	Medium loamy
PH	8	8.10
Macro elements :		
Total available nitrogen (%)	1.20	1.20
Total phosphate (Kg/ Acre)	10	12
Total potash (Kg/ Acre)	74	134

Table 8 continued.....

Parameters	M.P. A. C., Pune	Hadapsar field , Pune.
Micro elements (ppm) :		
Zinc	6.03	2.73
Copper	8.84	7.01
Iron	8.10	8.93
Manganese	36.68	21.23

*M.P. A. C. Mahatma Phule Agriculture, College, Pune. *This data is the mean of three experiments.

8.4.5. Effect of *Acinetobacter* bioinoculum on different crop plants :

Twelve different crop plants were tested for the effect of *Acinetobacter* bioinoculum. Effect of *Acinetobacter* bioinoculum was observed by measuring root and shoot length of these crop plants. Table 9, shows effect of *Acinetobacter* bioinoculum on 12 different crop plants. From table 9, it was found that bioinoculum of *A.* genospecies 3 A28 was proved best for 12 different crop plants those are Sorghum, (*Sorghum bicolor*), Pearl millet (*Pennisetum americanum*), Gram (*Cicer arietinum*) Rajma, (*Phaseolus vulgaris*), Mung,(*Vigna radiata*), Soybean (*Glycine max*), Maize (*Zea maize*), Black beans, (*Phaseolus vulgaris*), Groundnut (*Arachis hypogea*), Spinach (*Spinacia olerecea*), Tomato (*Lycopersicon esculentum*) and Coriander (*Coriander cilantro*). It was found that *Acinetobacter* bioinoculum significantly increased root and shoot length of each plant as compred to control plants.

Fig.3A

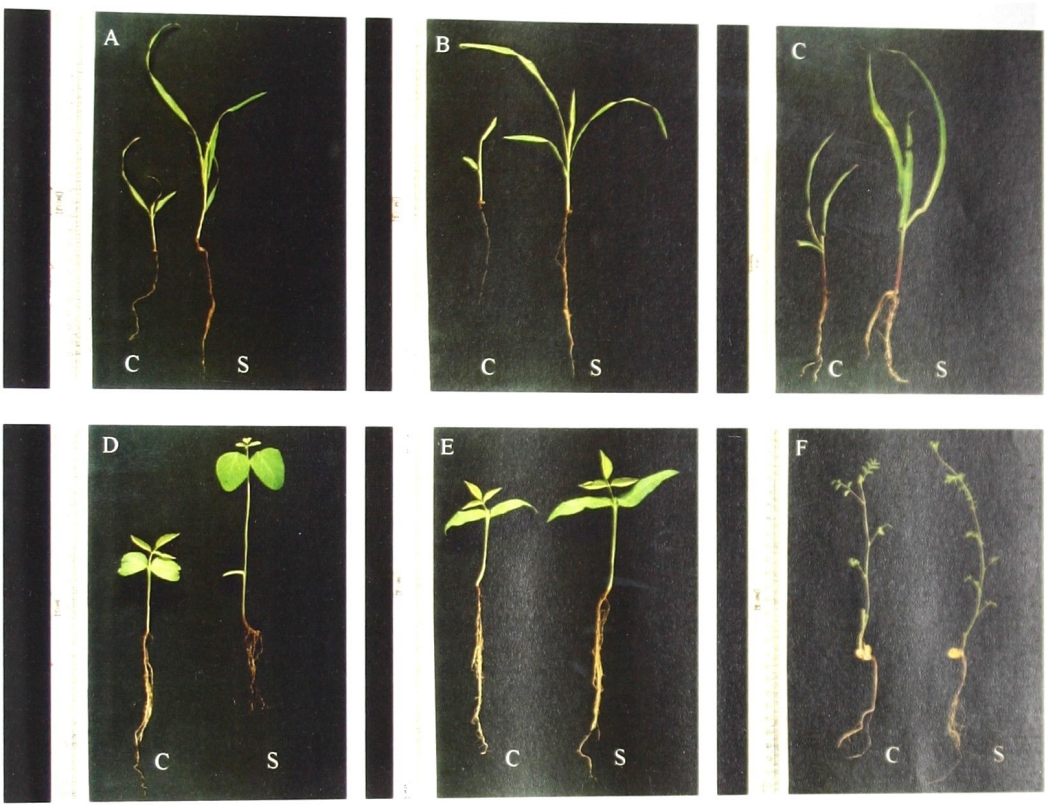

Fig.3A. Effect of *A.* genospecies 3 A28 bioinoculum on different crop plants.

A : Sorghum (*Sorghum bicolor*); B: Pearlmillet (*Pennisetum americanum*);

C : Maize (*Zea maize*); D:Soybean (*Glycine max*); E: Mung (*Vigna radiata*);

F : Gram (*Cicer arietinum*).

C : Control; S : Sample.

Fig.3B

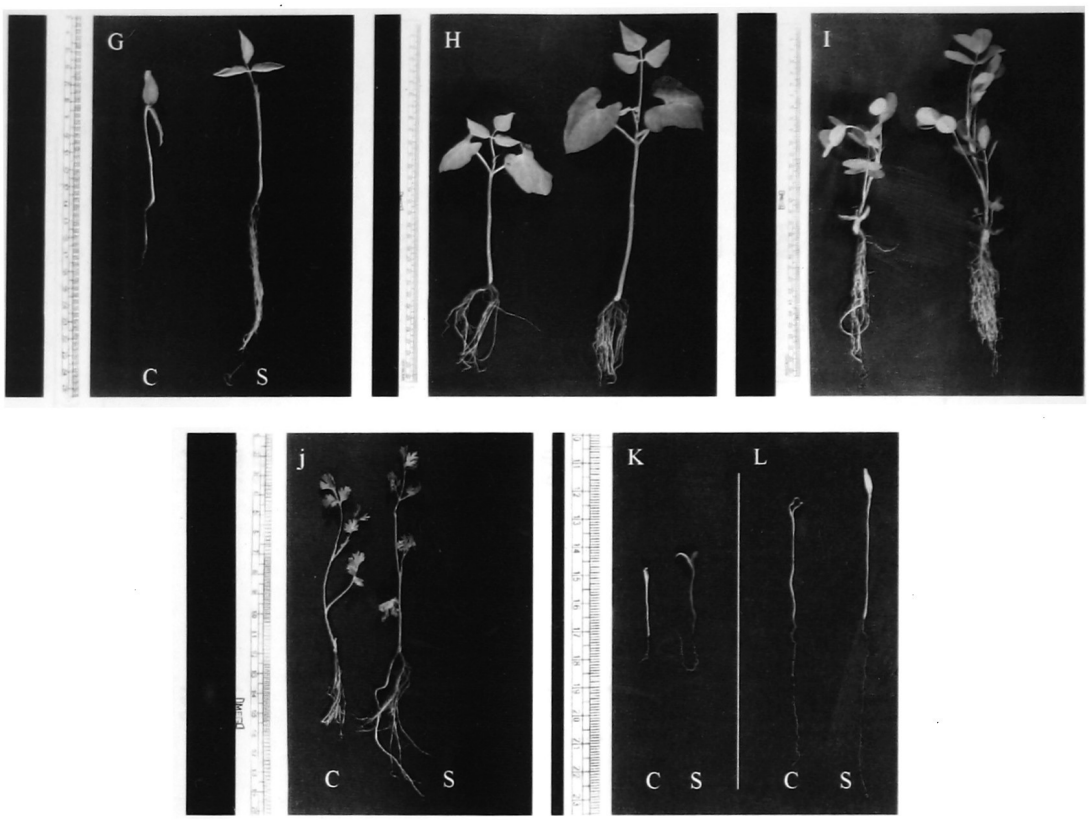

Fig.3B. Effect of *A.* genospecies 3A 28 bioinoculum on different crop plants.

G : Black bean (*Phaseolus vulgaris*); H : Rajama (*Phaseolus vulgaris*);

I : Groundnut (*Arachis hypogea*); J : Coriander (*Coriander Cilantro*);

K : Spinach (*Spinacia olerecea*); L : Tomato (*Lycopersicon esculentum*).

C : Control; S : Sample.

Table 9. Shoot and root length of plants treated with *Acinetobacter* genospecies3 A28 bioinoculum.*

Name of plant	Mean of Shoot length (cm)	Standard. Deviation	Mean of root length (cm)	Standard Deviation
Monocotyledons				
Zea maize (Maize)				
Sample	31.7	1.56	19.6	0.966
Control	27.6	0.84	16.4	1.075
Sorghum bicolor (Sorghum)				
Sample	21.8	0.67	7.80	1.22
Control	18.8	1.22	5.10	1.10
Spinacia olerecea (Spinach)				
Sample	4.80	0.42	4.05	0.36
Control	2.80	0.25	2.72	0.24
Lycopersicon esculentum (Tomato)				
Sample	6.60	0.51	10.4	15.3
Control	4.40	0.50	3.80	0.42
Coriander cilantro (Coriander)				
Sample	10.98	0.51	5.60	0.51
Control	9.10	0.87	4.10	0.56
Dicotyledons				
Phaseolus vulgaris (Rajma)	18.4	0.84	7.20	1.75
Sample	13.9	3.17	6.70	1.05
Control				
Arachis hypogea (Groundnut)				
Sample	16.40	0.966	12.6	1.07
Control	9.98	0.862	8.10	0.87
Cicer arietinum (Gram)				
Sample	15.6	0.44	10.9	0.92
Control	11.5	0.85	8.81	0.57
Vigna radiata (Mung)				
Sample	11.2	0.64	10.0	0.58
Control	8.65	0.36	6.88	0.82
Phaseolus vulgaris (Black bean)				
Sample	9.99	3.44	9.43	0.66
Control	8.83	0.48	8.24	1.16
Pennisetum americanum (Black millet)				
Sample	15.1	3.66	5.10	0.99
Control	11.4	0.73	3.50	0.52

*This result is mean of each 10 plants.

8.5. Discussion :

Indole 3 acetic acid (IAA) is a plant growth-promoting hormone which is produced by plant as well as bacteria (Glick, 1994 & 1995). IAA is thermo-sensitive and photosensitive. It enhances mitotic rate and there by increases the growth rate of plant. IAA and its derivatives are secreted extracellularly by many bacteria and fungi, in culture containing tryptophan (Glick, 1995; Day et al, 2000). There are many soil bacteria which are known to produce IAA such as *Pseudomonas fluorescens* , *Azospirillum* spp., *Azotobacter* spp. *Agrobacterium tumefaciens* and *Rhizobium* spp. (Subba Rao, 1986, Baraibar, et al, 1999; Gaur & Algawadi, 1992, Berge, 1990; Glick, 1995; Zimmerman, et al, 1989; Fay, 1983; Lehari & Malhotra, 1968; De Brito, et al, 1995; Radjacommare et al, 2002). Indole acetic acid production is one of the important means of plant growth promotion. Near about 80 % rhizosphere microorganisms produce IAA (Glick, 1994 & 1995).

Acinetobacter is not reported as a plant pathogen. There are preliminary reports of presence of *Acinetobacter* in wheat and maize rhizosphere and the production of IAA as well as phosphate solubilization was only reported by Leinhons & Vocek (1994) and Lippmann, et al (1995), from maize rhizosphere, however, no detail study was carried out about the presence and possible role of *Acinetobacter* in the rhizosphere of wheat and rye. Large number of studies have been carried out to find out the role of *Pseudomonas, Azospirillum, Azotobacter* and *Rhizobium* in the rhizosphere and they have been successfully used for bioinoculum preparation (Pandey & Kumar, 1989; Mishustin & Shilinikova, 1969; Reichardt, et al, 1997; Hegde, et al, 1999; Malik, et al, 1995; Rai & Brahmaprakash, 1993). *Azotobacter* spp. and *Azospirillum* spp., which are used for bioinoculum preparation, both, are known to produce IAA and fix atmospheric nitrogen. In both strains nitrogen fixation is considered important characteristic than IAA production for plant growth promotion and hence their bioinoculums are prepared mainly on the basis of nitrogen fixation.

Wheat is one of the most important crops in India. It has played important role in successful green revolution in India. In Maharashtra, 10.49 lakh hector field is employed for the cultivation of wheat (Rangaswami, 1998; Hegde, 1999). The average production of wheat in India is 26.2 quintal/ hectare (Rangaswami, 1998). However, Maharashtra state is much behind in wheat production as compared to national average production due to number of reasons as follows :

i.) Imbalanced or excessive use of chemical fertilizers.

ii.) Lack of water during growing stage of wheat.

iii.) Less duration of winter season.

However, by employing the modern agriculture biotechnological practices, it is possible to increase average yield per hectare in the state of Maharashtra. Thus to increase wheat yield in Maharashtra is very necessary (Hegde, 1999).

There are only 1 to 2 preliminary reports on presence and production of IAA by *Acinetobacter* in the rhizosphere but no detailed study is done, on the possible role of *Acinetobacter* in the rhizosphere. Therefore we have isolated *Acinetobacter* from rhizosphere of wheat and studied IAA production by this bacterium. We observed that *Acinetobacter* is present in significant amounts in the rhizosphere of wheat and effect of IAA production at different stages of wheat plant (Huddedar, et al, 2001).

Acinetobacter is not a plant pathogen, therefore, its role in the rhizosphere seems important. We have found that it has importance in plant growth promotion by producing IAA and also in protection, as *A. haemolyticus* A19 produced pyrrolnitrin an antibiotic with broad range of antimicrobial activity against plant pathogenic bacteria and fungi (described in Chapte 4). Other *Acinetobacter* spp. *Acinetobacter* genospecies 3 A28 is able to produce a pink colored pigment, which exhibits an antimicrobial activity against some plant pathogenic bacteria. By observing all these properties, we decided to develop a novel *Acinetobacter* bioinoculum for wheat and carried out field trials of *Acinetobacter* bioinoculum.

It is known that chemical fertilizers are very hazardous for the soil texture and overall health of soil. When compared to chemical fertilizers, biological fertilizers known as biofertilizers are very effective (Okon, 1985; Watanabe, 1986). They increase soil fertility and productivity without harming the texture of soil. They increase the nutrients in the soil to make nutrient rich soil better for the plant growth (Watanabe, 1986). Thus by considering these advantages of biofertilizers, over chemical fertilizer we prepared this novel *Acinetobacter* bioinoculum (biofertilizer).

We used lignite as a carrier for bioinoculum since it has a high water holding capacity and has high organic matter content which enhances growth of the microorganisms (Kandasamy & Prasad; 1971). Here we mainly studied the effect of IAA production, by bacteria, on the wheat plant. We observed that *Acinetobacter* has a symbiotic interaction with wheat plant and increased noticeable yield, weight and height of wheat plant and grains. Lignite based *Acinetobacter* bioinoculum was prepared and treated with wheat seeds. Different possible treatments of *Acinetobacter* bioinoculum, *Azotobacter* and *Azospirillum* bioinoculum and chemical fertilizers were given to wheat plants. It is a fact that biofertilizers can't be substituted for chemical fertilizers but can be used with chemical fertilizers to obtain reduction in its toxic effects and to obtain maximum yield (Gaskins, et al, 1985). In our study, we observed that treatments with only *Acinetobacter* bioinoculum increased the yield of wheat grains by 60 % while treatment of wheat seeds with *Acinetobacter* bioinoculum and half dose of chemical fertilizers increased the yield of wheat grains by 75 % which is recorded even more than standards. Standard bioinoculums used in the experiments such as treatment with only *Azotobacter* and *Azospirillum* bioinoculum increased the yield of wheat grains with 10 to 12 %. Until now mostly *Azotobacter, Azospirillium &*

Rhizobium bioinoculants were used as biofertilizers. It was observed by Wani (1992) that due to *Azospirillum* and *Azotobacter* bioinoculum in pearl millet, a maximum 20 % increase in grain yield was obtained. In case of sorghum, Tilak & Subba Rao, (1987) reported highest increase in grain yield was 33.67 % by application of co-inoculants *Azospirillum brasilense* and *Glomus fasciculatum*. In case of wheat & soybean the effect of inoculation with *Azospirillum brasilense* was also positive (Bashan, et al, 1990).

We carried out these experiments at two different sites and got similar results from both the fields. We also measured shoot length and number of tillers of wheat plants at four major life stages and found that length of the wheat plants, treated with *Acinetobacter* bioinoculum, as well as of *Acinetobacter* bioinoculum with half dose of chemical fertilizer showed increase in length than standards and controls. Increase in root and shoot length and number of tillers of wheat, maize and groundnut plants were observed by *Azotobacter* and *Azospirillum* spp. (Barabieri & Galli, 1993; Glick 1996; Dey, et al, 2000). *Rhizobium* spp. also reported for increase in root, shoot length and number of tillers of soybean plants (Baraibar, et al, 1999).

In our experiments we noticed one important observation, that leaves of wheat plants treated with *Acinetobacter* bioinoculum were broader than untreated & standards, this is important for the photosynthesis and in turn help in plant growth. However, the role of broad surfaces of plant leaves in plant growth promotion is not yet clear but it was observed by many workers (Forni, et al, 1992), that when the leaf surface was broad, growth of the plant was more. Furthermore it should be noted that IAA is a plant growth-promoting hormone, which increases the shoot and root length of plant, also increases number of roots as well as branches or tillers (Forni, et al, 1992; Glick 1996; Prikry, et al, 1985; Loper & Schroth, 1986; Fuentes-Ramirez et al, 1993, Leinhos & Vocek; 1984) which intern is responsible for increasing yield.

It was observed previously that, the introduced organism must colonize plant roots and demonstrate rhizosphere competence before its further utilization as biological control and /or plant growth promoting agent (Zodr & Anderson, 1992). When the proper bacterial strain is used, plant roots are extensively colonized by the introduced strain, which suggest a close bacterial plant association that allows the beneficial plant growth or disease protection (Schmidt, 1979; Lam & Gaffney, 1993; Nautiyal, 1997). To prove colonization of *Acinetobacter* to wheat roots we used bioinoculum of rif^r mutants of *Acinetobacter*. To serve this purpose we have isolated rif^r mutants (Lewis et al, 1987; Compeau, et al, 1988; Juhnke, et al, 1987, Scher et al, 1984; Turco, et al, 1986) of *Acinetobacter* and used them, to see colonization of wheat roots by *Acinetobacter* spp. in the field experiments. Colonization of *Acinetobacter* to wheat roots was done by root mapping method (Brown, 1962). It was noted that *Acinetobacter* can successfully colonize the wheat roots and are

responsible for plant growth promotion as well as for the increased yield. Similar results were noted for *Pseudomonas* spp. by Kloepper, et al (1980). They noted that, *Pseudomonas* successfully colonized the potato roots and is responsible for plant growth promotion as well as for the increased yield.

While studying we also observed that there were differences in colors of the treated and control plants. Further to confirm the difference we calculated total chlorophyll content. Chlorophylls are the essential compounds for photosynthesis and thus important in plant growth (Sadashivam & Manickam, 1991). We found that, there was significant difference in the chlorophyll contents of treated wheat plants and in non treated controls. It was noted that control without any fertilizer and with half dose of fertilizer showed lowest chlorophyll contents (0.065 and 0.80 mg/g) while the maximum chlorophyll content was observed by *Acinetobacter* bioinoculum (7.74 and 6.31 mg/g).

IAA oxidase (IAAO) is one of the important enzymes present in plants. IAAO is the enzyme involved in the catabolic degradation of IAA to 3 methylene oxidole. Thus it helps to utilize free IAA for the plant growth (Byrant & Lane, 1979). In present study we found that plants treated with *Acinetobacter* exhibited more IAA oxidase activity than other treatments, which proved that IAA is produced more, and also oxidized more by the plants treated with *Acinetobacter* bioinoculum. Maximum IAA oxidase activity was exhibited by plants treated with *A.* genospecies 3 A28 wild bioinoculum, and according to our previous results IAA was produced maximum by *A.* genospecies 3 A28 wild. Soil analysis for the two experimental fields had little difference in pH but both had alkaline pH and both had medium loamy type texture of soil.

Dry weight of the wheat plants treated with *Acinetobacter* bioinoculum was increased than their respective controls and standards. It was previously noted by Subba Rao, et al (1979a) that dry weight of two grasses were increased by *A. brasilense* with respect to their controls.

From the above results we conclude that, *Acinetobacter* bioinoculum is best and suitable for the growth of wheat plant. We found that with all other 12 crop plants *Acinetobacter* bioinoculum was effective and increased root and shoot length of all treated plants than controls. These plants were : i.) *Phaseolus vulgaris* ii.) *Sorghum bicolor* iii.) *Pennisetum americanum* iv.) *Zea maize* v.) *Arachis hypogea* vi.) *Vigna radiata* vii.) *Cicer arietinum* viii.) *Coriander cilantro* ix) *Spinacia olerecea* x.) *Lycopersicon esculentum* xi.) *Glycine max* and xii.) *Phaseolus vulgaris* . We found that all 12 plants showed increased shoot and root lengths. Thus, *Acinetobacter* bioinoculum also proved effective for above monitored 12 plants.

Overall, the role of *Acinetobacter* in the wheat rhizosphere is thus clear by these observations. It should also be taken in account that *A. haemolyticus* A19 produces pyrrolnitrin

(explained in chapter 4). It has broad-spectrum of antimicrobial activity against many plant pathogenic fungi and bacteria. Interestingly it also exhibited antimicrobial activity against human and plant pathogenic bacteria. Strain *A.* genospecies 3 A28 is also a pigment producer, produces pink colored pigment. This pigment also showed antimicrobial activity against some plant pathogenic bacteria. Currently we found that these bacteria were also able to solubilize phosphate (data not shown). Thus the role of *Acinetobacter* in the wheat rhizosphere becomes more clear and found that *Acinetobacter* is one of the plant growth promoting rhizobacteria and can be used as a effective biofertilizer for wheat and other plants, which needs further investigation in this area.

8.6. Conclusions:

- *Acinetobacter* bioinoculum was proved as one of the best bioinoculum for the growth of wheat plant.
- *Acinetobacter* bioinoculum was found to be best for 12 different crop plants.
- *Acinetobacter* applied in the form of bioinoculum was able to colonize wheat root successfully in the field.
- *Acinetobacter* bioinoculum increased grain yield of wheat by 60 % whereas *Acinetobacter* bioinoculum and half dose of fertilizer increased grain yield of wheat by 75 %.

8.7. Significance of this work :

- Overall, it is found that *Acinetobacter* is one of the important rhizobacteria present in the rhizosphere of wheat. By producing IAA, and antibiotic pyrrolnitrin *Acinetobacter* is able to promote plant growth and may play an important role in plant protection. Thus it has beneficial interaction with wheat plant. Bioinoculum of *Acinetobacter* spp. found to increase yield of wheat plant by 60 %. Thus *Acinetobacter* bioinoculum can be used successfully for increasing yield of wheat as well other crop plants.

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CHAPTER 9

Genetics of *Acinetobacter* genospecies Isolated from Rhizosphere of Wheat.

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Genetics of *Acinetobacter* genospecies isolated from rhizosphere of wheat.

9.1. Abstract

Plasmid profile of 37 *Acinetobacter* genospecies isolated from rhizosphere of wheat was studied. Five different methods were used for extraction and purification of plasmids. All 37 *Acinetobacter* genospecies harbor at least one plasmid of 40Kb in each. Plasmid pUPI126 (40Kb) was not cured by ethidium bromide, higher temperature (52^0C) and plumbagin. Rifampicin resistant mutants were isolated from *E. coli* HB101 at a frequency of 10 x 10^8 cfu/ml. These mutants were 95 % stable. MIC of rifampicin resistant mutants of *E. coli* HB101 was found to be 128μg/ml. Plasmid pUPI126 was conjugated by membrane filter technique at frequency of 5.9 x10^{-8} per recipient cells. Plasmid pUPI126 was transformed to E. coli HB101 at a frequency 5 x10^5 per ìg of DNA. Plasmid pUPI126 was 90 % stable in host *E. coli* HB101. *E. coli* HB101 rif ^{r} mutant harboring plasmid pUPI126 also showed pyrrolnitrin as well as IAA production as shown by *A. haemolyticus* A19. Transformation was also carried out with other IAA producing Acinetobacter genospecies and rifampicin resistant mutant of *E. coli* HB101. Frequencies for these transformations were, of *A. baumannii* : A13: 3 x10^5, A16 : 1x 10^7,A18: 4 x10^6, A30 :3 x 10^6 per μg of DNA respectively. For *A.* genospecies 3 A15 and A28 is 2 x10^6 & 1x 10^8 per μg of DNA and for *A. junii* A6 transformation frequency was 4 x10^6 per ìg of DNA respectively.

Key words : *Acinetobacter haemolyticus*, Wheat Rhizosphere, Conjugation, Curing, plasmid pUPI126, Transformation, Stability, Rifampicin resistant mutants.

9.2. Introduction :

Plasmids are extra chromosomal, double stranded circular DNA which have the capacity to replicate independently of the host chromosome, yet coexist with it. (Day, 1982). Plasmids are found in a wide variety of bacteria, and it is as difficult to generalize about plasmids as, it is to generalize about the bacteria that harbor them (Trevors, 1985; Trevors et al, 1985, Gerhardt, et al, 1994). The medical importance of plasmids that encode antibiotic resistance and specific virulence traits has been well documented. Plasmids have also been shown to influence significantly several properties contributing to the usefulness of the bacteria in agriculture and industry (Anderson & Mckay, 1983; Chakrabarty, 1976; Crosa, 1989;Crosa, et al, 1977; Helinski, et al, 1985; Nester & Kosuge, 1981; Nies et al, 1987; Palchaudhary, 1977; Woloj, et al, 1986). Some plasmids are stable and can be maintained through successive generations by being partitioned to each daughter cell during cell division. This allows each cell to receive at least one plasmid copy. In recent years, plasmids have been observed in a wide variety of bacteria. (Trevors, 1986). The major interest in bacterial plasmid/s has been focused on the antibiotic resistance as these are the serious concerns to

medical community, where as the metal resistance plasmid are of close concern to environmental hazards, which can affect our ecosystem and our food chain.

Very few studies have demonstrated gene exchange in soil (Reanney et al, 1982). Plasmids have been found in any genera of soil bacteria (Ranford et al, 1981, Friedrich, 1984, Trevors, et al , 1985, Anson & Mackinnon, 1984) and some have potential biotechnological applications. For example *Pseudomonas* spp. containing 100 Kb plasmid encoded the enzyme involved in aniline degradation (Anson & Mackinnon, 1984), a 2-4-dichlorophenoxyacetic acid metabolizing *Alcaligenes* spp. (Friedrich, et al, 1983, Don & Pemberton, 1985), and species of *Acinetobacter* and *Arthrobacter* contains a 53.7 megadalton plasmid that mediates the biodegradation of chlorinated biphenyls, have been described. Plasmid encoded genes often mediate bacterial resistance to heavy metals (Trevors, et al,1985). This suggests that resistance to metals in natural microbial communities could be enhanced by the spread of resistance plasmids. Cadmium, mercury and silver are mainly studied metals for bacterial resistance (Barkay et al, 1985; Radford et al, 1981; Shakibaie, et al 1999). The potential of gene transfer in the soil environment depends on the survival and transport of organisms in soil ecosystem (Smith et al , 1985). In soil environment gene transfer mainly takes place by transformation and conjugation. Conjugative plasmids may be the most important means of gene transfer in the environment (Reanney, et al, 1983).

In view of this background, it is necessary to find out the presence of plasmids in the *Acinetobacter* genospecies isolated from rhizosphere of wheat. Rhizosphere is a unique environment in the soil as it contains large number of microorganisms, their interactions, nutrients and their interactions with plant and root exudates (Glick, 1995). There are many reports on biocontrol of wheat plants by *Pseudomonas fluorescens* antibiotic production against many plant pathogenic fungi and bacteria (Phillip, 1986). Antibiotic production is one of the important tools for the plant protection and is ecologically very safe. Thus, to find out what exactly exist in the plasmids of *Acinetobacter* isolated from rhizosphere of wheat and their possible role in gene transfer, following study was carried out.

9.3. Materials and Methods :

9.3.1.1. Plasmid isolation by Kado & Liu Method (1981) :

Acinetobacter genospecies were grown overnight in Luria Bertani broth at 28 ^{0}C on shaker at 150 rpm. 3 ml of each culture was centrifuged at 10,000 rpm for 10 min. (RM12C, Microcentrifuge, REMI, Mumbai, India). The cell pellet was thoroughly suspended in 1 ml E. buffer (40mM Tris-acetate, 2mM sodium EDTA, pH 7.9 adjusted with glacial acetic acid). The cells were lysed by adding 2 ml of lysis solution (3% SDS, 50 mM Tris, pH 12.6) and mixed gently but thoroughly. The contents were then heated by incubation at 65^{0}C for 90 min in a water bath (modification).

Tubes were cooled at room temperature for 10-15 min followed by mixing of 2 volumes of phenol chloroform solution 1:1 (v/v). Tubes were gently inverted for 50 times and upper aqueous phase of each was separated by centrifugation at 10,000 rpm for 15 min. at room temperature. The upper aqueous phase was collected in a fresh tube while avoiding the precipitate at interphase (Kado & Liu, 1981). The extraction of aqueous phase with phenol chloroform solution was repeated twice to avoid suspended proteins. The aqueous phase was then mixed gently with equal volume of isopropanol (500µl) and incubated at room temperature for 1h. The precipitated DNA was collected by centrifugation at 10,000 rpm for 15 min., dried in the laminar air flow by inverting the tubes for 20 min and finally dissolved in 20µl of $T_{10}E_1$ buffer (10 mM Tris, 1mM EDTA, pH 8) and stored at -20^0C until further use.

9.3.1.2. Plasmid isolation by Holmes & Quigley method (1981) :

The cells were recovered from 3 ml overnight grown culture in Luria Bertani broth at 28^0C on shaker at 150 rpm and centrifuged by 10,000 rpm for 10 min. The pellet was resuspended in 350 µl of STET buffer (8 % sucrose, 5 % triton X-100, 50 mM Tris, pH8). The solution was then kept in boiling water bath for 40 seconds and centrifuged immediately at 10,000 rpm at room temperature. The clear supernatant was transferred into fresh tube and then mixed with two volumes of phenol chloroform solution, then mixed gently. The upper aqueous phase was separated and collected in a fresh tube. The equal volume of isopropanol (500µl) was added, mixed gently and incubated at room temperature for 1h. The precipitated DNA was collected by centrifugation at 10,000 rpm for 15 min., dried in the laminar air flow by inverting the tubes for 20 min and finally dissolved in 20 µl of $T_{10}E_1$ buffer (10mM Tris, 1mM EDTA, pH8) and stored at -20^0C until further use.

9.3.1.3. Plasmid isolation by Olsen method (1990) :

Plasmid DNA extraction was followed as per method described by Olsen, (1990). The cell pellet was obtained from 3ml overnight grown *Acinetobacter* culture as described above and resuspended in 40 µl solution I (50mM Tris, 10mM EDTA, pH8). Cell lysis was carried out by addition of 400 µl solution II (3 % SDS, 50mM Tris, pH 12.45) and incubation at 60^0Cfor 40 min. The 300 µl of solution III (1.5 M potassium acetate , pH 5.2) was added and incubated on ice for 20 min. The clear supernatant obtained by centrifugation at 10,000 rpm for 20 min and transferred in a fresh microfuge tube. Two volumes of phenol-chloroform solution was added and mixed gently. The upper aqueous phase was separated and collected in a fresh tube. The equal volume of isopropanol was added, mixed gently and incubated at room temperature for 1h. The precipitated DNA was collected by centrifugation at 10,000 rpm for 15 min, dried in the laminar air flow by inverting the tubes for 20 min at room temperature and finally dissolved in 20µl of $T_{10}E_1$ buffer (10mM Tris, 1mM EDTA, pH8) and stored at -20^0C until further use.

9.3.1.4. Plasmid isolation by Birnboim & Doly method (1979) :

This method was carried out as described by Birnboim & Doly (1979). The cell pellet was obtained from overnight grown *Acinetobacter* culture as described above. Each of cell pellet was suspended in 100 µl of suspension buffer (Reagent I : 50mM glucose, 10mM EDTA, 25 mM Tris-HCL, pH 8) and incubated on ice for 30 min. Then 200 µl of alkaline SDS solution (Reagent II : 0.2 N NaOH, 1% SDS) was added, mixed well and incubated on ice for 5 min. This resulted in formation of viscous solution to which 150 µl neutralization solution (Reagent III : 3 M sodium acetate, 5 M potassium acetate, pH 4.8 with glacial acetic acid), mixed thoroughly by inverting tubes and incubated on ice for 30 min. The contents were then centrifuged at 10,000 rpm for 15 min at room temperature. The clear supernatant was collected in fresh microfuge tube, mixed with two volumes of phenol- chloroform solution 1:1 (v/v), mixed gently and incubated at room temperature for 10 min. The upper aqueous phase was collected by centrifugation at 10,000 rpm for 15 min in a fresh tube. Two volumes of chilled ethanol (70 %)was added to aqueous supernatant and mixed gently by tube inversions for 50 times and then incubated at -20^0C for 1 h. The DNA precipitate was collected by centrifugation at 10,000 rpm for 15 min, DNA pellet was washed with two volumes of 70 % cool ethanol, centrifuged at 10,000 rpm, dried at room temperature, re-dissolved in 20 µl T_{10} E_1 buffer, and stored at -20^0C until further use.

9.3.1.5. Plasmid extraction by the method of Sambrook, et al (1989) :

- **Small-scale preparations of plasmid DNA :**

Mini preparations of plasmid DNA was obtained by alkaline lysis method (Maniatis et al , 1989). This method is a modification of the method of Birnboim & Doly (1979).

i. A single bacterial colony was transferred into 5 ml of LB medium, and incubated the culture overnight at 28^0C on shaker at 150 rpm.

ii. 1.5 ml of grown culture was added into a microfuge tube and centrifuged at 10,000 rpm for 10 min. at 4^0C .

iii. The medium was removed by inverting the microfuge tubes and bacterial pellet was dried at R.T.

iv. The bacterial pellet (obtained from step 3) was resuspended in 100 µl of ice cold solution I (50mM glucose, 25mM Tris. HCl, pH 8, 10 mM EDTA pH 8) and vortexed vigorously. Solution I was prepared in batches of approximately 100 ml, autoclaved for 15 min and stored at 4^0C.

v. 200 µl of freshly prepared solution II was added (0.2 N NaOH, freshly diluted from a 10 N stock , 1 % SDS). The tube was closed tightly, and the contents were mixed by inverting the tube rapidly five times. The care was taken that the entire surface of the tube must come in contact with solution II. The tube was stored on ice.

vi. 150 µl of ice cold solution III was added (5 M potassium acetate 60 ml, glacial acetic acid 11.5 ml, and d.H_2O 28.5 ml. The resulting solution is 3 M with respect to potassium and 5 M with respect to

acetate). The tube was closed and vortexed gently in an inverted position for 1-2 min to disperse solution III through the viscous bacterial lysate. The tube was stored in ice for 3-5 min.

vii. Centrifuged at 10,000 rpm for 10 min at 4^0C and transferred the supernatant to a fresh microfuge tube.

viii. Equal volume of phenol- chloroform 1:1 (v/v), was added and mixed by vortexing. Centrifuged at 10,000 rpm for 5 min. at 4^0C and transferred the supernatant in a fresh tube.

ix. The double–stranded DNA was precipitated with two volumes of ethanol at room temperature, the solution was mixed by gently agitation of hands and allowed to stand for 1 h at room temperature.

x. Centrifuged at 10,000 rpm for 10 min at 4^0C in a microfuge.

xi. The supernatant was discarded and the microfuge tube was kept in an inverted position on a paper towel in laminar air flow and allowed to all the fluid drain away.

xii. The pellet of DNA was rinsed with 1 ml of ice cold 70 % ethanol at 4^0C. Remove the supernatant and discard. Allow the pellet of nucleic acid to dry in laminar flow for 10 min. at R.T.

xiii. Redissolved the nucleic acid in 20 µl of T_{10} E_1 buffer, pH8 and stored the DNA at -20^0C until further use.

9.3.2. Agarose gel electrophoresis :

The plasmid DNA extracted by all above methods was subjected to agarose gel electrophoresis (Manniatis, et al, 1989). 0.7 % agarose (Hi-Media, Mumbai) was dissolved in 100 ml of TE buffer (10mM Tris, 1 mM sodium EDTA, pH8) by boiling , cooled to around 45-50^0C and poured in to the gel mould (7 x 13 cm) sealed at open ends by cellotape, holding comb of 8 teeth (5 mm lane width/teeth, Bangalore Geni, Ltd, Bangalore, India). After the agarose was solidified, comb was removed carefully taking precaution not to disturb the wells, the tape from the open ends of the tray was removed and tray was placed in electrophoresis tank filled with electrophoresis buffer (TAE buffer : 40 mM Tris-acetate, 1mM EDTA, pH8, adjusted with glacial acetic acid) with the wells towards the cathode. 10 µl of sample was mixed gently in a fresh microfugetube with 5 µl of 1x gel loading buffer (0.25 % bromophenol blue, sucrose in 40 % (w/v water) and loaded carefully in the agarose well. After loading, gel was run at 12 V (low volts) for 30 min then run 52 V for 6-7 h. When bromothymol blue was migrated completely from gel to loading buffer, power supply was switched off. The agarose gel was carefully removed and stained in ethidium bromide solution (0.5µg/ml in TAE buffer) for 15-20 min. Excess ethidium bromide was removed by washing with distilled water. Properly stained gels were observed and documented on gel documentation system. (Alpha Imager, 2000, Alpha Innotech Inc., USA).

9.3.3. Determination of molecular weight of plasmid :

For determination of molecular weight of plasmid/s extracted , 1 kb DNA Ladder (0.1 µg of ladder (40 kb, to 1Kb) per lane width) and high DNA mass ladder (10,000 to 1000 bp , GIBCO-BRL, Life

Technologies) and was also electrophoresed along with test plasmid DNA samples as described above. The molecular weight of each plasmid was determined by analysis of comparative mobility of each plasmid band with respect to the bands from the reference ladder close to it. The concentration of pure plasmid DNA was also determined by the absorbance spectra of 260-280 nm (Shimadzu Japan). The absorbance of DNA obtained from 260-280 nm spectra is multiplied by 50 and result obtained is the concentration of the plasmid DNA expressed in μg/ml.

9.3.4. Curing of Plasmid PUPI126 :

Plasmid curing was carried out by using various curing agents (Trevors, 1986). Plasmid pUPI126 (40Kb) isolated from *A. haemolyticus* A19 is mainly studied for curing. Curing of Plasmid pUI126 was carried out by heating at 45 and 52^{0}C. Curing was also done by ethidium bromide from 100 μg/ml up to 1024 μg/ml. Plasmid curing was also done by plumbagin (Sigma, USA) from 100-1024μg/ml. Combined treatment of ethidium bromide(128 μg/ml) and heat at 52^{0}C and plumbagin (512 μg/ml) and heat at 45^{0}C and 52^{0}C was also carried out for curing of plasmid.

9.3.5. Isolation of rifampicin resistance mutants of *E. coli* HB 101 :

Rifampicin resistant mutants were isolated by plating 100 μl of over night grown cell culture (1 x 10^8 cell/ml) of *E. coli* HB101 on Luria Bertani (LB) agar plates containing 100 μg/ ml of rifampicin dissolved in DMSO. Plates were incubated at 28^{0}C for 7 days. Control experiment was carried out by plating 100 μl of same culture on plane LB agar and plates were incubated at 28^{0}C for 7 days. Number of colony forming units from each plate was calculated. Rate of spontaneous mutation was determined by comparing sets of control and test plates.

9.3.5.1. Purification of rifampicin resistant mutants :

The colonies appeared on the selective media plates were selected and tested for their ability of re-growing on same selective media plates as LA + rifampicin (100μg/ ml). The purified colonies were tested for their stability and preserved at 4^{0}C.

9.3.6. Stability of rifampicin resistant mutants :

Stability testing for mutants was done by plating 100 μl of overnight grown culture of mutants (1 x 10^8 cells/ml) on plain LB agar and LB agar with rifampicin 100 μg/ml by spread plate technique. The inoculated plates were incubated at 37^{0}C for 24 h and colony count was recorded. Mutants stability was expressed as number of cells capable of forming colonies on antibiotic containing medium divided by total viable count on plane medium.

9.3.7. Conjugation between *Acinetobacter* genospecies and *E. coli* HB101 :

Interspecies conjugation was carried out to see whether the plasmid pUPI126 could transfer to *E. coli* HB101. Resistance markers taken into consideration during the selection of donor and recipient strains for conjugation were as follows :

A. haemolyticus A19 isolated from rhizosphere of wheat having selective markers as Te^r, and K^r, used as donor. Rifampicin resistant mutant of *E. coli* K12 HB101 having markers as Sm^r, Ap^-, Tc^- Cm^- and Lac^- ,used as a recipient.

- **Conjugation between *Acinetobacter* genospecies and *E. coli* HB101:**

Conjugation was performed by employing Membrane filter technique (MFT) method is described (Deshpande & Chopade, 1994) as follows :

- **Membrane filter technique (MFT) :**

Freshly prepared cultures of donor and recipient strains were added into 20 ml of LB broth, in 100 ml flask separately and incubated on shaker at 150 rpm for 18 h. Donor was grown in presence of tellurium100 μg/ml and kanamycin 50 μg/ml, to allow expression and replication of plasmid. Three ml of donor and two ml of recipient cells were mixed in sterile petriplate and with the help of 5 ml sterile syringe passed through sterile filter assembly containing 0.45 μm pore size membrane filter (Sartorius, Germany). Membrane was then removed aseptically and placed on the surface of Luria agar containing tellurium (100 μg/ml) and kanamycin (50 μg/ml). Plates were incubated at 37^{0}C for 24-48 h. Mating was disrupted by vigorous vortexing on cyclomixer in 5 ml of Ringers quarter strength solution or 0.8 % sterile saline and 100μl of each dilution was spread on LA containing tellurium 100 μg/ml and kanamycin 50 μg/ml. Plates were incubated for 24-48 h at 37^{0}C and frequency of conjugation was calculated as follows :

$$\text{Frequency of Conjugation} = \frac{\text{Number of Transconjugants}}{\text{Number of recipient cells}} \times \text{dilution factor.}$$

ii.) Replica plating :

To study co-transfer of antibiotic and metal resistant genes, patch test was carried out. Briefly, 25 well-isolated colonies of transconjugants from each selective plate were taken and master plate was prepared on non selective medium. From these 25 colonies were patched manually on to LB agar plates containing kanamycin (50μg/ml) and tellurium (100μg/ml) with the help of sterile toothpicks and incubated at 37^{0}C for 18-24 h. Number of colonies in presence of selection was then scored.

9.3.8. Transformation between *Acinetobacter* genospecies and *E. coli* HB101 :

Plasmid DNA from *Acinetobacter haemolyticus* A19 (pUPI126) was isolated by employing various methods. The quantification of pure DNA was done by employing absorbance spectra 260/280 nm by Shimadzu spectrophotometer and by standard high DNA mass ladder. The transformation was carried out by preparing competent cells of *E. coli* K12 HB101, rifampicin resistant mutant using $CaCl_2$-$MgCl_2$ method described by Sambrook, et al (1989). Transformation between other *Acinetobacter* genospecies and *E. coli* HB101 was also carried out. Plasmid DNA from *A. baumannii* A13, A16, A18, A30, *A.* genospecies 3 A15, A28 and *A. junii* A6 were also isolated and

quantification of these DNA was also done by above method. Frequency of transformation was calculated for per μg of DNA.

- **Preparation of competent cells of rifampicin resistant mutants of *E. coli* K12 HB101 by $CaCl_2$ method (Sambrook, et al, 1989) :**

A single colony of 2-3 mm diameter was picked from 16 h grown fresh culture at 37°C and transferred in to 50 ml of Luria broth in a 500 ml Ehrlenmeyer flask and incubated at 37°C for 3-4 h with vigorous shaking at 150 rpm. For efficient transformation, it is necessary that the number of viable cells should not exceed 10^8 cells/ml. To monitor growth of the culture O.D. (660 nm) was taken after every 30 min. Cells were aseptically transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes (Falcon 2070). Cultures were cooled to 0°C by storing the tubes on ice for 10 min. All the subsequent steps in this procedure were carried out aseptically. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C in a cooling centrifuge (REMI, India). Decanted the media from the cell pellets. Tubes were inverted for 1 min. to allow the last traces of media drain away. Each pellet was resuspended in 10 ml of ice cold 0.1M $CaCl_2$ and stored on ice for 10 min. Cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C in a cooling centrifuge. The media from the cell pellets was decanted. Tubes were inverted for 1 min. to allow the last traces of media drain away. Each pellet was resuspended in 5 ml of ice cold 0.1M $MgCl_2$ for each 50 ml of original culture and stored on ice. Recovered cells by centrifugation and media was removed from pellet as described above, and each pellet was then resuspended in 2 ml of ice cold 0.1M $CaCl_2$ for each 50 ml of original culture and stored on ice. For long time preservation, 50% sterile glycerol was added as cryo-protectant and vials were stored at –20°C.

9.3.8.2. Transformation of Plasmid pUPI126 to *E. coli* HB101:

Plasmid transformation of eight IAA producing *Acinetobacter* spp. was carried out. 1μg of plasmid DNA was mixed with 100 μl of competent *E. coli* K12 HB101 cells (10^8 cells/ml) in a 1.5 ml of microfuge tube and incubated on ice for 30 min. The microfuge tube was immediately transferred to water bath adjusted to 42°C for 2-3 min. and immediately kept on ice for 5 min. Finally 850 μl of double strength LB broth was added to above suspension and incubated at 37 °C for 1h in a water bath. Cultures were serially diluted up to 10^{-8} and 100μl of appropriate dilution including undiluted was inoculated on selective LB agar medium supplemented with kanamycin 50μg/ml, rifampicin 100μg/ml and tellurium 100μg/ml. The plates were incubated at 37°C for 24-48 h. The number of colonies appeared were observed and transformation frequency was determined by calculating number of colonies grown on selective agar medium as transformants obtained per μg of DNA. At the same time competent cells without plasmid DNA were used as control and plated on selective and non-selective agar medium. Transformation between other IAA producing *Acinetobacter*

genospecies such as *A. baumannii* (A13, A16, A18, A30), *A.* genospecies 3 (A15, A28) and *A. junii* (A6) with *E. coli* HB101 rif^r mutant was also carried out as described above.

Same markers, such as tellurium (100μg/ml), rifampicin (100μg/ml) and kanamycin (50μg/ml) was used for above each *Acinetobacter* strain as all strain as all are resistant to tellurium and kanamycin.

Preservation and maintenance of transconjugants and transformants :

Transconjugants and transformants of plasmid pUPI126 were preserved and maintained by streaking them on a selective medium and sub-cultured on Luria agar slants. The transconjugants and transformants were revived on Luria agar containing kanamycin (50μg/ml), rifampicin (100μg/ml) and tellurium (100μg/ml) and transferred on agar slants and sealed with wax and stored at 4^{0}C.

9.3.9. Stability of plasmids :

Stability of plasmids in *E. coli* and *Acinetobacter* was determined as per method described by Chopade, et al (1985). In brief : a single colony of plasmid carrying *A. haemolyticus* A19, grown in LB agar plate containing tellurium 1(00 μg/ml) and kanamycin (50 μg/ml)was used to inoculate 10 ml of LB containing same concentration (100 μg/ml of tellurium and 50 μg/ml of kanamycin) on shaker at 150 rpm for 37^{0}C for 7-8 h, till culture reached cell density of approximately 5 x 10^8 cells/ml. Portions of appropriate dilutions (in 0.85 % saline) of this culture was then spread on LB agar plates with and without tellurium and kanamycin. Plates were incubated at 37^{0}C for 24-48 h. Number of cells capable of forming colonies on each medium was then calculated as follows :

$$\% \text{ Stability} = \frac{\text{TVC on Luria agar + Tellurium (100 μg/ml) + rifampicin (100μg/ml) + Kanamycin (50 μg/ml)}}{\text{TVC on Luria agar.}}$$

9.3. Results :

9.4.1. Plasmid profile of *Acinetobacter* genospecies isolated from rhizosphere of wheat :

All 37 *Acinetobacter* strains belonging to 5 different *Acinetobacter* genospecies were tested. Interestingly all 37 *Acinetobacter* contain at least one plasmid of molecular weight of 40 Kb each. It is observed that plasmid extracted by 5 different methods showed presence of single plasmid in all strains with molecular weight 40 Kb. It should be noted that though the plasmid in each strain has same molecular weight they harbor different characteristics such as, some species for example, *A. haemolyticus* A19 showed plasmid encoded IAA and pyrrolnitrin production, other species such as *A.* genospecies 3 (A15 and A28), *A. baumannii* (A13, A16, A18 and A30) and *A. junii* (A6). showed plasmid encoded IAA production while the remaining are not able to produce IAA. Fig.1 shows the plasmid profile of *Acinetobacter* genospecies isolated from rhizosphere of wheat.

Fig.1A

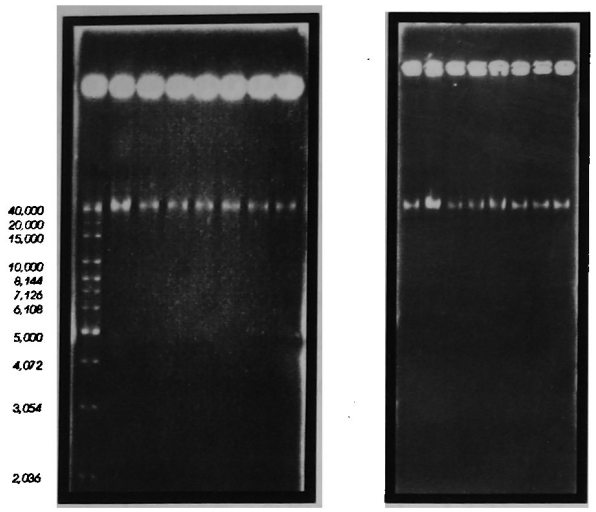

Fig.1B

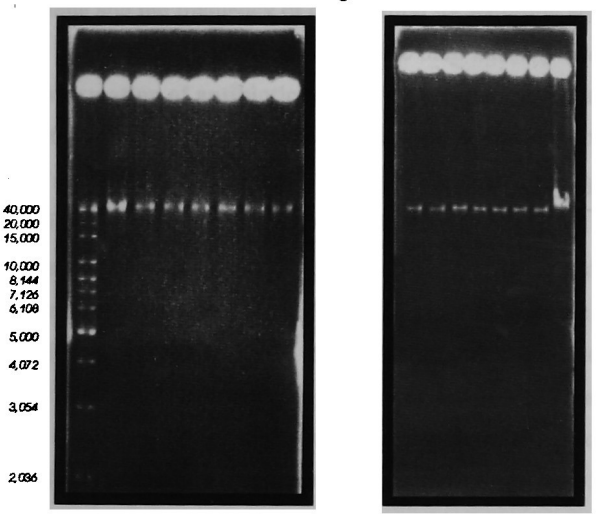

Fig.2

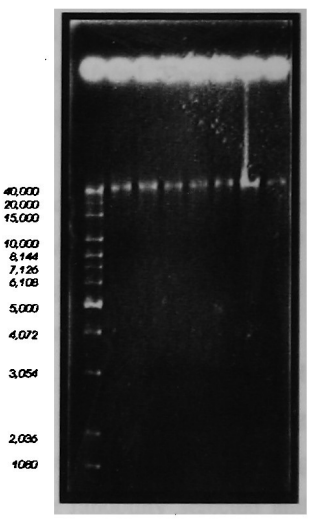

Fig.1.: Plasmid isolation of *Acinetobacter* genospecies 3 isolated from rhizosphere of wheat .

Fig.1A : Method used : Kado & Liu, (1981).

Lane 1 : 1 Kb DNA ladder; Lane 2 : *A. calcoaceticus* (A15); Lane 3 : *A. haemolyticus* A37

Lane 4 : *A. baumannii* (A25); Lane 5: *A. junii* (A7)

Lane 6,7&8 : *A.* genospecies 3 (A12, A15 & A28).

(same for each gel)

Fig.1B : Method used : Maniatis, et al (1981).

Lane 1 : 1 Kb DNA ladder; Lane 2 : *A. calcoaceticus* (A5); Lane 3 : *A. haemolyticus* (A37)

Lane 4 : *A. baumannii* (A25); Lane 5: *A. junii* (A7)

Lane 6,7&8 : *A.* genospecies 3 (A12, A15 & A28).

(same for each gel)

Fig.2 : Confirmation of conjugal transfer and transformation of plasmid pUPI126 from *A. haemolyticus* A19 to *E. coli* HB101.

Lane 1 : 1 Kb DNA ladder; Lane 2 & 8 : *A. haemolyticus* A19;

Lane 3 & 4 : transconjugants of *E. coli* HB101

Lane 5,6 & 7 : transformants of *E. coli* Hb101

9.4.2. Curing of plasmid pUPI126 :

Plasmid pUPI126, subjected for different curing treatments was not cured by ethidium bromide even at concentration of 1024 µg/ ml and also not by heat at 45 and 52 ^{0}C. It was also not cured by plumbagin at concentration of 1024 µg/ml. It can be concluded that these plasmids were refractory for curing.

9.4.3. Isolation of rifampicin resistant mutants of *E. coli* K12 HB101 and their stability :

E. coli K12 HB101 was exposed to 100 µg/ml of rifampicin for isolation of mutants. 10 colonies were obtained after 48 h and frequency calculated for mutants was 10 x 10^8 cfu/ml. These mutants were found 95 % stable. MIC of these mutants to rifampicin was found to be 1024 µg/ml.

9.4.4. Conjugal transfer of plasmid/s from *Acinetobacter* :

Conjugation between *E. coli* K12 HB101 and *A. haemolyticus* A19 carried out by membrane filter technique and the frequency of conjugation for tellurium and kanamycin was found to be 5.9 x 10^{-8} It can be concluded that plasmid pUPI126 of *A. haemolyticus* A19 is a conjugative plasmid.

9.4.5. Transformation of antibiotic and metal resistant genes from *Acinetobacter* spp. :

Transformation of plasmid pUPI126 between *A. haemolyticus* A19 and *E. coli* HB101 was carried out. Transformation frequency obtained for tellurium and kanamycin was found to be 5 x10^6 per µg of DNA. The control plate without addition of DNA did not form any colony on LB agar containing kanamycin (50 µg/ml) and tellurium (100 µg/ml). Transformants were randomly selected and purified on same selective medium and plasmid isolation was carried out. Presence of plasmid pUPI126 was detected in *E. coli* HB101 with same molecular weight plasmid (40Kb) after transformation as well as conjugation. *E. coli* HB 101 transformants as well as transconjugants were also checked for some special characters of *A. haemolyticus* A19 encoded by plasmid pUPI126 i.e. production of antibiotic and IAA. It was found that transconjugants as well as transformants showed production of antibiotic as well as IAA. Transformation between *A. baumannii* A13, A16, A18, A30, *A.* genospecies 3 A15, A28 and *A. junii* A6 with *E. coli* HB101 rif ^{r} mutant was also carried out. It should be noted here that transformation frequencies for all *Acinetobacter strains* were calculated for resistant markers i.e. tellurium and kanamycin. Tellurium and kanamycin in all above *Acinetobacter* strains were transformed at the same frequency. Frequency for each transformation was recorded as follows : Transformation frequency for *A. baumannii* A13 and *E. coli* HB101 was 3 x10^5 per µg of DNA, for *A. baumannii* A16, 1x 10^7 per µg of DNA, for *A. baumannii* A18, 4 x10^6 per µg of DNA and for *A. baumannii* A30, 3 x 10^6 per µg of DNA. Transformation frequency of *E. coli* HB101 and *A. genospecies* 3 A15 was 2 x10^6 and for A28 1x 10^8 per µg of DNA. In case of *A. junii* A6 transformation frequency was found to be 4 x10^6 per µg of DNA.

9.4.6. Stability of plasmid pUPI126 :

Plasmid pUPI126 was found to be highly stable (100 %) in its original host *A. haemolyticus* A19. Plasmid pUPI126 was stable without any selection pressure as well as in presence of kanamycin and tellurium. Plasmid pUPI126 was also 90 % stable in *E. coli* HB101.

Table 1 : Plasmid profiles of *Acinetobacter* genospecies isolated from rhizosphere of wheat :

Acinetobacter genospecies	No. of plasmids obtained by the methods used					Total No. of Plasmids	Molecular Weight Kb[a]
	Method[1]	Method[2]	Method[3]	Method[4]	Method[5]		
***A. calcoaceticus* (4)**							
A2, A3, A5 &A35	1	1	1	1	1	1	40
***A.* genospecies 3 (3)**							
A12, A15 &A28	1	1	1	1	1	1	40
***A. baumannii* (9)**							
A1, A13, A16,	1	1	1	1	1	1	40
A18, A23, A25,	1	1	1	1	1	1	40
A30, A32 &A33	1	1	1	1	1	1	40
***A. haemolyticus* (11)**							
A11, A17, A19,	1	1	1	1	1	1	40
A21, A22, A24	1	1	1	1	1	1	40
A27, A29, A34	1	1	1	1	1	1	40
A36 & A37	1	1	1	1	1	1	40
***A. junii* (10)**							
A4, A6, A7, A8	1	1	1	1	1	1	40
A9, A10, A14,	1	1	1	1	1	1	40
A20, A26 & A31	1	1	1	1	1	1	40

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a1000 Dalton = 1mega Dalton, 1000bp = 1Kb, 1 md = 1.5 Kb.

9.4. Discussion :

Plasmids may encode a wide variety of genetic determinants which permit their bacterial host to survive better in an adverse environment or to compete better with other microorganisms occupying the same ecological niche (Trevors, 1986). Gene transfer in bacteria mainly takes place by plasmid/s and resistance in bacteria to many antibiotics and toxic metals is known to be conferred by plasmids.(Trevors, 1986). Plasmid carrying genes encoding antibiotic and metal resistance in *Acinetobacter* genospecies have been studied in detail with clinical and environmental isolates (Dhakephalkar & Chopade, 1994). However, there is no report on rhizosphere isolates. A number of clinical isolates of *A. baumannii* have been reported to exhibit wide range of antibiotic resistance (Bergogne-Berezin &Towner, 1991). However, very few studies have demonstrated plasmid mediated transfer of resistance genes (Towner, 1991). Complex transfer frequencies of standard plasmids belonging to different incompatibility have been observed between *A. calcoaceticus* EBF65/65 *and E. coli* K12 (Chopade et al , 1985). Plasmid sizes range from 1 to more than 200bp (Gerhardt, et al, 1994) and even larger mega-plasmids were detected in *Rhizobium* spp. (Burkardt et al, 1987).

There is a growing interest in the fate of introduced bacteria and its gene transfer potential in soil (van Elsas, 1987), as there are many possible applications for agronomically important microorganisms that have been genetically modified to improve their beneficial performance in crop production, biodegradation of xenobiotics or as biological pesticides. However, information on gene transfer in soil is scarce in spite of considerable knowledge gathered with in vitro mating systems (van Elsas, 1987). In soil and rhizosphere environment, plasmid isolation and gene transfer was studied mostly in *Pseudomonas, Agrobacterium E. coli, Klebsiella, Enterobacter, Bacillus,* and *Rhizobium* spp (Maria-Isabel, et al, 1991; Inze, et al, 1984; Weinberg & Stotzky, 1972; Henschke, & Friederich, 1990; Graham & Istock, 1978; Burkardt, et al,1987).

In the present study we have isolated plasmid/s from 37 *Acinetobacter* spp., isolated from rhizosphere of wheat. It was important to note that each of *Acinetobacter* strain contain one mega plasmid of molecular weight of 40 Kb. Typing of *Acinetobacter* strains in different clusters on the basis of plasmid profile has been widely used and observed to be a rapid and simple method (Alexander, et al, 1988; Gerner-Smith, 1989; Johnson, et al, 1992; Gerner-Smidth & Tjenberg, 1993; Kropec, et al, 1993; Seifert, et al, 1994). It was observed that number and size of Plasmids in *Acinetobacter* varies (Deshpande & Chopade, 1994). Interesting we noted that all 37 *Acinetobacter* spp. showed one plasmid of molecular weight of 40 Kb. From the molecular weight of these plasmid, it is clear that it is a megaplasmid. From the MIC values of antibiotics and metal salts it was also indicated that *Acinetobacter* spp. isolated from rhizosphere of wheat were highly resistant to most of the antibiotics such as chloramphenicol, streptomycin, penicillin and heavy metal salts

such as tellurium, selenium, arsenate, arsenite and lead. These resistant markers maybe present on the plasmid in these strains. Previously, Dhakephalkar & Chopade (1994), found that environmental isolates of *Acinetobacter* were resistant to lead, zinc, cadmium, arsenate, nickel, selenium and tungsten. Shakibaie et al, (1999), isolated *Acinetobacter* strains from clinical environment found that *Acinetobacter baumannii* BL-54 was resistant to silver and ampicillin, tetracyclin and chloramphenicol used successfully for removing silver from photographic west. Thus from above result one can use these plasmids in the use of such environments polluted by tellurium and other heavy metals to reduce the pollution by removing the toxic metals (Shakibaie et al, 1999).

Plasmid curing is one of the important methods for the detection of resistant genes encoded by bacteria (Trevors, 1985; 1986). It allows direct comparison between plasmid containing and plasmid cured strains. Curing requires the use of curing agents or other procedures (elevated growth temperature, thiamine starvation) to increase the frequency of spontaneous segregation (Caro, et al, 1984). The usefulness of curing agents is unpredictable in many bacterial strains, as there are no standard protocols applicable to all plasmids. However, there are some procedures that have provided good results with certain species. *A. haemolyticus* A19 contained one plasmid pUPI126 of molecular weight 40Kb. The strain showed resistance to metals like tellurium, selenum, arsenite, arsinate and antibiotics like kanamycin, penicillin and chloramphenicol in addition to production of pyrrolnitrin and IAA. Therefore to confirm the location of genes coding these different characteristics curing was attempted by different curing agents like ethidium bromide and plumbagin individual and in combination with elevated temperature. Plumbagin and acridine orange had been proved to be useful curing agent for *Acinetobacter*. Plumbagin has been used for curing antibiotic and metal resistant plasmids from clinical isolates of *Acinetobacter* (Chopade, et al, 1994). Soil isolate, *A. baumannii* BL88, containing plasmid pUPI199 was also cured by plumbagin (Deshpande & Chopade, 1994). Acridine orange had been used for curing of plasmid present in *A. calcoaceticus* RA57 and *Acinetobacter* spp. strain YAA, both coding for hydrocarbon degradation (Fujii, et al, 1997). In case of plasmid pUPI126 it was not cured with any curing agent. However, the usefulness of curing agent is unpredictable in many bacterial strains, as there are no standard protocols applicable to all plasmids (Trevors, 1986). We observed that with the individual curing agents plasmid pUPI126 was not cured, however it was cured by combination of plumbagin and heat (45^0C).

Gene transfer mechanism between bacteria, in soil and rhizosphere environment mainly takes place by conjugation and transformation. Conjugation is an important gene transfer mechanism for soil and rhizosphere bacteria (Fry & Day, 1990; Smit, et al, 1991; Smit, et al, 1993) and the genes responsible for mating-aggregate formation and DNA transfer are often carried out by self transmissible plasmids. Different bacterial plasmids posses resistance to different metals and antibiotics. In the present study conjugation was carried out between *Acinetobacter haemolyticus*

A19 and *E. coli* HB101. Plasmid pUPI126 from *A. haemolyticus* A19 has two additional characters such as antibiotic production and IAA production. Transconjugants of *A. haemolyticus* A19 were observed for antibiotic and IAA production, which exhibited positive results. Thus, from these results one may expect the gene transfer between *Acinetobacter* and other gram negative soil bacteria such as *Pseudomonas fluorescens, Pseudomonas* spp., *Serratia marcescens, Azospirillum* spp., *Azotobacter* spp. etc. may takes place in rhizosphere of wheat environment and their genes can be transferred to other bacteria by conjugation. Similar results were obtained by *E. col.* from soil by conjugation. In case of *Pseudomonas putida*, isolated from soil, can able to transconjugate TOL plasmids in other soil isolates. It was observed that plasmid pUPI126 is 95 % stable in *E. coli* HB101 and 100 % stable in *A. haemolyticus* A19. Thus plasmid pUPI126 can replicate and remain stable in the original host as well as in transconjugants and transformants.

Uptake of exogenous DNA from the environment was the first mechanism of genetic exchange reported in bacteria (Postendorfer, et al, 1997). Today a large number of representatives of gram negative and gram positive bacteria are known to undergo natural transformation. Since transformation dose not involve direct cell to cell interaction, it has been suggested that natural transformation most probably accounts for the exchange of DNA between distantly related microorganisms, providing a major mechanism for acquiring novel metabolic capabilities and adapting to changing environmental conditions (Postendorfer, et al, 1997).

In the present study, transformation was carried out between *Acinetobacter* genospecies and *E. coli* HB101. All transconjugants also exhibited presence of plasmid DNA in them and also showed IAA production. Transconjugants of *A. haemolyticus*A19 contains plasmid pUPI126 were also checked for antibiotic production, which also exhibited positive results for antibiotic production.

The significant findings of the present study demonstrate that rhizosphere *Acinetobacter* possess mega-plasmids with different interesting characteristics such as antibiotic production (*A. haemolyticus* A19), IAA production and metal and antibiotic resistance. We can use these plasmids in gene cloning to make a super plasmid containing strains and can be used in genetic transfer studies of *Acinetobacter* spp. to other microorganisms in rhizosphere environment.

9.5. Conclusions:

- *A. haemolyticus* A19 isolated from rhizosphere environment contains plasmid pUPI126 which encodes genes for pyrrolnitrin and IAA production.
- Plasmids from *Acinetobacter* spp. isolated from rhizosphere of wheat exhibited high antibiotic and metal resistance.

- Plasmids from *Acinetobacter* spp. are found to conjugative and transformable to other bacterial spp. such as *E. coli* from rhizosphere environment.
- It can be concluded from the present study that rhizosphere is a unique environment for the isolation of plasmids and gene transfer.

9.6. Significance of this work :

- Overall, it can be conclude that rhizosphere Acinetobacter spp. contains plasmid which may code important markers for resistance to heavy metal and antibiotics tellurium and chloramphenicol. It is also important to note that *Acinetobacter haemolyticus* A19 isolated from rhizosphere of wheat able to produce IAA and pyrrolnitrin and genes responsible for production are encoded on plasmid pUPI126. Thus, it was found that rhizosphere is a unique environment for the isolation of *Acinetobacter* spp. showing heavy metal and antibiotic resistance which can be further used in control of environmental pollution.

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CHAPTER 10

Concluding Remarks

Concluding Remarks

From the work presented in this thesis it can be concluded that *Acinetobacter* was found to be one of the dominant bacteria (31.3 %) next to *Pseudomonas* (35.7 %) in the rhizosphere of wheat. *Moraxella* was also found to be dominant (28.0 %) next to *Acinetobacter* in the rhizosphere of wheat. Maximum number of *Acinetobacter* spp. (23) were found in rhizosphere area. Growth stages of wheat plant showed changes in the population of these bacterial genera and it was observed that at inflorescence and fruiting stages, count of all four bacterial genera was increased. This fact suggest that root exudates of wheat plant may affect natural population of bacteria in the rhizosphere. It was also observed that *Acinetobacter* spp. were able to colonize roots of wheat (10 %) though *Pseudomonas* was found to be dominant (75 %) in rhizoplane. This is the first report on distribution of *Acinetobacter* in rhizosphere of wheat. This suggests that members of genus *Acinetobacter* are one of the important bacteria in rhizosphere environment of wheat.

Characterization of heterogeneous strains of *Acinetobacter* genospecies into subgroups or biotypes, in addition to utilization of carbon and nitrogen source, growth at different temperatures, and properties like salt tolerance were also found to be useful. It is also found that *Acinetobacter* genospecies isolated from rhizosphere of wheat produce different enzymes such as chitinase, which play an important role in plant protection against fungal pathogens. L- asparginase, which play an important role in medicine and lipase, an industrially important enzyme. Almost all *Acinetobacter* strains were found to be resistant to β-lactam group of antibiotics such as Pn, Ap, Ce, Am, Cf and Cl. They were found to be sensitive to aminoglycoside group of antibiotics. Moreover, a diverse resistance pattern to toxic metals like arsenate, arsenite, tellurium, selenium and lead is another important character shown by *Acinetobacter*. However, more studies on *Acinetobacter* are needed to improve our knowledge of ecology of different *Acinetobacter* genospecies from rhizosphere of wheat, which in turn will help us to understand the role of *Acinetobacter* in the rhizosphere of wheat and other crop plants.

It was found that, *Acinetobacter* spp, isolated from rhizosphere of wheat produced pyrrolnitrin antibiotic, which exhibited broad-spectrum antimicrobial activity. Pyrrolnitrin production in *Acinetobacter haemolyticus* A19 was found to be encoded on plasmid pUPI126 of molecular weight 40kb which is a conjugative plasmid. Plasmid pUPI126 is conjugally transferred to *E. coli* HB101 at the frequency of 5.9×10^{-8} per recipient cells. Plasmid pUPI126 is also able to transform to *E. coli* HB101 at frequency 5×10^{5} per μg of DNA. This is the first report of plasmid encoded pyrrolnitrin production by any known microorganisms as well as in the genus *Acinetobacter*. Literature search recorded that pyrrolnitrin proved as a biocontrol agent against many fungal diseases. Thus, *Acinetobacter* producing pyrrolnitrin would probably play an important role in plant protection.

Acinetobacter spp. isolated from rhizosphere of wheat showed bioemulsifier production. This bioemulsifier named as "Chopadesan" has unique characteristics. It consist of protein-polysaccharide (85 : 15) and has excellent emulsification activity, and very stable at room temperature. The production of this bioemulsifier is constitutive with highest yield of 6.6.g/liter. It also exhibited good cleaning activity and reduced surface tension of water by 10 dynes/cm and CMC of this bioemulsifier was recorded as 14mg/ml. Thus, Chopadesan can be used in different fields such as in agriculture, medicine and in industry. This is the first report on novel protein polysaccharide bioemulsifier production by *Acinetobacter* spp. from rhizosphere of wheat.

Indole acetic acid (IAA) was produced by eight *Acinetobacter* spp. isolated from rhizosphere of wheat. IAA produced by *Acinetobacter* spp. played an important role in wheat plant growth promotion. IAA produced by *Acinetobacter*, was encoded by plasmid pUPI126 molecular weight of plasmid pUPI126, was 40kb. Plasmid pUPI126 was conjugally transferred at frequency 5.9 x 10^8 per recipient cells. Plasmid pUPI26 can transform to *E. coli* HB101 at frequency 5 x 10^5 per μg of DNA. Plasmid pUPI126 also encoded resistance for tellurium and kanamycin. This is the first report on plasmid pUPI126 encoded IAA production in the genus *Acinetobacter*. Effect of IAA production was observed by inoculating *Acinetobacter* spp with wheat plant and increase in root and shoot length was observed. It was found that *Acinetobacter* significantly increased root and shoot length of wheat plant. Thus IAA production showed the role of *Acinetobacter* in plant growth promotion.

Rifampicin resistant mutants of *Acinetobacter* were isolated and could also produce IAA. These mutants survived best up to four months in lignite carrier at room temperature (28-30^{0}C). After 4 months counts of wild as well as rif^r mutants of *Acinetobacter* present in lignite carrier was found to be decreased. *Acinetobacter* and its rif ^{r} mutants used for the preparation of lignite based *Acinetobacter* bioinoculum and used in field experiments showed 62 % increase in wheat grain yield without any fertilizer and 75 % increase in the wheat grain yield with half dose of fertilizers. These results suggest that *Acinetobacter* bioinoculum was found to be excellent for the wheat plant. This is also the first report on novel bioinoculum preparation of *Acinetobacter* genospecies isolated from rhizosphere of wheat. Effect of *Acinetobacter* bioinoculum on 12 different plants such as i. *Phaseolus vulgaris* ii. *Sorghum bicolor* iii. *Pennisetum americanum* iv. *Zea maize* v. *Arachis hypogea* vi. *Vigna radiata* vii. *Cicer arietinum* viii. *Coriander cilantro* ix *Spinacia olerecea* x. *Lycopersicon esculentum* xi. *Glycine max* and xii. *Phaseolus vulgaris* were also observed and found that *Acinetobacter* bioinoculum was best for all 12 plants.

Overall, this research work has revealed the role of *Acinetobacter* in rhizosphere of wheat. *Acinetobacter* helps plant in different ways such as i.) it promotes plant growth by producing plant growth promoting hormones such as IAA, ii.) it can produce pyrrolnitrin, an antibiotic, which has broad

range of antimicrobial activity, specially against fungi. It was also observed that these *Acinetobacter* spp. are able to solubilize phosphate, also can produce chitinase and other important enzymes. They are also able to produce bioemulsifier. IAA production and antibiotic production in *Acinetobacter* spp. was encoded on plasmid pUPI126.

Thus, *Acinetobacter* would act as novel promising bacterium for plant growth promotion and in biocontrol. This thesis describes the role of *Acinetobacter* in the rhizosphere of wheat, which is studied for the first time. This work has opened a new field of research in biology of *Acinetobacter* and *Acinetobacter* has been established as a novel rhizobacteria. This work has lead the foundation, based on which further studies can be carried out.

- **Significant findings of this work :**

1. *Acinetobacter* is significantly present in rhizosphere of wheat as compare to other gram-negative bacteria. *Acinetobacter* genospecies, isolated from rhizosphere of wheat showed interesting biochemical characteristics.
2. This is the first report on plasmid pUPI126 encoded indole acetic acid (IAA) and pyrrolnitrin production by genus *Acinetobacter.*
3. This is also the first report on IAA production by species of *A. baumannii, A. haemolyticus, A. junii* and *A.* genospecies 3.
4. This is the first report on novel protein-polysaccharide bioemulsifier production by *A. baumannii* A25, isolated from rhizosphere environment.
5. This is the first report on development of novel *Acinetobacter* bioinoculum for plants.
6. Lignite based *Acinetobacter* bioinoculum and used in field experiments showed 62 % increase in wheat grain yield without any fertilizer and 75 % increase in the wheat grain yield with half dose of fertilizers.

- Overall, this work has established *Acinetobacter* spp. as a novel rhizobacteria.

- **Deposition of Microorganism :**

Out of 37 *Acinetobacter* spp isolated from rhizosphere of wheat, 10 strains, showing important characteristics, have been deposited with the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India and have been assigned accession number of NCIM. These are as follows :

Name of bacteria	NCIM number	Presence of plasmid	Important characteristics
Acinetobacter haemolyticus A19	NCIM 5155	One (PUPI126,40kb)	Pyr^+, IAA^+, Te^r, Km^r
A. genospecies 3 A28	NCIM 5159	One (40kb)	IAA^+, Te^r, Km^r
A. genospecies 3 A15	NCIM 5151	One (40kb)	IAA^+, Te^r, Km^r
A. baumannii A13	NCIM 5158	One (40kb)	IAA^+, Te^r, Km^r
A. baumannii A16	NCIM 5156	One (40kb)	IAA^+, Te^r, Km^r
A. baumannii A18	NCIM 5157	One (40kb)	IAA^+, Te^r, Km^r
A. baumannii A30	NCIM 5154	One (40kb)	IAA^+, Te^r, Km^r
A. junii A6	NCIM 5153	One (40kb)	IAA^+, Te^r, Km^r
A. junii A7	NCIM 5160	One (40kb)	IAA^+, Te^r, Km^r
A. baumannii A25	NCIM 5152	One (40kb)	ND

Pyr: Pyrrolnitrin, IAA : indole acetic acid, Te: tellurium, Km: kanamycin, + : positive, ND: not done.

*All (37) cultures submitted to Prof. Dr. B. A. Chopade, Research Guide Department of Microbiology, University of Pune.