Studies on production of Cyclodextrin glycosyl transferase (CGTase) enzyme using alkaliphilic bacteria

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DOCTOR OF PHILOSOPHY

In

MICROBIOLOGY

(FACULTY OF SCIENCE)

By

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Under the guidance of

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at

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Pune, 411004. India

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This thesis is dedicated to my dear grandmother,

Late Mrs. Shalini Arthur Choramble.

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My gratitude to my God, my *El-Shaddai* without whom this would have been impossible.

Mrs. Rebecca Sandeep Thombre

FORM A

CERTIFIED that the work incorporated in the thesis "Studies on production of Cyclodextrin glycosyl transferase (CGTase) enzyme using alkaliphilic bacteria" submitted by Mrs. Rebecca Sandeep Thombre was carried out by the candidate under my supervision/ guidance. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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Declaration by the Candidate

I declare that the thesis entitled "Studies on production of Cyclodextrin glycosyl transferase (CGTase) enzyme using alkaliphilic bacteria" submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me during the period from November 2008 to April 2012 under the guidance of Dr. Pradnya P. Kanekar and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institution of Higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

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Abstract of the Thesis entitled

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November 2012

Studies on production of Cyclodextrin glycosyl transferase (CGTase) enzyme using alkaliphilic bacteria

Cyclodextrin glycosyl transferase (CGTase; EC 2.4.1.19) is the enzyme that converts starch into cyclodextrins (CD's) which are closed-ring structures having six or more glucose units joined by means of α-1, 4 glucosidic bonds. CGTases are classified in the α-amylase family and are known to catalyze four different transferase reactions: cyclisation, coupling, disproportionation, and hydrolysis .Three major types of Cyclodextrins are produced by CGTases depending on number of glucose units, α – CD, β- CD and γ- CD. CGTase is used for industrial production of cyclodextrin and for biotransformation. Cyclodextrins have a variety of applications in food, pharmaceutical and cosmetic industry. Cyclodextrins are used as drug carriers and tabletting vehicles.

It is evident from the above mentioned applications that CGTase enzyme is industrially a very important enzyme because of its application in production of CD's. However, its application is still significantly limited because of its low yield and extravagant price. CGTase from alkaliphilic *Bacillus* sp. overcame all these problems and lead to mass production of α – CD, β- CD and γ- CD .Since then it is known that alkaliphiles are candidate organisms for CGTase production.

In India, Lonar Lake (Buldhana Dist., Maharashtra State) is a well known alkaline lake formed by meteorite impact in basalt. The cultivable bacterial diversity of Lonar lake and screening of organisms for industrially important compounds with biotechnological applications (PHA, EPS, proteases) has been studied previously but there is no report on the occurrence of CGTase in organisms isolated from Lonar Lake. Thus studies on alkaliphilic bacteria from Lonar Lake for production of CGTase may pave way for discovery of this extremozyme with huge biotechnological potential and application for production of CD's.

The present investigation resulted in following findings:

Part 1

Aerobic alkaliphilic bacteria previously isolated and identified from Lonar Lake were screened for starch hydrolysis using starch medium and for production of CGTase on phenolphthalein methyl orange medium. 15 isolates from Lonar lake showed starch hydrolytic activity and 6 were positive for CGTase production on phenolphthalein methyl orange medium. The results of CGTase production were confirmed by phenolphthalein assay. The six isolates showing positive CGTase activity were *Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1.To the best of our knowledge, this is the first report of alkaliphilic *Exiguobacterium aurantiacum,* Lake Bogoria isolate 25 B1 and *Paenibacillus* sp L55 from Lonar lake for production of CGTase. *Bacillus* sp is a common producer of CGTase hence *Exiguobacterium aurantiacum* and *Paenibacillus sp.*L55 were selected for production of CGTase.

Part 2

The preliminary studies on optimization of fermentation parameters using one parameter at a time and two parameters at a time at multi levels were performed. Taguchi Design of Experiments (DOE) was used for optimization of medium composition. In Taguchi DOE, a standard L9 orthogonal array was used to examine 4 factors at 3 levels. Taguchi DOE revealed that starch is the most significant factor for the CGTase production by *Paenibacillus* sp. and *E. aurantiacum* followed by peptone and yeast extract. pH showed the least impact among the factors studied with the assigned variance of values. The error observed was very low which indicated the accuracy of the experimentation. Based on the equation for prediction, the CGTase activity by *E. aurantiacum* can be increased from 4.19 U/ml (which is average activity obtained from the nine trials) in an optimized batch submerged shake flask level fermentation to 6.15 U/ml. CGTase activity of 6.15 U/ml which is 96 % of the predicted >6.4 U/ml with the modified culture conditions. Similarly the CGTase activity by *Paenibacillus* sp. was enhanced from 3.5 U/ml to 5.15 U/ ml. *Exiguobacterium aurantiacum*

demonstrated higher activity of CGTase with a faster growth rate and was selected for scale up studies.

Part 3

After optimization of production medium in shake flask, the production was scaled up in 3 L fermentor where aeration, agitation and aspect ratio were optimized. The production was further scaled up in a 14 L SS stirred tank in-situ sterilizable Fermentor. The yield of CGTase was enhanced from 6.15 U/ml in 250 ml shake flask to 7.7 U/ml in 3 L and upto 8.2 U/ml in 14 L fermentor. The CGTase production was attained in 4 hours in 14 L fermentor which demonstrates that the production process is of a remarkably short time which is industrially significant. An optimized and rapid fermentative process for production of CGTase by *E. aurantiacum* was thus developed successfully using starch based medium.

Part 4

CGTase produced by *Exiguobacterium aurantiacum* was successfully purified using starch adsorption chromatography with 25 fold purification. A single band of 77.84 kDa on SDS PAGE indicates the homogeneity and possible molecular weight. CGTase demonstrated pH Optimum 9, pH stability8 – 9, temperature optimum 50°C and temperature Stability 50 – 70 °C. CGTase was strongly inhibited with complete loss in activity by Fe+3 and partially inhibited by Mn+2, Zn+2, Cu+2, DTT and PMSF. The K_m, V_{max} and K_{cat} value for CGTase produced by *E. aurantiacum* was calculated as 0.0225 g/L, 57 µg/ml/min and 316.7 s-1. MALDI TOF –MS revealed its homology to glycosylase produced by *Colwellia* spp. The isoelectric potential was predicted as 9.7 by MALDI TOF-MS.

Part 5

Cyclodextrins were produced by action of CGTase from E. aurantiacum on raw potato starch, corn starch, soluble potato starch, wheat starch, rice starch and sago starch. The cyclodextrins were detected by HPLC and FEG-SEM.

CGTase was produced by immobilized cells of E. aurantiacum entrapped in calcium alginate beads in a packed bed reactor and was compared to CGTase produced by free cells. For economical production of CGTase, agro-waste like

potato-peel waste was used as the sole carbon substrate. The immobilized cells were effectively used for the production of β -Cyclodextrins from potato peel waste with an activity of 5 .1 U/ml as compared to 6 U/ml for free cells. The β – Cyclodextrins produced were detected by HPLC, microscopy and colorimetric estimation. Thus immobilized whole cells can be effectively used for economical production of cyclodextrins from agro –waste based materials.

CGTase produced by *E .aurantiacum* demonstrated moderate thermostability due to which it can be used as an antistaling enzyme in bread making. The firmness and textural properties of bread containing CGTase were studied. It was observed that loaf volume and texture of bread with CGTase was greater than control bread. The parameters like hardness, fracturability, adhesiveness, springiness, cohesiveness, gumminess, chewiness and resilience of bread were studied using TA XT2i Texture Profile analyzer. The pore size analysis of bread was also studied. CGTase from *E. aurantiacum* can thus be used to enhance texture and freshness of bread and has a potential application in bakery industry.

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LIST OF SYMBOLS/ABBREVIATIONS

Å - Angstrom

°C - degree Celcius

% - percentage

A - absorbance

Ala - alanine

Arg - arginine

Asn - asparagine

Asp - aspartic acid

au- Atomic units

blast - Basic local alignment search tool

BSA - Bovine Serum Albumin

bp - base pairs

Cys - cysteine

CD - Cyclodextrin

CDs- Cyclodextrins

CGTase - Cyclodextrin glucanotransferase

DNA - deoxyribonucleic acid

DNase - deoxyribonuclease

DMSO - dimethyl sulphoxide

E.coli - *Escherichia coli*

EC - Enzyme Commission

EDTA - ethylene diamine tetraactic acid

g – gram

Gln – glutamine

Glu - Glutamic acid

Gly - Glycine

HCl - hydrochloric acid

His - Histidine

HPLC - High Performance Liquid Chromatography

kDa - kilo Dalton

L - Litre

Lys - Lysine

M - Molar

MBS - Maltose Binding Site

Met - Methionine

mg - milligram

min - minute(s)

ml - Milliliter

MW - Molecular Weight

m/z – Charge /mass ratio

µg - microgram

µm - micrometer

NaCl - sodium chloride

NaOH - sodium hydroxide

NCBI - National Center for Biotechnology Information

ng - nanogram

OD - optical density

pmol - picomole

PAGE - polyacrylamide gel electrophoresis

Tris – Tris amino methane

Chapter 1

Introduction and Review of Literature

1.1 Introduction

The increasing industrialization poses a great threat to the environment because of use of harsh chemicals. Biological enzymes provide a solution to develop an eco-friendly and sustainable process for industrial applications. Enzymes are proteins that are used in food, detergent, paper, leather and other industries. The common enzymes used in industry are amylase, protease, lipase, nuclease, ligase, phytase, cellulase, and xylanase. Horikoshi (1999) expected that production of enzymes used in detergent industry may increase to 60%. However, the major share in the market is of enzymes used in food industry viz. amylase, pullulanase, pectinase and invertase. Amylases account for 30 % of the world's enzyme production (Sivaramakrishnan *et al*., 2006). Cyclodextrin glycosyltransferase (CGTase) is a common starch degrading enzyme produced by bacteria. It is a member of the largest family of glycoside hydrolases i.e., glycoside hydrolyase family 13 that acts on starch and related α-glucans (Svensson *et al.*, 1994). Biwer et al. (2002) have reported that cyclodextrin glycosyl transferase produced by *Bacillus macerans* has the highest market share. This enzyme is important because of its application in the industrial production of cyclodextrin that is used in pharmaceutical, food and cosmetic industry.

1.2 Discovery of Cyclodextrin glycosyl transferase (CGTase) and Cyclodextrin

Villiers (1891) reported the production of a precipitate by *Bacillus amylobacter* and called it cellulosine. It was later discovered by Franz Schardinger that the precipitate was a crystalline dextrin produced from starch by the action of *Bacillus macerans.* It was Tilden and Hudson (1939) who reported that a cell free enzyme of *B. macerans* converts starch to Schradingers dextrins i.e. Cyclodextrin (CD). Hence the enzyme was known as *B. macerans amylase* (BMA). Schwimmer (1953) reported the first evidence on purity of CGTase which he then called as Schardingers dextrinogenase.

It was during this period that the focus of all researchers was on CD's but the key enzyme CGTase still remained overshadowed. French (1957) published the first review on CDs. In 1969, Corn Products International started producing CD's using

CGTase from *Bacillus macerans* (Horikoshi, 1999). The first International Symposium on cyclodextrins was organized in 1981, but the structural and genetic studies on CGTase were reported only in the late 1980's. Kaneko *et al.* (1988) elucidated the nucleotide sequence and studied the cloning of genes coding for CGTase. Maekalea *et al.* (1988) purified and studied the enzyme in the same year.

Nakamura *et al*. (1993) investigated the active residues in the substrate binding sites of CGTase and identified them as three histidine residues. Haga *et al.* (1994) crystallised CGTase from alkaliphilic *Bacillus* and performed some preliminary experiments on X - Ray Diffraction studies. New Cyclodextrin-based technologies are constantly being developed and, thus, even 100 years after their discovery cyclodextrins are still regarded as novel excipients of unexplored potential (Loftsson and Duchêne *et al*., 2007).

1.3 Physiological function of CGTase

Bacillus species secrete a variety of important industrial enzymes. One of these enzymes is cyclodextrin glucanotransferase. The function of CGTase is to generate CD's as illustrated in Fig. 1.1.

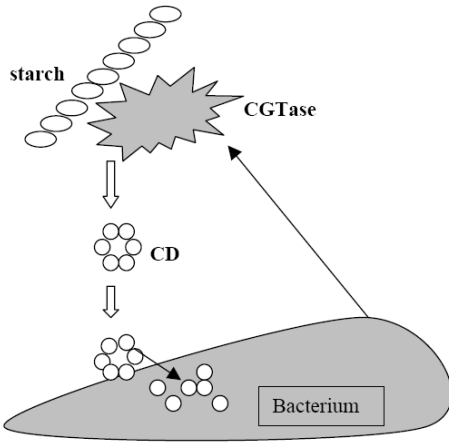


Fig. 1.1 : Schematic representation of function of CGTase (Aeckersberg *et al*., 1991)

CGTase cleaves α-(1, 4) bond in starch molecule, linking the reducing and non-reducing end to produce a cyclic molecule known as cyclodextrin (CD). The

bacterium builds up an external storage of glucose by producing CD which is not accessible for other organisms because they are not able to metabolize CD (Fig.1.1). The CD's protect the bacteria from toxic compounds by forming inclusion complex with it (Aeckersberg *et al*., 1991). The production of CGTase, its uptake and proposed utilization is outline in Fig. 1.2.

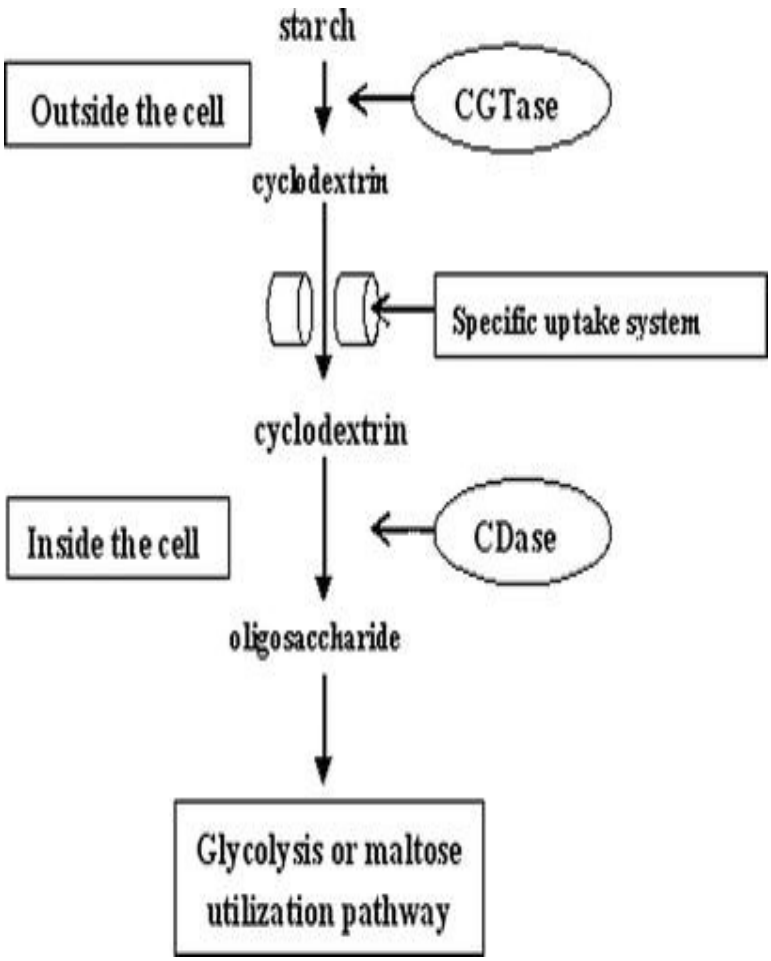


Fig. 1.2 : Proposed substrate starch utilization pathway involving CD formation (Qi *et al.*, 2005)

The CD's produced by CGTase outside the cell are transported by tranporter proteins inside the cell. The CD's are hydrolysed by cyclodextrinase (CDase) during starvation to release glucose molecules which are metabolised by the EMP pathway.

1.4 Characteristics and functions of CGTase (catalytic mechanism)

CGTases have a molecular weight varying from 60 -110 kDa and consist of about 700 amino acids. Most require calcium as a protective agent against heat denaturation (Wind *et al.*, 1998) and slightly acidic range at pH values 4.5-7.0 but alkalophilic CGTases display an optimum pH of 9-10. Maximal temperature for most bacterial CGTases range from 40°C to 85°C. Most of the CGTases are strongly inhibited by Zn^{2+}, Cu^{2+} and Fe^{2+} (Tonkova, 1998). CGTases produce a mixture of three types of CDs, α-, β- and γ-CDs, with specific equilibrium distributions. CGTases are classified in the α-amylase family and are known to catalyze four different transferase reactions: cyclization (A), coupling (B), disproportionation (C), and hydrolysis (D) (Wind *et al*., 1995) as illustrated in Fig. 1.3.

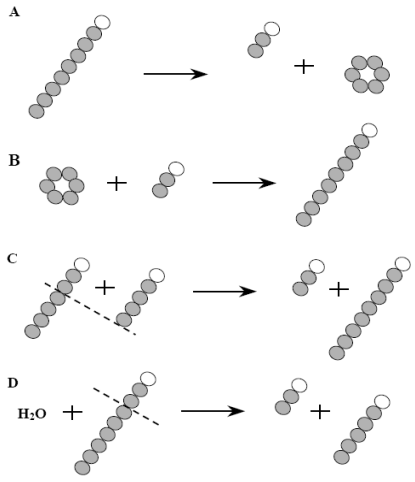


Fig. 1.3 : The reactions catalysed by CGTase (van der Veen *et al*, 2000).

The disproportionation reaction is also performed by several other enzymes of the amylase family. The mechanism of catalysis of CGTases is depicted in Fig 1.4.

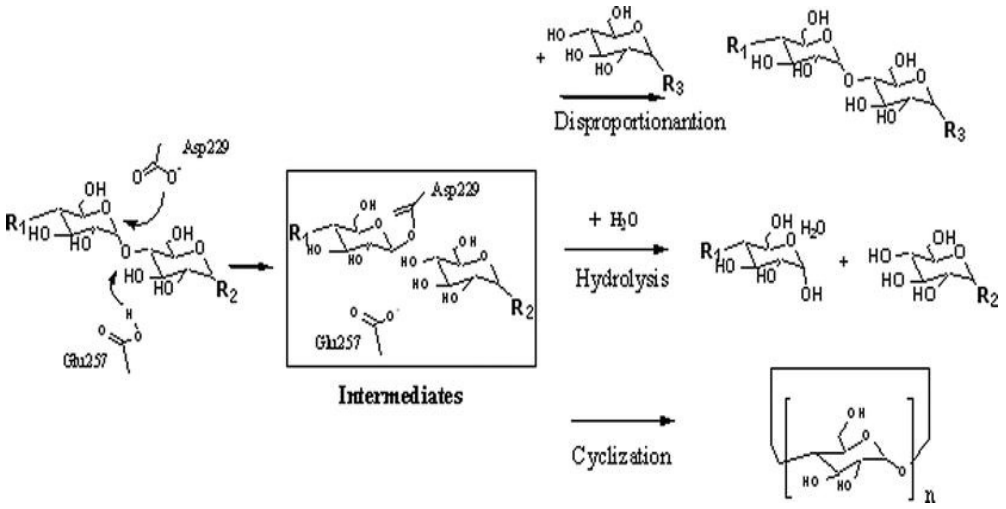


Fig. 1.4 : Reaction mechanism of CGTase (R1, R2, R3 are glucose molecules and n is no. of glucose molecules)

Cyclization i.e. formation of CD, is the characteristic property of CGTase in which the acceptor is a part of the cleaved donor. The reverse reaction is called as the coupling reaction. The next reaction of CGTase is hydrolysis. The most common substrate of CGTase is starch.

Starch is one of the largest molecules in nature found in potato, sweet potato, corn, wheat, barley, potato, rice, oat, tapioca and sago. It is carbohydrate polymer composed of two high molecular weight compounds amylopectin (75-85%) and amylose (15-25%) comprising of glucose units linked by α- (1, 4) or α- (1, 6) glucosidic bonds. The structure of amylose and amylopectin is represented in Fig. 1.5 a and b.

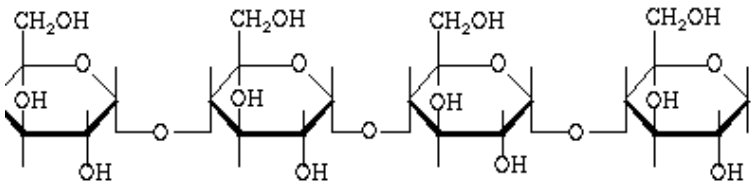


Fig. 1.5 a : Structure of amylose

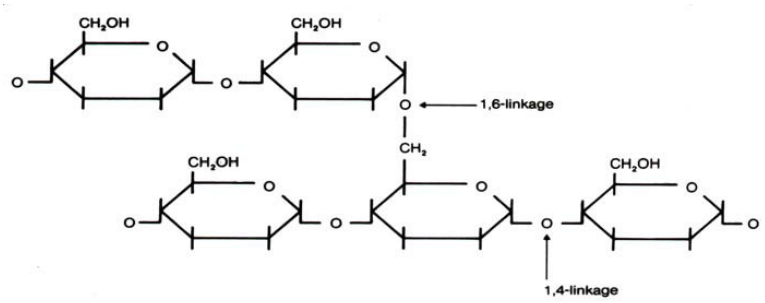


Fig. 1.5 b : Structure of amylopectin

Starch has a highly branched structure, which consists of short amylose chains. Amylose is a linear polymer containing up to 6000 glucose units and is connected by α-(1,4) linkages. Amylopectin chains are linked to each other by α-(1,6) bonds.

The major application of starch hydrolysing enzymes like cyclodextrin glucanotransferase (CGTase), glucoamylase, α-amylase, pullulanase, and α-glucosidase is in the production of low molecular weight oligosaccharides by complete breakdown of starch (Leemhuis *et al.*, 2003). The action of various starch hydrolyzing enzymes is outlined in Fig. 1.6.

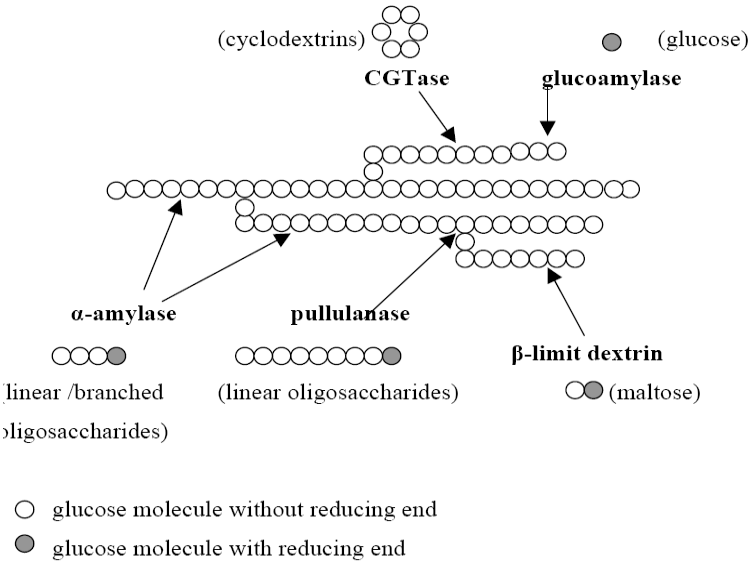


Fig. 1.6 : Action of α-amylase family enzymes involved in degradation of starch. Arrows indicate points in the starch molecule where the enzyme attacks (Takata *et al*., 1992).

It can be concluded that starch hydrolyzing enzymes release different types of oligosaccahrides according to their difference in mode of action. The α-amylase family is a large enzyme family that consists about 20 different reaction and product specificities, including exo/endo specificity. Takata *et al.* (1992) defined the α-amylase family as enzymes that hydrolyze or transfer α-glycosidic bonds and have four conserved sequence motifs which have the catalytic sites containing Asp, Glu and Asp residues.

1.5 Three Dimensional Structures of the α-amylase Family

α-amylase from *Aspergillus oryzae* was the first α-amylase with the three dimensional structure that has been solved (Matsuura *et al*., 1984). α-amylase family contains mainly three domains which are known as domain A, B and C. Domain A is the catalytic $(\alpha/\beta)_8$ domain, present in entire α-amylase family (Fig. 1.7). This domain contains 300-400 amino acid residues and the catalytic residues are located at the C-terminal ends of the β strands in domain A. Four short conserved regions typical for the α-amylase family in this domain (Fig.1.8). Domain B is an extended loop region inserted after the third β-strand of domain A. This domain consists of 44 -133 amino acid residues and contributes to substrate binding. Domain C has an antiparallel β-sandwich fold. It is known to be a maltose binding site observed in the structure derived from maltose dependent crystals (Lawson *et al*., 1994). This maltose binding site is involved in raw starch binding (Penninga *et al.,* 1996), suggesting a role of the C-domain in substrate binding. There are a few other domains such as domain D and domain E for some of the enzymes in α-amylase family as represented in Fig. 1.7.

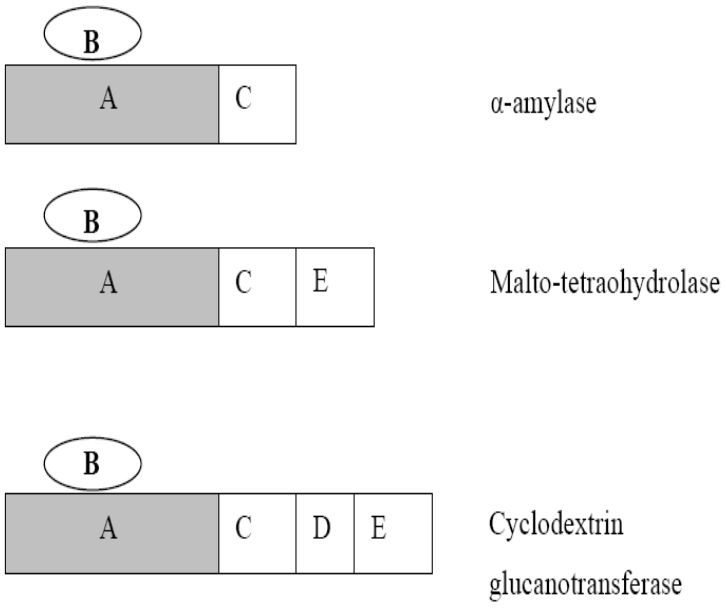


Fig. 1.7 : The domain organizations of several α-amylase families. α-amylase from *Aspergillus oryzae*; Malto-tetraohydrolase from *Pseudomonas saccharophila*; Cyclodextrin glucanotransferase from *Bacillus circulans* strain 251. A: Domain A; B: Domain B; C: Domain C; D: Domain D; E: Domain E.

The overall sequence similarity within the α-amylase family enzymes is relatively low, less than 30% (Tao, 1991). However there are four highly conserved regions that have been identified in this family (Nakajima *et al.*, 1986).An amino acid sequence alignment showing these four conserved regions for diverse members of the α-amylase family is presented in Fig.1.8.

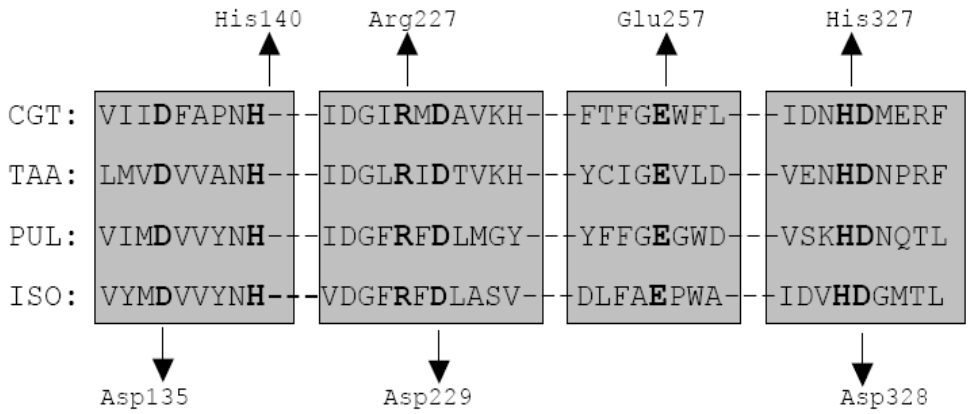


Fig. 1.8 : Amino acid sequence alignment of the four conserved region for different members of the α-amylase family. Bolded residues were the seven residues strictly conserved in this enzyme family; **CGT**: cyclodextrin glucanotransferase from *Bacillus circulans* strain 251(Lawson *et al*., 1994); **TAA**: α-amylase from *Aspergillus oryzae* (Matsuura *et al*., 1984); **PUL**: pullulanase from *Klebsiella aerogenes* (Katsuragi *et al*., 1987); **ISO**: isoamylase from *Pseudomonas amyloderamosa* (Amemura et al., 1988). The residues are numbered according to the CGTase from *Bacillus circulans* strain 251

All four regions are directly involved in catalysis, either through substrate binding, bond cleavage or transition stabilization or as ligands of a calcium binding site present near the active site. Three carboxylic acid groups, one glutamic acid and two aspartic acid residues, were found to be essential for catalytic activity in α-amylase family. The amino acids are equivalent to Asp 229, Glu 257 and Asp 328 for CGTase from *Bacillus circulans* strain 251 (Klein *et al*., 1992). Two conserved histidine residues; His 140 and His 327 are involved in substrate binding and transition state stabilization (Nakamura *et al*., 1993; Uitdehaag *et al*., 1999). Lastly, the third histidine His 233 is present only in some α-amylase and CGTase, is involved in substrate binding and acts as calcium ligand with its carbonyl oxygen (Lawson *et al*., 1994).

1.6 Detailed Three-dimensional structure of CGTase

There are reports on the structure of five CGTases viz. CGTase from *B. circulans* 251, *B. circulans*, *Bacillus* sp. 1011, *B. stearothermophilus* and *Thermoanaerobacterium thermosulfugenes* EM1. CGTase has five domains A–E. All enzymes of the amylase family have domain A and E (Janecek, 1997). The conserved catalytic and substrate- binding sites are also present in domain A (Klein *et al*., 1992; Knegtel *et al*., 1995). Domain E is responsible for hydrolysis activity of all amylases and CGTases. Substrate binding and catalytic sites are present in domains B and C (Knegtel *et al*., 1996; Penninga *et al*., 1996). Maltose binding site is also present in domain C of CGTases (Strokopytov *et al*., 1995). The function of the D domain, which is almost exclusively found in CGTases, is still unknown. The 3-D structure of CGTase is outlined in Fig. 1.9.

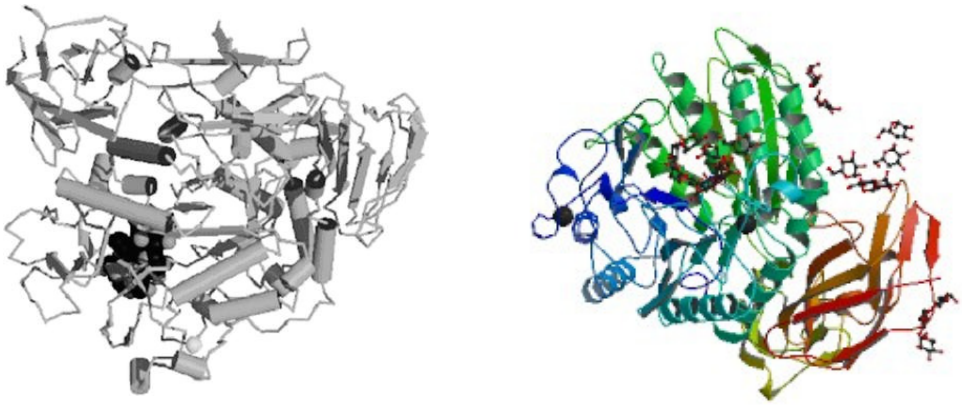


Fig. 1.9 : Structure of CGTase from *B. circulans* complexed with its main product, CD_7 (Knegtel *et al*., 1995)

The active site of CGTase has nine sugar binding subsites in the substrate binding groove (Davies *et al*., *1997*). It is believed that Asp196 present in the active site is responsible for the cyclization mechanism (Nakamura *et al.,* 1992; Schmidt *et al., 1998*; Uitdehaag *et al*., *1999*).

1.7 Mechanism of CD formation by CGTase

CGTase hydrolyses any α-1,4-linkage within amylose molecule and then transfers the newly formed reducing end of the oligosaccharide to its own non-reducing end (Terada *et al.,* 1997). During longer reaction times, the larger CDs will be subsequently converted to small CDs (CD_6–CD_8), due to their susceptibility to the coupling and hydrolytic reactions of the enzyme.

This mechanism of CD formation can be divided into five steps as described by van der Maarel *et al.* (2002) as represented in Fig. 1.10.

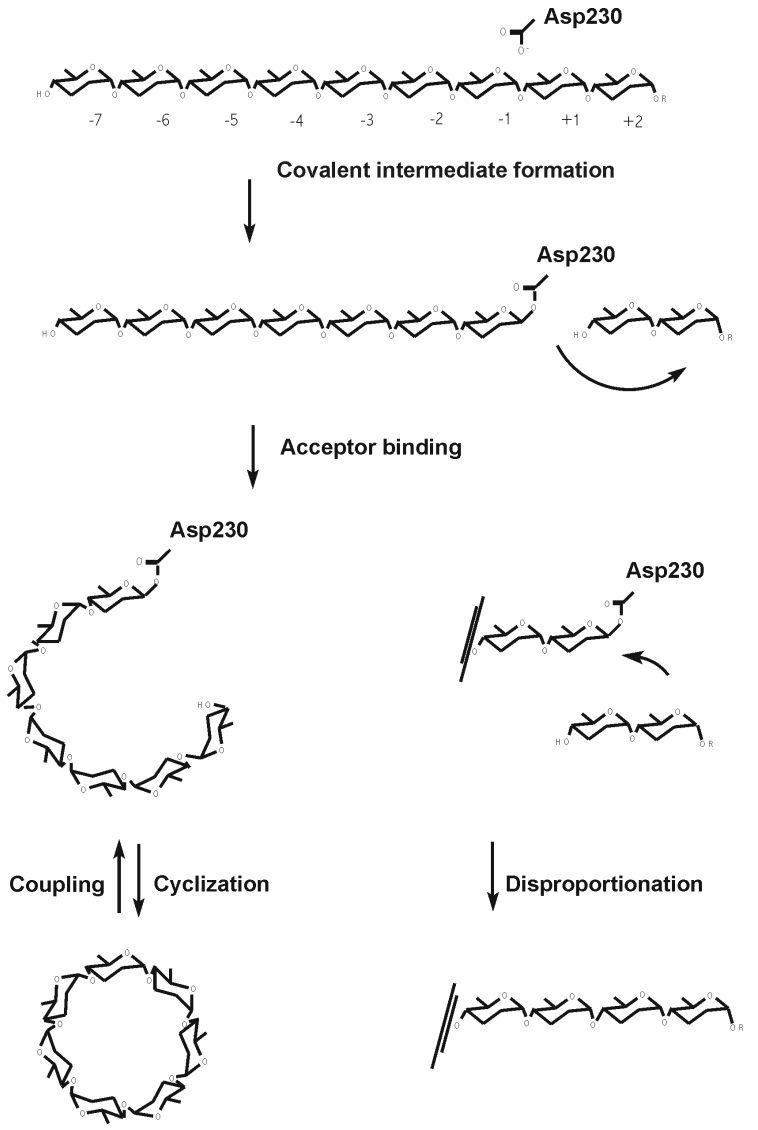


Fig. 1.10 : Schematic representation of mechanism of CD formation (van der Maarel *et al.,* 2002).

In the first step of the reaction (bond cleavage) a covalently linked oligosaccharide intermediate is formed. In the second reaction step this oligosaccharide is transferred to an acceptor molecule.

The five steps in mechanism are:

(1) The substrate binds to the active site and glutamic acid transfers a proton to the glycosidic bond oxygen, while the nucleophilic aspartate residue attacks the C1 of glucose at subsite –1.

(2) There is formation of oxocarbonium ion-like transition state after which a covalent intermediate formed.

(3) The protonated molecule of glucose at subsite +1 leaves the active site and another acceptor glucose molecule attacks the covalent bond between the glucose molecule at subsite –1 and the aspartate residue.

(4) There is reformation of oxocarbonium ion-like transition state (Uitdehaag *et al.,* 1999).

(5) The glutamate accepts a hydrogen ion from glucose molecule at subsite +1 and the oxygen of the incoming glucose molecule at subsite +1 replaces the oxocarbonium bond between the glucose molecule at subsite –1 and the aspartate residue leading to formation of a new hydroxyl group at the C1 position of the new glycosidic bond between the glucose at subsite –1 and +1.

The role of the three important carboxylic amino acids in this mechanism was clarified with acarbose, a potent pseudotetraose inhibitor, bound at the active site. Glu257 is the general acid catalyst, acting as proton donor, Asp229 serves as the nucleophile, stabilizing the intermediate, and Asp328 has an important role in substrate-binding (Klein *et al*., 1992; Nakamura *et al*., 1992, 1993).

1.8 The product of CGTase: Cyclodextrin

Cyclodextrins (CD's also known as cellulosines or Schardingers dextrins) are produced from starch by means of enzymatic conversion by cyclodextrin glycosyl transferase enzyme. The conversion of starch to CD by CGTase is represented in Fig.1.11.

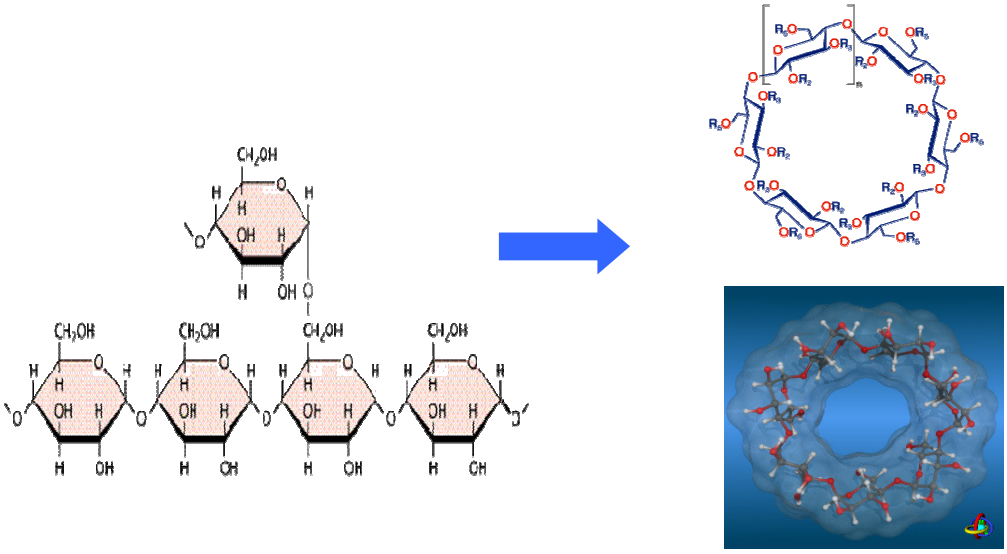
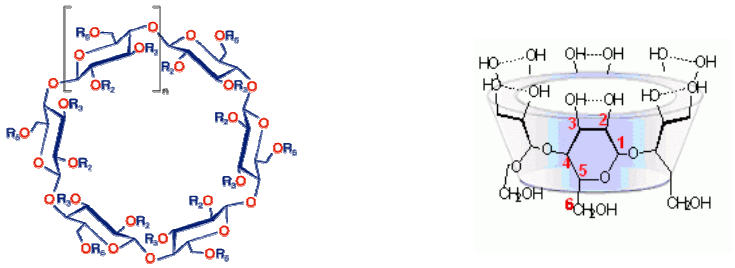


Fig. 1.11 : Conversion of starch to cyclodextrin

The CD's produced by CGTase are also called as cycloamyloses and are cyclic oligosaccharides composed of 5 or more α-D-glucopyranoside units. The interior of the cyclodextrin is not hydrophobic, but considerably less hydrophilic than the aqueous environment and thus able to host other hydrophobic molecules as outline in Fig. 1.12.



a) b)

Fig. 1.12 a : Structure of cyclodextrin; b:Cyclodextrin showing hydrophilic hydroxyl group

In contrast, the exterior is sufficiently hydrophilic to impart water solubility to cyclodextrins or their complexes (Li *et al*., 2007). Typical cyclodextrins contain a number of glucose monomers ranging from six to eight units in a ring, thus denoting α-cyclodextrin: six sugar ring molecule, β-cyclodextrin: seven sugar ring

molecule and γ-cyclodextrin: eight sugar ring molecule. Now a days, a cyclodextrin containing 9 glucose monomer is termed as theta- cyclodextrin. Structure containing more than 10 glucose monomers is called as Large Ring Cyclodextrins (LR- CD's). Cyclodextrin containing upto 60 glucose monomers are also reported. The three basic types of cyclodextrins are presented in Fig. 1.13.

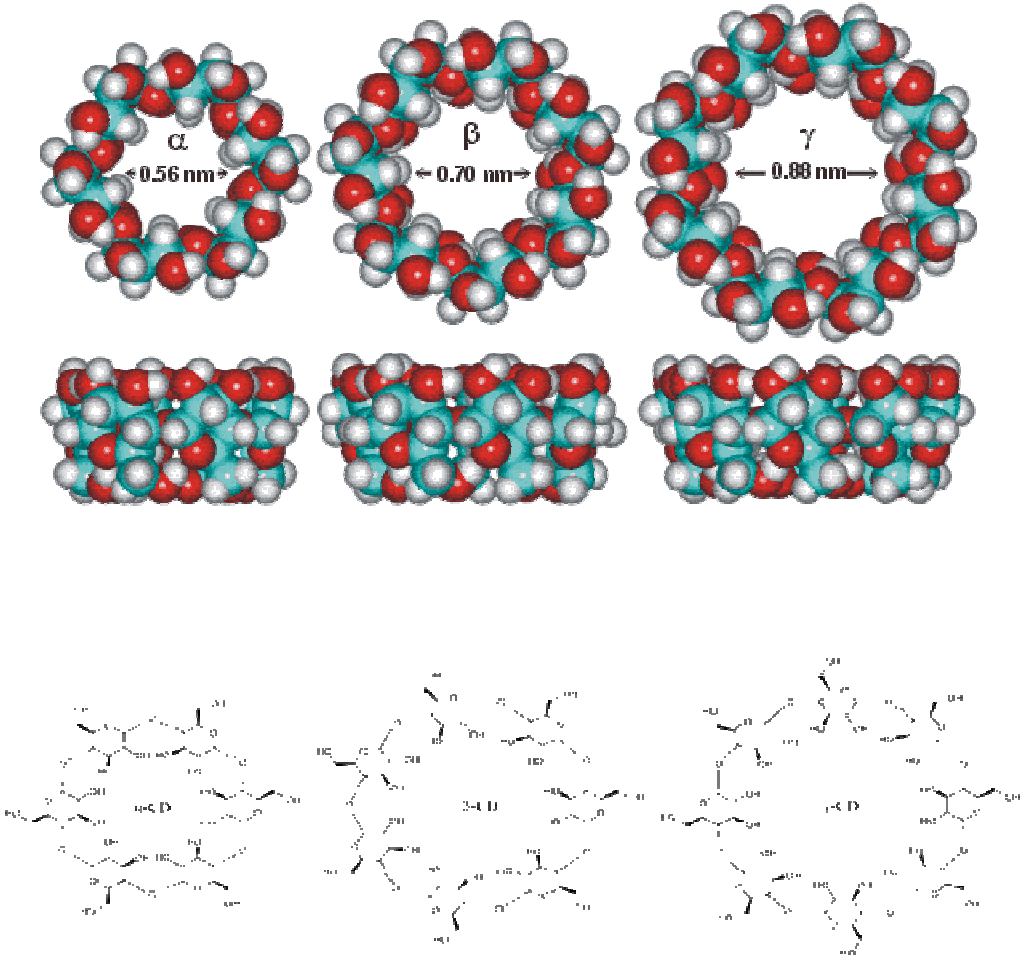


Fig. 1.13 : Different types of cyclodextrins (*Copyright images reproduced with permission of Prof. Martin Chaplin, London*)

The cyclodextrins are soluble in water and have an inner cavity suitable for complexation.

The various properties of cyclodextrin are outlined in Table 1.1.

Table 1.1 : Properties of cyclodextrin

Properties	α –CD	β-CD	γ –CD
Number of glucose units	6	7	8
Molecular weight	927	1134	1296
Approximate inner cavity diameter (pm)	500	620	800
Approximate outer diameter (pm)	1460	1540	1750
Approximate volume of cavity ($10^6 pm^3$)	174	262	427
Solubility in water (RT., g/100ml)	14.5	1.85	23.2
Surface tension	71	71	71
Melting temperature range (^0C)	255-260	255-265	240-245
Water molecules in cavity	6	11	17

(*adapted from Thorsteinn Loftsson, University of Iceland, European CyclodextrinSociety,http://www.eurocdsoc.com/index.php)*

The cyclodextrin cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges. The hydrophobic and hydrophilic regions of CD's are depicted in Fig 1.14.

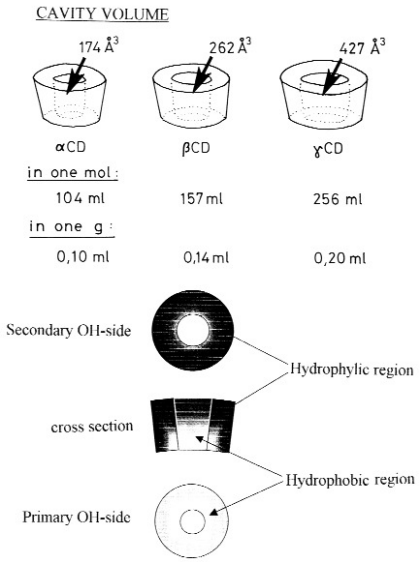


Fig. 1.14 : Dimensions and hydrophilic/hydrophobic regions of the CD molecules (Szejtli , 2004)

The nonbonding electron pairs of the glycosidic-oxygen bridges are placed in the direction of the inside of the cavity producing a high electron density rendering it is a Lewis-base property. The topology of CD's is like a toroid. The intramolecular hydrogen bond formation decreases the water solubility of CDs.

The molecular structure and form of CD's confers the ability to act as molecular containers by entrapping guest molecules in their interior cavity as represented in Fig. 1.15 (Szejtli, 2004).

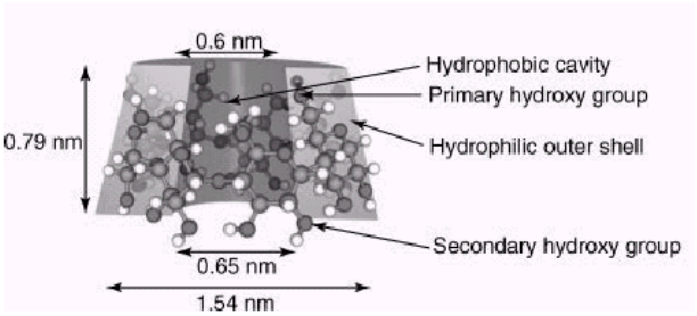
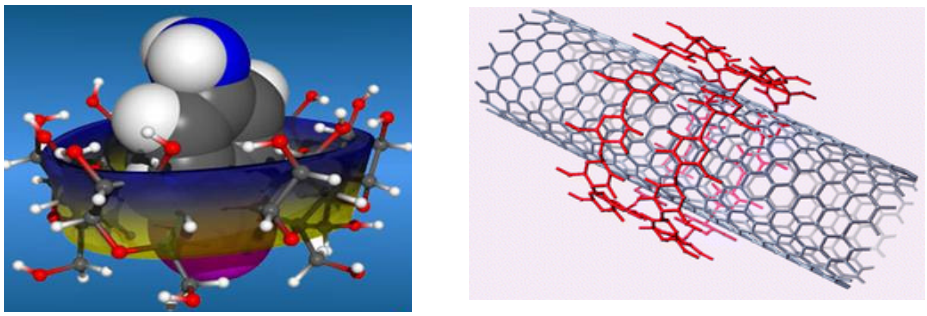


Fig. 1.15 : 3-D structure of β-cyclodextrin (Duchene, 1987)

CD molecules have hydrophobic interior cavity and hydrophilic exterior surface and thus can form complexes with hydrophobic guest molecules. These inclusion complexes are formed with drugs, dyes, insoluble compounds, volatile chemicals and pharmaceutical compounds. The various applications of CD's in encapsulation of pharmaceutical drugs and nanotubes are illustrated in Fig 1.16.



a) b)

Fig. 1.16 : a) Cyclodextrin demonstrating Host – guest molecular relation, b) Complexation of carbon nanotube with CD 12

The property of CD's of encapsulating guest molecules inside their cavity is called "molecular encapsulation" (Fig. 1.16 a). Nowadays, nanoparticles are also encapsulated in the cavity of CD's leading to the development of a new field of nanoencapsulation (Fig. 1.16 b).

1.9 Production of CGTase and CD

CGTase is an industrially important extracellular enzyme which is used for the production of CD. There are many methods for the industrial production of CGTase (Biwer *et al.,* 2002). The production of CGTase is very similar to other enzyme-manufacturing processes (Choi *et al.,* 1996; Gawande and Patkar, 2001).

To enhance the production of active CGTase, several methods have been applied. The first method comprises an optimization of the cultivation conditions of the CGTase-producing bacterial strain, including optimization of the growth medium (Jamuna *et al.,* 1993; Gawande and Patkar, 1998; Rosso *et al*., 2002). Heterologous expression of the CGTase gene in a another method used to enhance CGTase production. The production of the enzyme can also be increased by the overexpression of CGTase gene. It is estimated that half of the total commercial CGTase production processes use heterologous production. A CGTase production which was three times higher than the extracellular production of the enzyme by the wild-type *Bacillus* sp. 1011 was observed in a combination of the trp promoter and the CGTase gene starting from the 48 nucleotide position in the presence of the inducer IAA (Kimura *et al.,* 1990).

Two types of CD production processes are generally used at an industrial scale (Schmid, 1996; Biwer *et al*., 2002). The solvent process, which is mainly used, requires an organic complexing agent to precipitate a specific CD selectively and to obtain one main product. A non-solvent process does not require complexing agents and produces a mixture of CDs. The total yield of CD produced by CGTase depends on the enzyme employed and the chosen reaction conditions (Kitahata, 1995). The addition of debranching enzymes such as pullulanases and isoamylases to the synthesis reaction medium can increase the yield by 4–6% (Schmid, 1996; Rendleman, 2000). Lima *et al*. (1998) proposed a CD production process where the CGTase synthesis reaction is combined with yeast fermentation.

The yeast consumes compounds inhibitory for CD synthesis by CGTase, which are produced by the enzymatic conversion, e.g. glucose or maltose, while the ethanol produced by the yeast further increases the yield of CD. CGTases can also be employed to produce large-ring CDs in high yields, by adjusting the reaction conditions to prevent their conversion to small CDs (Terada *et al*., 1997; Qi *et al.,* 2004).

1.10 Applications of CGTase in biotechnology

The most important application of CGTases is the synthesis of CDs (Goel and Nene., 1995; Mori *et al*., 1995; Gawande and Patkar 1998; Wong *et al*., 2003). CGTase can also be used to synthesize linear oligosaccharides and their derivatives by its coupling and disproportionating reactions. It has been shown that CGTase can use a variety of carbohydrates and other compounds as acceptors in transglcosylation reactions (Kobayashi, 1996). CGTase is used to for the enzymatic transglycosylation and to improve solubility of compounds like rutin, curcumin, stevioside and naringin. Lee *et al.* (2002) have reported the use of CGTase produced by *B. stearothermophilus* as an antistaling enzyme. Gujral *et al.* (2003) have improved the quality of rice bread using CGTase.

CDs form inclusion complexes with a wide variety of hydrophobic guest molecules due to the unique molecular structure. The properties like solubility and stability of the guest molecule can be modified after encapsulation in the CDs. Therefore, there are immense applications of CDs in cosmetic, pharmaceutical, food industry and agriculture (Szejtli, 1988). Cyclodextrins can improve the stability of active pharmaceutical ingredients and increase the shelf life of drugs (Szejtli, 2004). They can improve the cord strength of polyester fibres used for reinforcement of rubbers (Szejtli, 2004). Fava *et al*. (1998) found that γ-Cyclodextrin has the potential of being successfully used in the bioremediation of chronically polychlorinated biphenyl-contaminated soils. It has been proposed that CDs can be used in all kinds of food and nutraceutical applications as a food ingredient and additive. Cyclodextrin can also stabilize emulsions of fats and oils. This property is useful for the preparation of bread spreads, dairy ice creams and breads (Munro *et al*., 2004).

1.11 Source of CGTase

Bacteria are the only known sources of CGTases. Besides mesophiles, extremophiles e.g. psychrophiles, alkaliphiles and thermophiles also secrete CGTase. *Bacillus macerans* (Takano *et al.,* 1986; Fujiwara *et al.,* 1992), *Thermoanaerobacterium thermosulfurigenes* EM1 (Wind *et al*., 1995) and *Bacillus stearothermophilus* (Fujiwara *et al.,* 1992) are known to be producers of α-CGTase while *Bacillus circulans* strain 251 (Lawson *et al.,* 1994), *Bacillus ohbensis* (Sin *et al.,* 1991), alkalophilic *Bacillus* sp. 38-2 (Horikoshi *et al.,* 1999) and *Bacillus* sp. 1011 (Kimura *et al.,* 1987) are known to be producers of β-CGTase. Bacteria that produce γ-CGTase from starch are *Bacillus* sp. AL-6 (Fujita *et al*., 1990) and *Brevibacillus brevis* CD162 (Myung *et al.,* 1998). Most of the CGTase producing bacteria belong to the genus *Bacillus* and most *Bacillus* sp. produce extracellular CGTase (Yong *et al*., 1996). Some of the *Bacillus* sp. producing CGTase are listed in Table 1.2.

Table 1.2 : Some of the *Bacillus* sp. reported to produce CGTase

Organism	CGTase type	References
Alkalophilic *Bacillus* sp. 1011	α	Kimura *et al.,* 1987
Alkalophilic *Bacillus* sp. 38-2	β	Horikoshi , 1999
Bacillus ohbensis	β/ γ	Sin *et al.,* 1991
Bacillus circulans 251	β	Lawson *et al.,* 1994
Bacillus sp. E-1	γ	Yong *et al.,* 1996
Brevibacillus brevis CD162	γ	Myung *et al.,* 1998
Bacillus macerans	α	Horikoshi, 1999
Bacillus agaradhaerens strain LS-3C	β	Martins *et al.,* 2003

A CGTase produced by *Thermoanaerobacte*r sp. has been reported by Norman and Jorgensen (1992). CGTases from thermophilic actinomycetes have also been reported (Abelain *et al*., 2002). Gawande and Patkar (1998) have described

production of cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* AS-22 and *Bacillus firmus*.

Thatai *et al*. (1999) have studied CGTase enzyme produced by *Bacillus* isolated from soil. The enzyme has been reported from *Paenibacillus pabuli*, *P. graminis* and *Thermoanaerobicum* spp. Antranikian *et al*. (2009) have isolated and identified gene for CGTase production from extremophile *Anaerobranca gottschalkii* an extreme thermo alkaliphile. CGTase production had also been reported by halophilic archeon *Haloferax medditerrani* (Bautista *et al*., 2012) and alkaliphilic *Amphibacillus* spp. (Ibrahim *et al*., 2012). Thus it is evident that there are still many unidentified CGTase producing organisms, and they need to be studied as the panorama of applications of CD's keeps extending daily.

1.12 Alkaliphiles as source of CGTase

Corn Products International Co. (United States) started the industrial production of *B. macerans* CGTase in 1969. Similarly, Teijin Ltd. (Japan) inititated the manufacture of β-CD using CGTase produced by *B. macerans* in a pilot plant. Two major technical issues were encountered during the production of CGTase. The yield of CD was low as a mixture of all the three CDs was produced. Thus the cost of the product was high. Secondly, the recovery of product was difficult from the mixture of CD's and various harmful solvents were used for precipitation. The recovery of product using hazardous and toxic chemicals was a threat to the environment.

The CGTase of alkaliphilic *Bacillus* sp. strain 38-2 produced 85 to 90% CD from amylose due to high conversion rate. The CDs could be directly crystallized from the fermentation broth without the addition of toxic precipitating agents. The industrial production of CD by this alkaliphile decreased the production cost drastically and lead to mass production of CD. The application of CD's increased as their cost decreased. The application of CD's increased extensively in foodstuffs, chemicals, and pharmaceuticals. Since then it is known that alkaliphiles are candidate organisms for CGTase production especially since they have greater specificity and more yield (Horikoshi, 1999).

1.13 Alkaliphiles – Classification and Ecology

Alkaliphiles (*alcali* "Al- Qaly "in Arabic, soda ash, phile, loving) is generally restricted to those micro-organisms that actually require alkaline media (presence of carbonate or bicarbonate) for growth (Ulukanli, 2002) and pH of at least 8.5 to 9 for their survival. Alkaliphiles were used since ancient times in Japan, especially in Indigo fermentation. However the discovery of alkaliphiles was fairly recent. Koki Horikoshi started his work on alkaliphiles in 1968 and published the first paper concerning an alkaline protease in 1971. It was only after the discovery of the industrial applications of these microorganisms that research on alkaliphiles has gained more impetus (Horikoshi, 1999).

Koki Horikoshi (1999) has distributed alkaliphiles in two physiological groups; alkaliphiles and haloalkaliphiles. Alkaliphiles can be classified based on tolerance to alkali and other environmental factors as alkali tolerant, moderate alkaliphiles, extreme alkaliphiles, haloalkaliphiles and thermoalkaliphiles as represented schematically in Fig. 1.17.

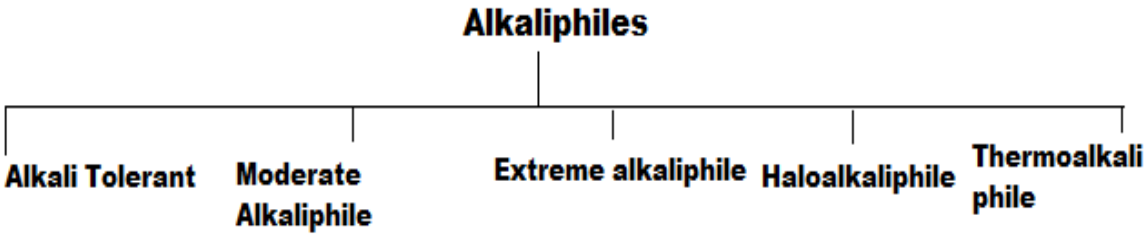


Fig. 1.17 : Classification of alkaliphiles

A large number of diverse environments exist that support the growth of these alkaliphiles. There are two types of alkaline environments, naturally occurring and artificially occurring environments. Artificially occurring environments result due to human activities. They could be manufacture of alkalis, cement, alkaline insecticide or herbicide manufacture processes or the traditional Japanese Indigo fermentation. Diverse industrial activities including food processing (KOH mediated removal of potato skins), cement manufacture (or casting), alkaline electroplating, leather tanning, paper and board manufacture, indigo fermentation

and rayon manufacture, and herbicide manufacture generate anthropogenic sources of alkaline type (Ulukanli, 2002).

Naturally occurring alkaline environments are created over the years as a result of intense geographical and climatic changes. Soda lakes, deserts and ground water rich in calcium hydroxide are naturally occurring alkaline environments.

Soda lakes are probably the most productive naturally occurring environments.

The alkaline soda lake (Latitude 19° 58′, Longitude 76° 36′) located in Lonar, India (Fig. 1.18) is believed to be 50 to 60 thousand year old unique basaltic rock meteorite impact crater, ranking third in the world (Kanekar *et al.,* 2002).

Fig. 1.18 : Lonar lake located in Buldana, Maharastra, India.

1.14 Biodiversity and significance of alkaliphilic bacteria from Lonar lake

Literature survey indicates that major emphasis in research has been on limnological analysis of Lonar Lake rather than microbiological (Kanekar *et al.,* 2002). An exhaustive account of identification of aerobic bacterial diversity of Lonar lake was given by Joshi *et al.* (2008), while Wani *et al.*(2006) reported the microbial diversity of Lonar lake using culture independent approach.

Over the past few decades, a huge amount of research is underway on the applications of these extremophiles in industry. Especially after the historic discovery of the enzyme Taq polymerase (from the extremophile *Thermus*

aquatus) used for molecular biology by (Chien *et al.,* 1980), maximum research of extremophiles now focuses on screening these organisms for production of extremozymes, industrially important biomaterials and novel antibacterial agents.

The study conducted by Joshi *et al.* (2008) revealed that most of the Lonar lake isolates were alkaliphilic while a few alkalitolerant. Majority of these isolates were halotolerant while a few halophilic. Among all the genera, *Bacillus* was found to be predominant and more diverse followed by *Halomonas*. Joshi *et al*. (2008) have also conducted a preliminary study on biotechnological potential of the bacterial isolates. There are reports on screening of Lonar lake isolates for production of industrially important compounds with biotechnological applications like protease (Kanekar *et al*., 2002) and poly hydroxyl alkanoates (Kulkarni *et al*., 2011). However there is no report on CGTase producers from Lonar lake.

1.15 Critical Views

Bacillus sp, *Klebsiella*, *Brevibacterium* and *Paenibacillus* are known to produce CGTase enzyme. Agharkar Research Institute has a collection of microorganisms isolated and identified from Lonar Lake previously (Joshi *et al.,* 2008). It is evident that there are still many unidentified CGTase producing organisms, and they need to be studied as the panorama of applications of CD's keeps extending from time to time.

Besides the organisms mentioned above, different unusual genera like *Exiguobacterium*, *Vagococcus*, *Halomonas*, *Arthrobacter*, *Marinobacter*, Lake Bogoria isolate and *Alkalimonas* isolated from Lonar lake will be included in the screening for CGTase production.

There is no record of production of this enzyme from the above mentioned organisms. If any of these organisms show promising results for the CGTase production, it will be a novel finding and it is expected that the enzyme may also have novel properties as compared to CGTase produced by the conventional organisms.

It is worthy to be mentioned here, that CGTases produced by extremophiles such as *Paenibacillus* are considered better enzymes for industrial applications as compared to the conventional CGTases from *Bacillus* sp. (Jemli *et al* ., 2008).

The CD's generated as a product of the CGTase producer will have immense biotechnological potential. Depending on the type of CGTase, the CD will have a lot of application in food, pharmaceutical or cosmetic industry.

1.16 Definition of problem

The aim of this research work is to produce CGTase by alkaliphiles for industrial application. CD's are extremely expensive because of low yield. Extremophiles are reported to be good sources of CGTases as the enzyme from such extremophiles, especially alkaliphiles have greater specificity and more yield. Alkaliphiles isolated from Lonar Lake have never been screened for CGTase production. This will be the first report of the presence of CGTase producing organisms from an alkaline lake, which is the only lake in the world formed by meteorite impact in basalt. Since alkaliphilic bacteria from Lonar Lake are being explored for production of CGTase, the enzyme isolated may be novel. The application of CD's depends on the structure of CD (α, β- and γ) which in turn is dependant on kind of CGTase. Thus the enzyme produced by the Lonar lake isolate will have potential applications in biotechnology.

1.17 Objectives of the present investigation

- To screen alkaliphiles from Lonar lake for production of CGTase
- To produce CGTase in flask level and select an appropriate CGTase producer from the organisms showing positive CGTase activity
- To optimise various parameters influencing production of CGTase
- To produce CGTase and Cyclodextrin using laboratory scale fermentor
- To perform purification and characterisation of the enzyme
- To study applications of CGTase

Chapter 2

Screening of alkaliphilic bacteria for production of Cyclodextrin glycosyl transferase

2.1 Introduction

Cyclodextrin glycosyl transferase (CGTase) is a microbial enzyme that generates cyclodextrin (CD) from starch. CD's and their derivatives are used in pharmaceutical, food and cosmetic industry. Most of the CGTase producing organisms belong to the genera *Bacillus* and *Klebsiella.* The screening of microorganisms for production of CGTase is performed by conventional methods. The general approach used is primary screening on starch agar plate followed by secondary screening on phenolphthalein methyl orange medium (PMO medium). This medium was developed by Park *et al*. (1989) by incorporation of the dyes phenolphthalein and methyl orange in Horikoshi medium. The CGTase production can also be detected colorimetrically using phenolphthalein assay as described by Goel and Nene (1995).

2.1.1 Principle of phenolphthalein assay

The decolorization of phenolphthalein solution in two variants cannot be attributed to the shift in pH towards the acid range, since the suspension or solution is demonstrably alkaline (pH = 10 – 11). When the magenta phenolphthalein solution is added to β-cyclodextrin or to the β-cyclodextrin solution, the cone-shaped β-cyclodextrin forms a host-guest complex as seen in Fig. 2.1 with the guest molecule phenolphthalein, due to van der Waals interactions.

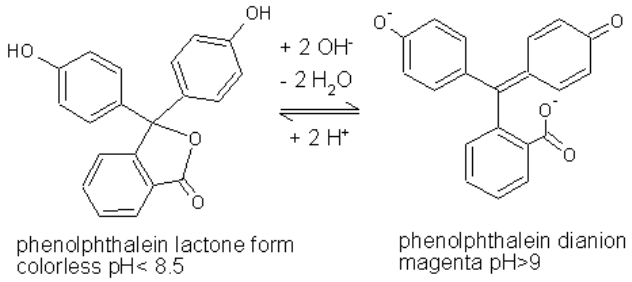


Fig. 2.1 : Molecular structures of phenolphthalein at pH < 8.5 and pH > 9

Since the molecular structure is non-ionic and therefore less polar in acid and neutral conditions, it can be assumed that the phenolphthalein is complexated in this form, which would also explain the decolorization of the solution. UV-VIS

spectroscopy of different substances and complexes has shown that the phenolphthalein is present in the cyclodextrin in the dianionic form. During the formation of the host-guest complex, the phenolphthalein dianion is complexated by the formation of three hydrogen bonds to the cyclodextrin molecule. Therefore the van der Waals forces between the guest molecule and the non-polar cavity of the cyclodextrin are not the significant forces in this complex.

Thus when CGTase is produced in starch containing nutrient medium, the enzyme breaks down starch to release cyclodextrin molecules. When phenolphthalein reagent is added to the reaction mixture in alkaline condition, cyclodextrin forms a complex with the pink dye and the reaction mixture becomes colorless.

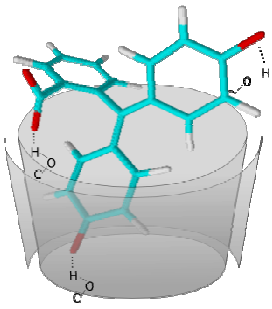


Fig. 2.2 : Schematic diagram of the hydrogen bonds between the β-cyclodextrin molecule and the phenolphthalein dianion

Higher the concentration of CD, lesser is the absorbance of reaction mixture. A standard curve of decrease in absorbance versus increasing standard solution of cyclodextrin (20- 200 µg/ml) can be plotted and the concentration of unknown solution can be extrapolated from the standard graph.

Researchers have used phenolphthalein plate assay for screening of bacteria for production of CGTase. Noi *et al*. (2008) have screened bacteria from soil samples using Horikoshi medium containing phenolphthalein by plate assay. Bonilha *et al.* (2006) have used phenolphthalein plate assay for qualitative analysis of CGTase from *B. licheniformis*. Aziz *et al.* (2007) have developed a rapid and modified phenolphthalein plate assay for screening CGTase producing organisms from soil.

In the present studies, alkaliphilic bacteria isolated from alkaline Lonar lake, India were screened for production of CGTase using starch hydrolysis plate assay, Horikoshi medium containing phenolphthalein and methyl orange (Park *et al*., 1989) and phenolphthalein colorimetric assay (Goel and Nene, 1995).

2.2 Materials and Methods

2.2.1 Screening of alkaliphilic bacteria from Lonar Lake for production of CGTase

The protocol for screening of CGTase producers from Lonar lake is outlined in Fig. 2.3.

Aerobic alkaliphilic bacteria previously isolated and identified from Lonar Lake were screened for hydrolysis of starch using Nutrient agar containing 1 % starch. Organisms showing starch hydrolytic activity were further screened for CGTase production on Phenolphthalein methyl orange agar medium containing (g/L) soluble starch 10, polypeptone 5, yeast extract 5, K_2HPO_4 1, $MgSO_4$. $7H_2O$ 0.2, Na_2CO_3 10, agar 15, phenolphthalein 0.3 and methyl orange 0.1. The organisms showing yellow halo on phenolphthalein methyl orange agar medium were selected for production of CGTase which was confirmed by CGTase assay using phenolphthalein reagent assay (PHP assay) as described by Goel and Nene (1995).

2.2.2 Production of CGTase in shake flask

The alkaliphilic bacteria were inoculated in Nutrient medium containing (g/L) soluble potato starch 10, peptone 10, yeast extract 5 and sodium chloride 5 for production of CGTase. The pH was maintained as 10 by addition of sterile 10 % sodium carbonate after autoclaving. The organism was cultivated in 250 ml flasks containing 50 ml medium incubated in an orbital shaker at 150 rpm at 30°C for 24 hours. After 24 h, the broth was centrifuged at 10,000 g at 4°C for 20 min. The cell free supernatant was used as crude enzyme and the enzyme activity was estimated as described below.

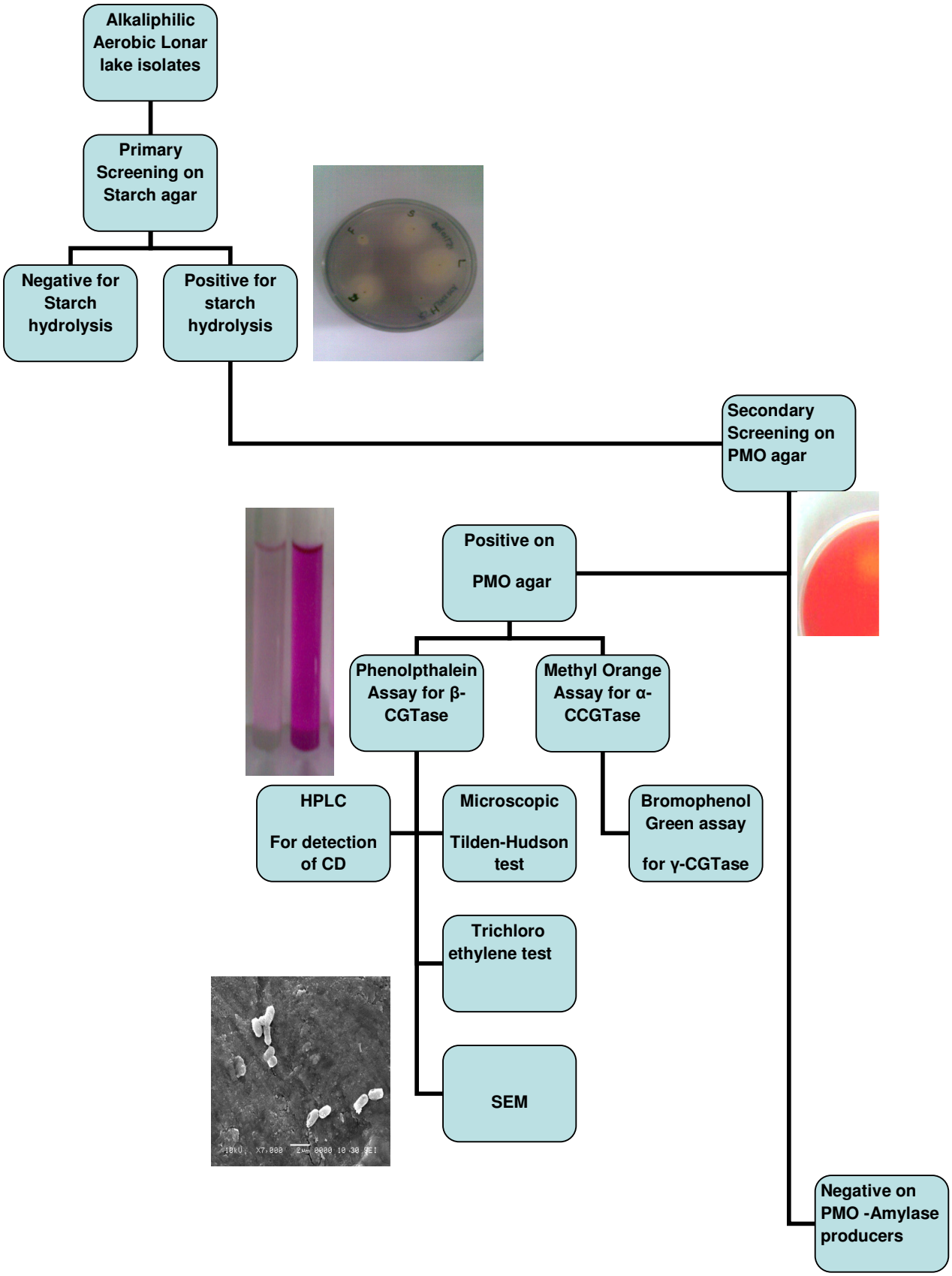


Fig 2.3 : Flowchart of screening of alkaliphilic bacteria for production of CGTase

2.2.3 Enzyme Assay

The CGTase assay was performed using phenolphthalein reagent as described by Goel and Nene (1995). 100 µl of crude enzyme extract was added to 1 ml of 1 % soluble potato starch in 0.005 M Tris Cl buffer pH 8.5 and incubated at 60^{o}C for 20 min. After incubation, this reaction mixture was cooled in ice. 4 ml of 1mM phenolphthalein reagent was added to the tubes and the absorbance measured immediately at 550 nm. The amount of β-CD produced is estimated from standard graph of 50 – 200 µg /ml of standard β-CD (Sigma-Aldrich) against decrease in absorbance. One unit of CGTase activity is defined as an amount of enzyme required to produce 1 µg of β-CD /ml/min.

The protein content was determined using bovine serum albumin as standard by the method described by Bradford (1976).

2.2.4 Tilden and Hudson Microscopic Test

Starch substrate is prepared by adding 3 % soluble potato starch in 0.05 M Tris-HCl buffer of pH 7.2. Five hundred µl of appropriately diluted enzyme was incubated with 1 ml of soluble starch and incubated at 60°C. Three drops of reaction mixture (approx 300 µl) are withdrawn every 5 minutes and mixed with 1 drop of Iodine and observed under a light microscope under high power (Tilden and Hudson, 1939).

2.2.5 Trichloro ethylene Test

The enzyme is diluted serially in 50 mM Tris HCl buffer of pH 8.5. One ml of enzyme is added to 5 ml of 2 % starch prepared in above mentioned buffer. The reaction mixture is incubated at 60°C. After 48 h, 2.5 ml of Trichloroethylene (TCE) is added to the reaction mixture. The contents are mixed vigorously and kept overnight. White precipitate of CD-TCE complex confirms the presence of CGTase. Maximum enzyme dilution which gave distinct precipitation was defined as enzyme activity (Nomoto *et al*., 1986).

2.2.6 Detection of Cyclodextrins by HPLC

The amount of β- Cyclodextrin produced by the CGTase enzyme in shake flask fermentation was estimated by HPLC. Perkin Elmer Amino column (5 µm, 250 X 4.6) was used. Mobile phase was acetonitrile: water (65:35) and detection was by online Perkin Elmer series-200 Refractive Index detector. The reaction mixture consisted of 100 microliters of enzyme and 1 ml of 1 % starch. The mixture was incubated at 60°C for 20 min.0.5 ml of this reaction mixture was mixed with equal volumes of HPLC grade Acetonitrile (Merck) and centrifuged at 20,000 g for 15 min at room temperature and then filtered through a 0.45 µ filter (Millipore, USA). 10 µl was injected in the column with a syringe and the pressure was maintained between 2800 to 3070 psi. The solutions of standard cyclodextrins were prepared in HPLC grade water. The output was read using a Total Chrom Navigator Software.

2.3 Results

2.3.1 Screening of alkaliphilic bacteria for CGTase producers

15 representative out of total 40 alkaliphilic starch hydrolyzing isolates from Lonar lake were screened for starch hydrolytic activity and CGTase activity. Six isolates were found positive for CGTase production on phenolphthalein methyl orange medium. The results of confirmation of CGTase production by phenolphthalein colorimetric assay are presented in Table 2.1.

Table 2.1 : Screening of alkaliphilic bacteria from Lonar lake for production of CGTase

No.	MCM number	Name of organism	Starch Hydrolysis (Amylase)	PHP Assay (CGTase)
1	MCM B-1001	*Bacillus cereus*	+	--
2	**MCM B-1016**	***B. firmus***	**+**	**+**
3	MCM B-1036	*B. flexus*	+	--
4	**MCM B-1044**	***B. fusiformis***	**+**	**+**
5	**MCM B-1010**	***B. licheniformis***	**+**	**+**
6	MCM B-1035	*B. benzoevorans*	+	--
7	MCM B-1038	*B. cohnii*	+	--
8	MCM B-1041	*Alkalibacillus haloalkaliphilus*	+	--
9	**MCM B-1034**	***Paenibacillus sp L 55***	**+**	**+**
10	MCM B-1018	*Vagococcus carniphilus*	+	--
11	MCM B-1027	*Halomonas campisalis*	+	--
12	**MCM B-1025**	**Lake Bogoria isolate 25 B 1**	**+**	**+**
13	MCM B-1046	*Alkalimonas delamerensis*	+	--
14	**MCM B-1021**	***Exiguobacterium aurantiacum***	**+**	**+**
15	MCM B-1006	*Arthrobacter mysorens*	+	-

The six isolates showing positive CGTase activity are *Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1. To the best of our knowledge this is the first report of alkaliphilic *Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 for CGTase production.

2.3.2 Production of CGTase in shake flask culture

CGTase was produced in 250 ml flask under shake culture condition. After 24 h, the broth was centrifuged and assessed for production of CGTase by colorimetric phenolphthalein assay (Fig. 2.4) showing disappearance of pink colour.

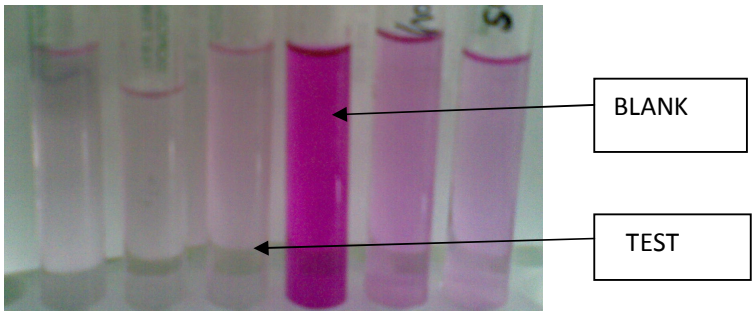


Fig. 2.4 : Colorimetric phenolphthalein assay showing disappearance of pink colour in test

The cyclodextrin produced by CGTase forms a complex with the phenolphthalein leading to disappearance of pink colour of phenolphthalein in alkaline condition.The results of estimation of β – cyclodextrin produced and specific activity of the CGTase are presented in Fig. 2.5.

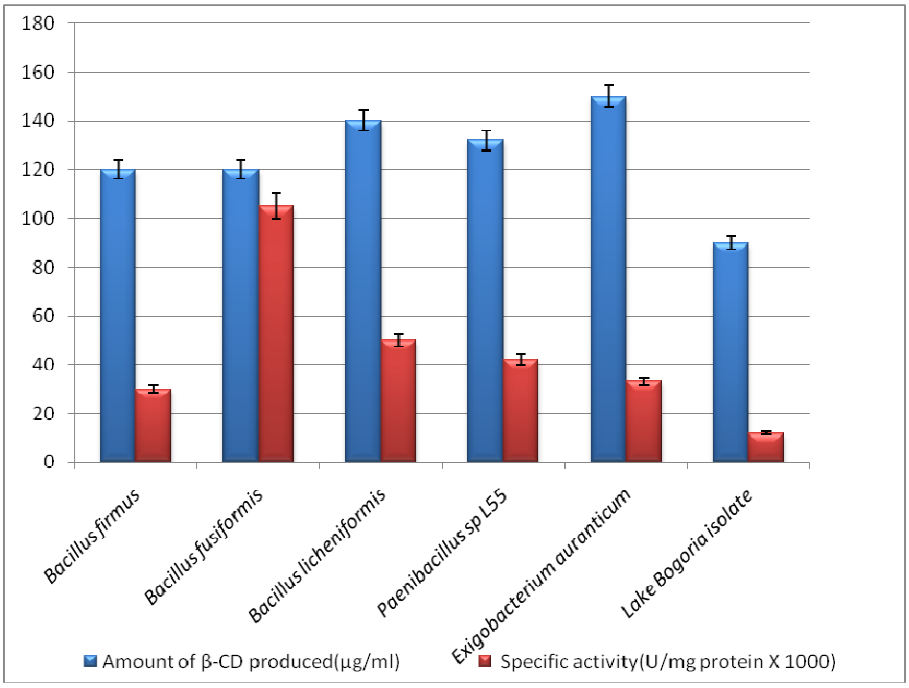


Fig. 2.5 : Amount of β-CD produced and specific activity of Lonar lake isolates

It was observed that *Exiguobacterium aurantiacum* produced maximum β-cyclodextrin (150µg/ml) followed by *Bacillus licheniformis* and *Paenibacillus* sp. L55 (Fig. 2.5). *B. licheniformis* isolated from cassava culture soil has been reported by Bonilha *et al.* (2006). Higuti *et al.* (2004) have reported production of CGTase from *Bacillus firmus.* However there is no report on production of CGTase from *Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 for CGTase production.

2.3.3 Detection of cyclodextrins by microscopic and precipitation tests

Tilden and Hudson test is one of the oldest and classical methods of measuring CGTase enzyme activity. The results of the test are observed microscopically and presented in Fig. 2.6.

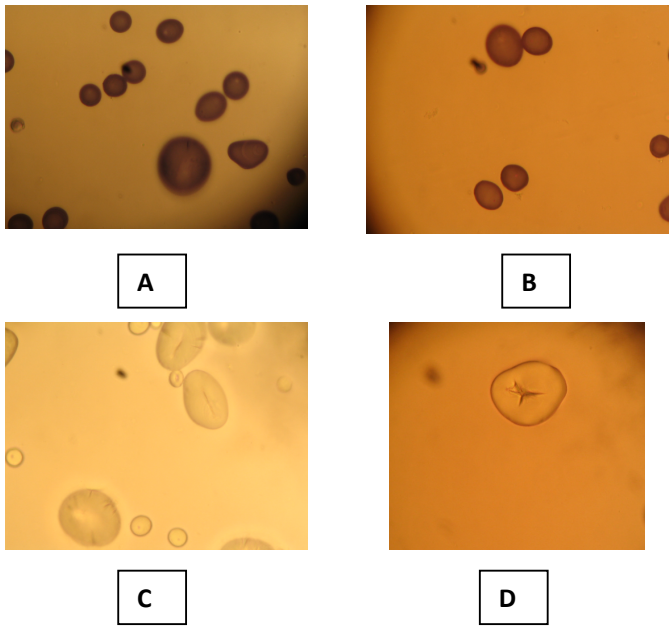


Fig. 2.6 : Detection of CD using microscopic assay. A) Starch granules (Control), B) Starch with CGTase (0 min), C) Colorless starch granules with CGTase (10 min) and D) Starch granules with CGTase showing typical dichoric crystals (30 min).

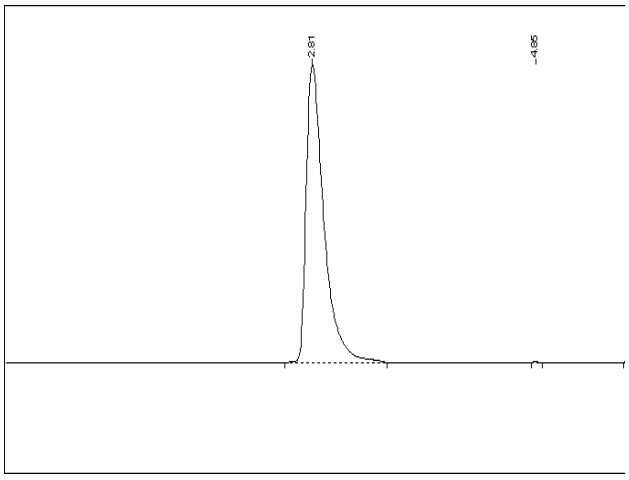
For Tilden and Hudson test, 3 % starch was incubated with crude cell free supernatant of *B. licheniformis, Paenibacillus* sp. L55 and *E. aurantiacum*. This was a time bound test and the end point was conversion of blue colored starch

granules to colorless in 30 min. The end point of CGTase activity for *Paenibacillus* sp. L55 and *B. licheniformis* was observed by addition of concentrated enzyme (2- 3 U/ml) while end point of CGTase from *E. aurantiacum* was observed by addition of 1:2 diluted crude enzyme with activity of 1.5 U/ml. Typical hexagonal and dichoric crystals of starch-iodine complex were observed in reaction mixture containing CGTase from *E. aurantiacum* as described by Szejtli (1988).

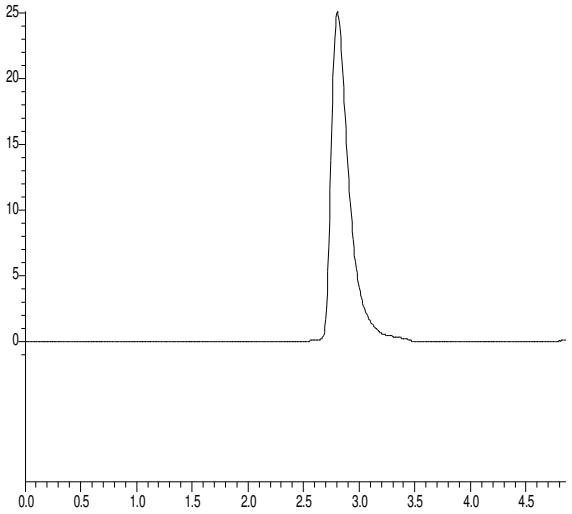
The Trichlororethylene (TCE) test was used to observe typical precipitation pattern. TCE assay is used to qualitatively determine the concentration of CGTase in two fold which produces CD that form TCE-CD complex precipitate. The enzyme activity of crude CGTase of all the six isolates varied from 2^2 to 2^{10} dilutions for all strains which are similar to results obtained by Higuti *et al*. (2004).

2.3.4 Detection of cyclodextrins by HPLC

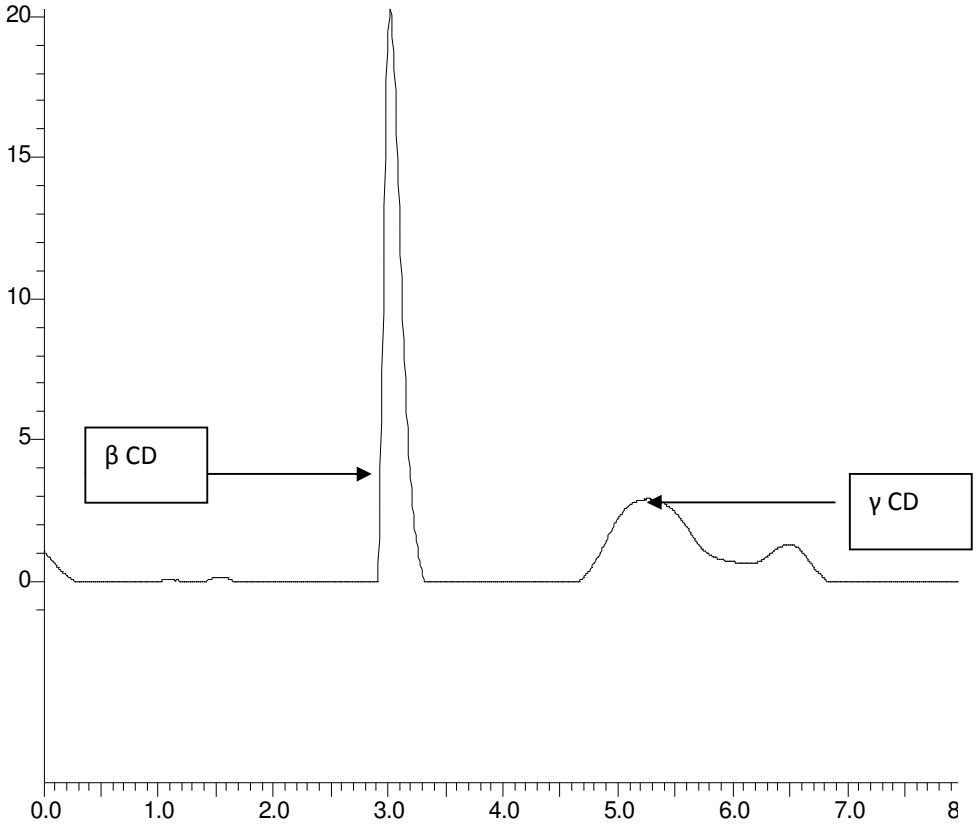
CGTase activity can be confirmed by isolation and identification of the endproducts, i.e. cyclodextrins. Cyclodextrins were detected by HPLC. Standard α -CD *(*Fluka) showed a distinct peak at 2.8 min. Standard β cyclodextrin showed a peak at 3.1 min. The mixture of standards showed distinct peak of β and γ CD at 3.1 and 5 min respectively (Fig. 2.7, a-c).



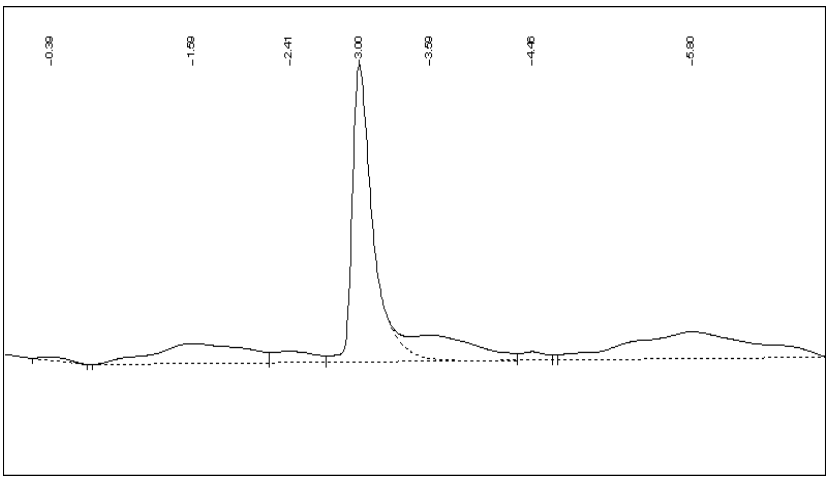
a) HPLC analysis showing standard α cyclodextrin at retention time 2.8 min.



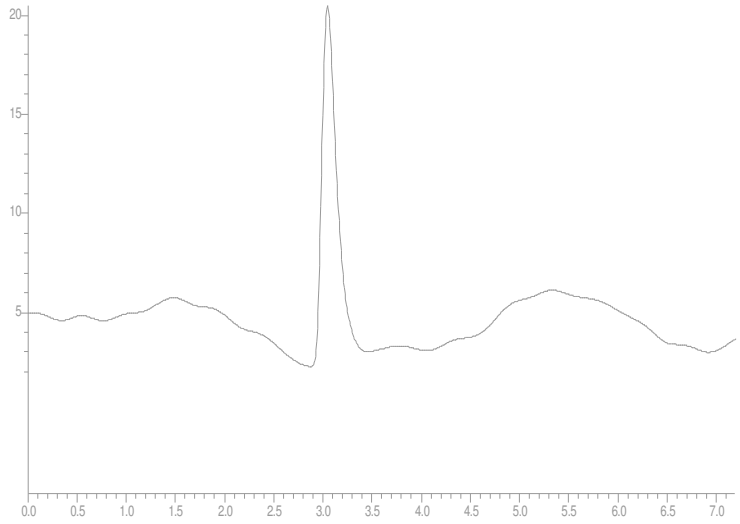
b) HPLC analysis showing Standard β cyclodextrin at retention time 3.1 min.



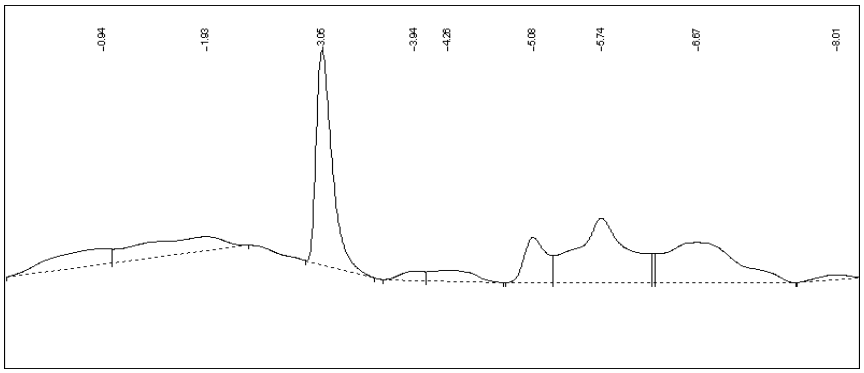
c) HPLC analysis of mixture of β and γ cyclodextrin showing clear separation at retention time 3.1 min and 5 min respectively.



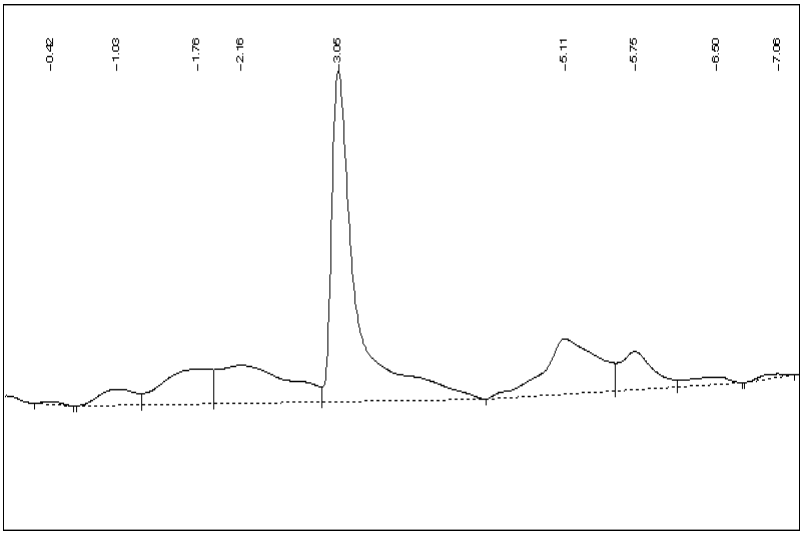
d) β- CD produced by *E. aurantiacum*



e) Mixture of CD's produced by *B. licheniformis*



f) Mixture of CD's produced by *Paenibacillus sp.L55*



g) Mixture of CD's produced by Lake Bogoria isolate 25 B

Fig. 2.7 : Detection of CD by HPLC

HPLC analysis of CD produced by *E.aurantiacum*, *B.licheniformis*, *Paenibacillus* sp.L 55 and Lake Bogoria isolate 25 B is presented in (Fig. 2.7, d-g). All the six isolates produced β-CD which was detected at 3.1 min (Table 2.2).

Table 2.2 : Estimation of β-cyclodextrin by HPLC

S.No	**Organism**	**Retention time (min)**
Standard 1	0.1 ppm β-CD standard in Acetonitrile	3.1
Standard 2	0.1 ppm β-CD standard in Acetonitrile	3.1
1	*Bacillus firmus*	3.2
2	*Bacillus fusiformis*	3.160
3	*Bacillus licheniformis*	3.1
4	*Paenibacillus sp L55*	3.179
5	*Exigobacterium auranticum*	3.13
6	*Lake Bogoria isolate*	3.18

E .aurantiacum showed single peak of β-CD while lake Bogoria isolate showed many peaks of α and γ CD with probable peaks of G1, G2 and G3 in addition to β-CD. The baseline of the samples showed many peaks as the culture filtrates were used, which may contain other oligosaccharides and traces of small peptides and sugars. Nene and Goel (1995) have also reported similar chromatographic peaks for α, β and γ CD.

2.4 Discussion

Rapid screening method like phenolphthalein plate assay helps in detection of microorganisms producing CGTase. Incorporation of phenolphthalein in Horikoshi medium was used by Park *et al.* (1989) for screening of CGTase producers.

Most of the producers of CGTase belong to *genera Bacillus*, *Klebsiella*, *Paenibacillus* and *Thermoanaerobacterium* (Wind *et al*., 1995) and researchers are exploring extremophiles from various habitat for CGTase producers. Alkaliphilic bacteria isolated from alkaline soda lake of Lonar, India were assessed for CGTase production. Six isolates *Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 were found to produce CGTase. Horikoshi (1999) have reported for the first time the alkaliphilic *Bacillus* sp. 38-2 for production of CGTase. Bonilha *et al.* (2006) have reported alkaliphilic *Bacillus licheniformis* for production of CGTase.

To the best of our knowledge, this is the first report of production of CGTase from alkaliphilic Lake Bogoria isolate 25 B, *Exiguobacterium aurantiacum* and *Paenibacillus* sp L55 from soda lake of Lonar, India. Lake Bogoria isolate demonstrated very low production of CGTase with lower specific activity. The specific activity of *B. fusiformis* is highest followed by *B. licheniformis* and *Paenibacillus* sp. L55. CGTase production has been previously reported from organisms like *Paenibacillus azotofixans*, *Paenibacillus macerans* and *Paenibacillus* sp RB01 (Zhou *et al*., 2012, Zheng *et al*., 2011, Yenpetch *et al*., 2011).

Generally bacteria produce a mixture of α, β and γ CD's. *Thermoanaerobacterium thermosulfurigenes* EM1 (Wind *et al*., 1995) and *Bacillus stearothermophilus* (Fujiwara *et al.,* 1992) are known to be α-CGTase producers, while *Bacillus*

circulans strain 251 (Lawson *et al.,* 1994), *Bacillus ohbensis* (Sin *et al.,* 1991) and *Bacillus* sp. 1011 (Kimura *et al.,* 1987) are known to be β-CGTase producers. Lake Bogoria isolate and *B. licheniformis* produces a mixture of CD's which is not suitable for industrial production. *Bacillus* sp. are known to produce a mixture of amylolytic enzymes with CGTase which makes the purification of the enzyme difficult. *Paenibacillus* sp. L55 and *E. aurantiacum* produce high amount of cyclodextrins and both these bacteria have not been reported for CGTase production. *Paenibacillus* sp. L55 and *E. aurantiacum* can thus be considered for further studies on production of CGTase.

2.5 Conclusions

Phenolphthalein plate and colorimetric assay were found to be efficient methods for screening of microorganisms for production of CGTase. Six isolates namely *Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 from Lonar lake were found to produce CGTase using phenolphthalein assay. Two isolates, *E. aurantiacum* and *Paenibacillus* sp L55 demonstrated promising results in terms of enzyme activity, specific activity and CD production and were selected for further studies. To the best of our knowledge this is the first report of alkaliphilic *Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 for CGTase production and also the first report of CGTase producing *Paenibacillus* sp L55 from Lonar lake, India.

Chapter 3

Optimisation of production of Cyclodextrin glycosyl transferase from *Exiguobacterium aurantiacum* and *Paenibacillus* sp L55

3.1 Introduction

3.2 Materials and methods

3.2.1 Microorganism and growth condition

3.2.2 Inoculum

3.2.3 Growth curve of *E. aurantiacum* and *Paenibacillus* sp. L55 in CG Tase production medium

3.2.4 Production of CGTase by *E. aurantiacum* and *Paenibacillus* sp. L55

3.2.5 Effect of environmental conditions on production of CGTase

3.2.6 Optimisation of production of CGTase by varying 'two factors at a time'

3.2.7 Statistical Analysis

3.2.8 Effect of different carbon and nitrogen sources on production of CGTase

3.2.9 Statistical Optimisation of production of CGTase using Taguchi Design of Experiments (DOE)

3.2.10 Production of CGTase under all optimum conditions

3.3 Results

3.3.1 Microorganism

3.3.2 Growth curve of *E. aurantiacum*

3.3.3 Growth curve of *Paenibacillus* sp L55

3.3.4 Effect of environmental conditions on production of CGTase

3.3.5 Optimisation of production of CGTase by varying 'two factors at a time'

3.3.6 Effect of different carbon and nitrogen sources on CGTase production

3.3.7 Optimisation of production of CGTase using Taguchi Design of Experiments (DOE)

3.3.8 Production of CGTase under optimised conditions

3.4 Discussion

3.5 Conclusions

3.1 Introduction

The fermentation medium and process conditions play a critical role in industrial fermentation process because they influence the formation, concentration and yield of the product and thus the process economics. It is therefore important to consider optimization of fermentation medium and process parameters to maximise profit from fermentation process (Scmidt *et al*., 1998). In optimization of fermentation, different combinations of process parameters and medium components need to be investigated to determine the optimum growth condition that will produce biomass with the physiological state which is best suited for the formation of product (Stanbury *et al.,* 1995). Media optimization using statistical experimental design has been reported by Casas *et al.* (1997), Lee and Chen (1997), Pujari and Chandra (2000), Dey *et al*. (2001), Liu *et al*. (2001), Cockshott and Sullivan (2001), Hujanen *et al*. (2001), Chen *et al*. (2002) and Li *et al*. (2002) in optimizing either biomass growth, enzymes, certain extracellular proteins and bioactive metabolites. The production of CGTase can be optimized by manipulating environmental factors and medium components. The most commonly used method of optimization is the conventional method involving changing one parameter at a time while holding all other factors constant. This approach is effective, but laborious and poorly suited for identifying interactions between multiple factors. Conventional method of optimization of one parameter at a time has been used by Bonilha *et al.* (2006) and Rosso *et al.* (2002). Other methods used are Factorial Design method and Response surface methodology (RSM). Gawande and Patkar (2001) have used factorial design for optimization of CGTase from *Klebsiella pneumoniae* AS-22. A better approach is to use statistical experimental design that enables the significance and studies the effect of multiple factors on the process. In Taguchi Design of Experiments (DOE), independant variables called factors are studied at different levels. Dr. Genichi Taguchi combined statistics and principles of engineering to develop methods for product design and manufacturing processes. The design of experiments developed by Dr. G. Taguchi were aimed at developing high quality with less error and have wide applications in engineering and biotechnology. Taguchi matrices are derived from classical full factorial arrays. Taguchi DOE is a statistical experimental method which uses Orthogonal Arrays (OA) to optimize independent variables (factors) at different levels with

minimized experimental error, greater efficiency and reproducibility (Dasu *et al.*, 2003; Krishna *et al*., 2005). Taguchi's emphasis on minimizing deviation from target led him to develop measures of the process output that incorporate both the location of the output as well as the variation. These measures are called as signal to noise ratios. The signal to noise ratio provides a measure of the impact of noise factors on performance. The larger the Signal /Noise, the more robust the product is against noise.The signal to noise ratio can be calculated using the following equation (and Bayrak, 2007) :

$$\text{Larger-the better} \quad S/N = -10\log_{10}\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{Y_i^2}\right), \tag{1}$$

$$\text{Smaller-the better} \quad S/N = -10\log_{10}\left(\frac{1}{n}\sum_{i=1}^{n}Y_i^2\right), \tag{2}$$

$$\text{Nominal-the better} \quad S/N = -10\log_{10}\left(\frac{1}{n}\sum_{i=1}^{n}(Y_i - Y_0)^2\right), \tag{3}$$

The advantages of using the Taguchi method are that many more factors can be screened and optimized simultaneously and much quantitative information can be extracted by only a few experimental trials. Therefore, these methods have been extensively applied in parameter optimization and process control.

This chapter describes production and optimisation of CGTase by *Exiguobacterium aurantiacum* MCM-B 1021 and *Paenibacillus* sp L55 isolated from Lonar Lake, India.

3.2 Materials and Methods

3.2.1 Microorganism and growth condition

Exiguobacterium aurantiacum MCM-B 1021 and *Paenibacillus* sp L55 MCM- B 1034 isolated from Lonar lake, India (Joshi *et al.,* 2008) were employed for the present studies. *E. aurantiacum* was maintained on nutrient agar pH 10 supplemented with sodium carbonate and *Paenibacillus* sp. L 55 was maintained on Horokoshi medium no. 1 (Appendix).

3.2.2 Inoculum

The inoculum for production of CGTase was prepared by growing the culture in Nutrient broth containing (g/L) soluble potato starch 10, peptone 10, yeast extract 5 and sodium chloride 5. The pH was maintained as 10 by addition of sterile 10 % sodium carbonate after autoclaving. The organism was cultivated in 250 ml flasks containing 50 ml medium incubated in an orbital shaker at 150 rpm at 37° C for 24 hours. After incubation, the cells were suspended in saline and cell density was measured spectrophotometrically (Schimadzu, Japan) at 540 nm. The optical density was adjusted to 1.0 which corresponded to 10^8 CFU/ml as enumerated by standard plate count using spread plate method. The medium used for production of CGTase was same as the medium used for preparation of inoculum.

3.2.3 Growth curve of *E. aurantiacum* and *Paenibacillus* sp. L55 in CGTase production medium

The growth curve and enzyme activity of both the organisms was studied in production medium. 10 % (v/v) seed inoculum having optical density of 1.0 at 540 nm was inoculated in 50 ml of production medium in 250 ml flask. The cultures were incubated at 37°C for 30 h on orbital shaker at 150 rev/min. The samples were withdrawn from 0 h till 30 h at 4 h interval to check growth in terms of optical density and production of CGTase by phenolphthalein assay. A growth curve of absorbance versus time was plotted. The exactly doubled points from the absorbance readings were recorded and, the points were extrapolated to meet the respective time axis. Generation time was found out by using following equations:
Generation Time = (Time in minutes to obtain the absorbance OD2 (double of OD 1) – (Time in minutes to obtain the absorbance OD1)

Let No = the initial population number, Nt = population at time t and n = the number of generations in time t.

Therefore, $Nt = No \times 2^n$ ……(1)

log Nt = log No + nlog 2

Therefore, n = (log Nt – logNo)/log 2

n= (log Nt – logNo)/ 0.301 …….(2)

The growth rate can be expressed in terms of mean growth rate constant (k), the number of generations per unit time.

K = n/t

K = (log Nt – logNo)/ 0.301 X t ……..(3)

Mean generation time or mean doubling time (g), is the time taken to double its size.

Therefore, Nt = 2 No…… (4)

Substituting equation 4 in 3, we get ,

K= ((log Nt – logNo)/ 0.301 X t = (log 2No- logNo)/ 0.301 X t = log 2 + (log No-Log No)/ 0.301 g. (Since the population doubles, t = g)

Therefore, K = 1/g

Mean growth rate constant, K = 1/g and Mean generation time, g = 1/k.

The mean growth rate and generation time can thus be calculated using the above equations.

3.2.4 Production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55

CGTase was produced in 250 ml Erlenmeyer flask containing 50 ml production medium. The inoculum (10 % v/v) having cell density of 10^8 CFU/ml was inoculated in the production medium and the flasks were incubated in an orbital shaker at 37°C at 150 rev/min. Growth of the organisms was observed in terms of

absorbance at 540 nm. After 24 h, sample was withdrawn, centrifuged at 10,000 g for 30 min (Kubota, Japan) and the CGTase activity in supernatant was estimated using phenolphthalein assay (Goel and Nene, 1995).

3.2.5 Effect of environmental conditions on production of CGTase

Production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55 was carried out under different conditions viz. agitation (100, 130, 150 rpm), temperature (25°C, 30°C, 37°C, 40°C), pH (8.0 to 11.0 of production medium), Inoculum density (0.1, 0.5,1.0 OD at 540 nm), age of inoculum (16 h, 18 h, 24 h), Inoculum size (1 %, 5 %, 10 %) and Inoculum medium (nutrient medium, starch medium, horikoshi medium) by 'one variable at a time' approach while keeping other conditions constant. After appropriate incubation, sample was withdrawn, centrifuged at 10,000 g for 30 min (Kubota, Japan) and the CGTase activity in supernatant was estimated using phenolphthalein assay (Goel and Nene, 1995).

3.2.5.1 Effect of agitation on production of CGTase

To study the effect of agitation on production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55, 10 % (v/v) seed inoculum of culture having 10^8 CFU/ml was inoculated in 50 ml production medium with pH set as 10. The culture was incubated at 37°C for 24 h under static condition and at 37°C in an orbital shaker set at 100, 130 and 150 rpm. After incubation, cells were centrifuged at 10,000 g for 30 min and CGTase activity was estimated in the supernatant by phenolphthalein assay.

3.2.5.2 Effect of temperature on production of CGTase

To study the effect of incubation temperature on CGTase production, 10 % (v/v) seed inoculum of *E. aurantiacum* and *Paenibacillus* sp L55 having 10^8 CFU/ml was inoculated in 50 ml production medium with pH set as 10. The cultures were incubated at different temperatures viz. 25°C, 30°C, 37°C and 40°C for 24 h on an orbital shaker at 150 rpm. After incubation, cells were centrifuged at 10,000 rpm for 30 min and CGTase activity was estimated in the supernatant by phenolphthalein assay.

3.2.5.3 Effect of pH on production of CGTase

To study the effect of pH on production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55, 10 % (v/v) seed inoculum of *E. aurantiacum* and *Paenibacillus* sp L55 having 10^8 CFU/ml was inoculated in 50 ml production medium with pH set from 8.0 to 11.0. The culture was incubated at 37°C for 24 h in static condition and at 37°C in an orbital shaker set at 150 rpm. After incubation, cells were centrifuged at 10,000 g for 30 min and CGTase activity was estimated in the supernatant by phenolphthalein assay.

3.2.5.4 Effect of inoculum density on production of CGTase

To study the effect of inoculum density on CGTase production, saline suspension of *E .aurantiacum* and *Paenibacillus* sp L55 was prepared by suspending 18 h growth culture from nutrient medium (adjusted to pH 10 using 10 % sodium carbonate) slant to sterile saline. Absorbance was measured at 540 nm and 10 % (v/v) seed inoculum of *E. aurantiacum* and *Paenibacillus* sp L55 having absorbance 0.1, 0.5 and 1.0 at 540 nm was inoculated in 50 ml production medium with pH set at 10. The culture was incubated at 37°C in an orbital shaker set at 150 rpm for 24 h. After incubation, cells were centrifuged at 10,000 rpm for 30 min and CGTase activity was estimated in the supernatant by phenolphthalein assay.

3.2.6 Optimisation of production of CGTase by varying 'two factors at a time'

Production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55 was optimised by varying two factors at a time approach keeping all other conditions constant. The levels for inoculum density were selected on the basis of previous optimisation experiments conducted by 'one factor at a time' approach. The levels of time of fermentation were selected on the basis of growth curve and CGTase production during growth curve.

3.2.6.1 Combined effect of inoculum density and medium volume: flask volume ratio on CGTase production

To study the combined effect of inoculum density and Medium volume : Flask volume ratio on production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55, 10 % (v/v) seed inoculum of the culture was inoculated in production medium with pH set as 10. The 3 levels of inoculum densities selected were 10^6, 10^7 and 10^8 CFU/ ml and Medium volume: Flask volume ratio selected were 1:10, 1:5 and 2:5 which corresponded to 25 ml, 50 ml and 100 ml medium in a 250 ml Erlenmeyer flask as outlined in Table no. 3.1. The culture was incubated at 37°C for 24 h under static condition and at 37°C in an orbital shaker set at 150 rpm. After incubation, cells were centrifuged at 10,000 rpm for 30 min and CGTase activity was estimated in the supernatant by phenolphthalein assay.

3.2.6.2 Combined effect of temperature and time of fermentation (incubation period) on CGTase production

To study the combined effect of temperature and time of fermentation on production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55, 10 % (v/v) seed inoculum of culture having 10^8 CFU/ml was inoculated in production medium with pH set as 10. The cultures were incubated at different temperatures viz. Room temperature (28±2°C), 30°C, 37°C and 40°C for 24 h on a orbital shaker at 150 rpm and for different period of fermentation viz. 12 h, 16 h and 24 h. After incubation, cells were centrifuged at 10,000 rpm for 30 min and CGTase activity was estimated in the supernatant by phenolphthalein assay.

3.2.7 Statistical Analysis

Mean and standard deviation was calculated using MS EXCEL for 'one factor at a time approach'. The data for 'two factors at a time ' was analysed statistically by two way Analysis of Variance (ANOVA) using Minitab® software.

3.2.8 Effect of different carbon and nitrogen sources on production of CGTase

CGTase production primarily depends on carbon source and nitrogen source. Production of CGTase was carried out using different carbon sources viz. glucose, fructose, mannitol, mannose, xylose, arabinose, raffinose, rhamnose, galactose, trehalose and soluble starch (concentration of 10 g/L) incorporated in production medium i.e. Nutrient broth. Effect of different nitrogen sources like peptone, tryptone, casein hydrolysate, beef extract, yeast extract, soyabean meal, ammonium nitrate, ammonium sulphate, ammonium chloride and ammonium phosphate on CGTase production (concentration of 1 g/L) incorporated in production medium i.e. Nutrient broth was studied.

3.2.9 Statistical Optimisation of CGTase production using Taguchi Design of Experiments (DOE)

The preliminary studies on optimisation of environmental conditions for CGTase production revealed that the medium components viz. starch, peptone, yeast extract and initial pH of the medium play a significant role in enzyme production. These factors were thus selected for studying their role in CGTase production using Taguchi DOE. Taguchi DOE involves establishment of large number of experimental situations described as Orthogonal Array (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments. This helps to determine the components of culture medium that have critical effect on the CGTase production. The study was carried out in 9 well defined sets with 4 variables at three levels in a standard L9 Orthogonal Array (OA). The experiments were performed in 250 ml flasks with 50 ml medium inoculated with 5ml of 18 hour old inoculum (10^8 CFU/ml) and incubated at 37°C at 150 rpm. All experiments were performed in duplicate. After incubation, cells were centrifuged at 10,000 rpm for 30 min and CGTase activity was estimated in the supernatant by phenolphthalein assay. The enzyme activity was calculated and an average of 6 readings was recorded. The levels of factors and the L9 orthogonal array are shown in Table 3.1 and Table 3.2.

The experimental data is analysed statistically to study the contribution of each factor to CGTase production according to Phadke (1989). In Taguchi method, the quality Loss Function, L(y) is used to demonstrate the impact of deviation from target. The deviation from target is called as noise. The signal to noise ratio provides a measure of the impact of noise factors on performance.

The quality loss function, L(y) is derived as follows:

$L(y) = k\ (y - m)^2$

Where y = value of quality characteristic, m = target value for y and k = constant that is a function of the financial importance of quality characteristic.

Calculation of the S/N ratio depends on the experimental objective which in the present study is Bigger – the better. The larger the S/N ratio, the more robust the product is against noise. The S/N ratio can be calculated by following equation:

$S/N = -\ 10 \log \{\sum(1/y^2\}$

The above equation can be simplified and written as follows:

$n = -10\ Log_{10}$ [mean of sum squares of reciprocal of measured data], where measured data is CGTase activity.

Calculation of the S/N ratio depends on the experimental objective which in the present study is Bigger-the better. The larger the S/N, the more robust the product is against noise.

The above formula was used to calculate signal/noise ratio where n = number of levels, and Y = yield of individual reactions. Analysis of variance (ANOVA) was applied to the data to identify the significant factors using signal to noise ratio (S/N ratio).

Table 3.1 : Factors and their levels for L9 Orthogonal Array

Factor	Level 1	Level 2	Level 3
Starch (g %)	0.5	1	5
Peptone (g %)	0.5	1	2.5
Yeast Extract (g %)	0.1	0.3	1
pH	8	9	10

Table 3.2 : L9 Orthogonal Array for CGTase production

Expt. No	Factor 1	Factor 2	Factor 3	Factor 4
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

3.2.10 Production of CGTase under all optimum conditions

After optimising all the environmental conditions and cultural condition by one and two factors at a time approach and Taguchi DOE, CGTase production was carried out under all optimum conditions. The conditions for *E. aurantiacum* were set as pH 10, temperature 37°, medium volume of 50 ml, inoculum volume 10 %, inoculum density as 10^8 and incubation period as 24 h. The conditions of CGTase production by *Paenibacillus* sp.L55 were temperature as 30°C and the environmental parameters were similar to *E. aurantiacum.*

3.3 Results

3.3.1 Microorganism

Exiguobacterium aurantiacum MCM B-1021 belongs to the Bacillaceae family and Bacillales order within Firmicutes division under kingdom Bacteria. The cells are Gram positive, non acid fast, non sporing, motile and produce light orange pigment (Fig. 3.1). Cells are coccoid in shape during stationary phase (Fig. 3.2) and form short coccobacilli in actively growing exponential phase. The Scanning electron micrograph of cells shows typical shorts rods in exponential phase (Fig. 3.3).

Fig. 3.1 : Typical light orange colonies of *E. auranticum* on starch agar pH 10

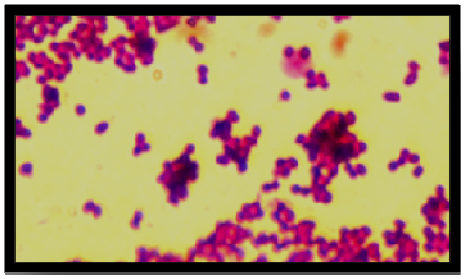


Fig. 3.2 : Gram positive cocci of *E. aurantiacum* grown in Nutrient broth (pH 10 adjusted with 10 % sodium carbonate) for 18 h, magnification X 1000

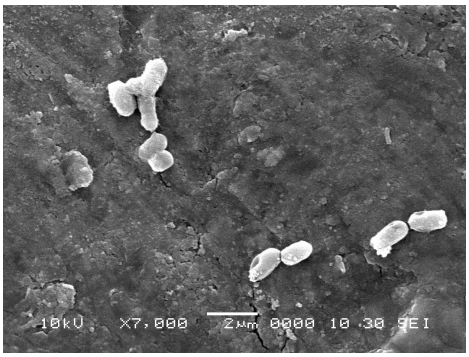
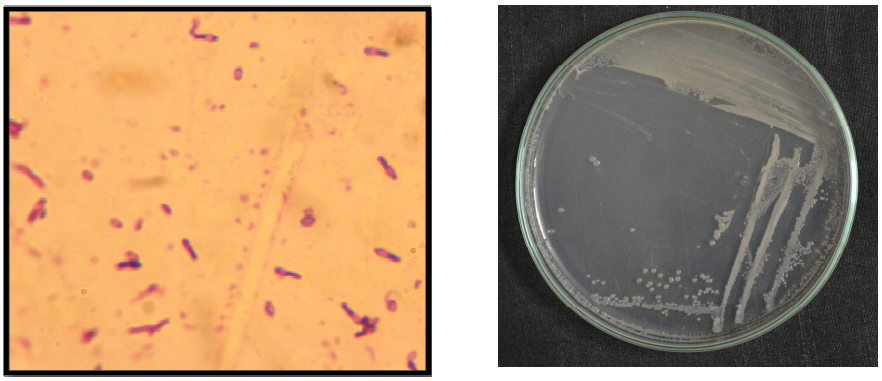


Fig. 3.3 : SEM of *E. aurantiacum* showing coccobacilli during log phase

The colonies of *E. aurantiacum* are orange, circular, convex, butyrous, opaque and easily emulsified. The organism is moderately alkaliphilic and halophilic and grows at pH 7.0 to 12.0 with optimum pH at 11. The organism was found to grow in presence of sodium chloride up to 3.44 M with optimum salt concentration as 0.86 M.

Paenibacillus sp L55 belongs to Paenibacillaceae family and Bacillales order within Firmicutes division under kingdom Bacteria They are Gram positive, sporulating and non motile rod shaped bacteria (Fig.3.4). Cells are rod shaped with a spore at subterminal position during stationary phase and form short rods in actively growing exponential phase. The Scanning electron micrograph of cells shows a single typical rod in exponential phase (Fig. 3.5).



a) b)

Fig. 3.4 : a) Gram positive rods of *Paenibacillus* sp L55 grown in Nutrient broth (pH 10 adjusted with 10 % sodium carbonate) for 18 h, b) Typical white colonies of *Paenibacillus* sp L55 isolated on nutrient agar (pH 10 adjusted with 10 % sodium carbonate) for 24 h.

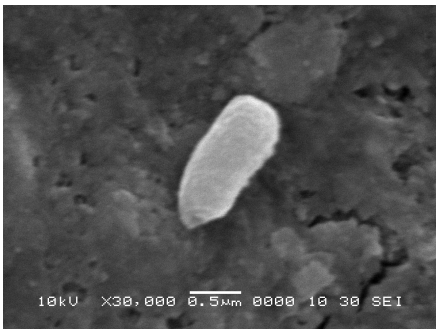


Fig. 3.5 : Scanning Electron micrograph of *Paenibacillus* sp L55 in exponential phase

The colonies of *Paenibacillus* sp L55 are white, circular, convex, opaque and easily emulsified (Fig. 3.4 b). The organism isolated from sediment sample of lonar lake is a moderate alkaliphile that grows at pH 7.0 to 10.0 with optimum pH at 9. The growth of the organism was not dependant on sodium chloride however it was found to grow in presence of sodium chloride up to 1.72 M with optimum growth at 0.86 M sodium chloride concentration (Joshi *et al*., 2008).

3.3.2 Growth curve of *E. aurantiacum*

Growth pattern of the organism is shown in Fig 3.6. It was observed that the organism had less or no lag phase and exponential growth phase was from the 1 h till 8 h. After 8 h, the organism entered stationary phase and from 20 h of incubation till 30 h, death phase was observed.

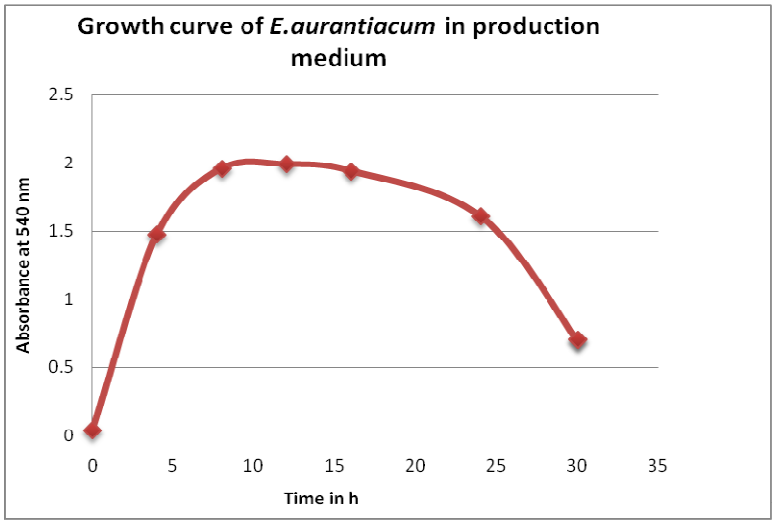


Fig. 3.6 : Growth curve of *E. aurantiacum* in production medium pH 10

The doubling time of *E. aurantiacum* in nutrient medium pH 10 was determined from Fig. 3.6 as 24 min. Harada *et al.* (2012) have reported the doubling time of *Exiguobacterium undae* as 35 min at 30°C which increased to 45 min at 37°C. The *E. aurantiacum* strain isolated from Lonar lake was a fast grower which reached stationary phase within 8h.

3.3.3 Growth curve of *Paenibacillus* sp L55

Growth pattern of the organism is shown in Fig 3.7. It was observed that the organism had a significant lag phase and exponential growth phase was observed from the 12 h till 16 h. After 16h, the organism entered stationary phase and from 30 h of incubation till 32 h, death phase was observed.

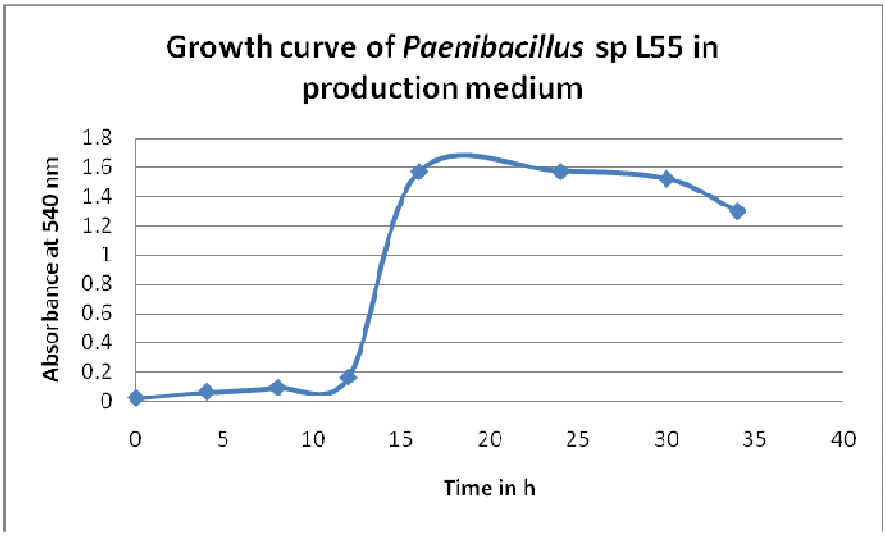


Fig. 3.7 : Growth curve of *Paenibacillus* sp L55 in production medium pH 10

The doubling time of *Paenibacillus s*p L55 in nutrient medium pH 10 was determined from Fig. 3.7 as 40 min. Bosshard *et al.* (2002) have reported the doubling time of *Paenibacillus turicensis* as 45 min at 37°C.

3.3.4 Effect of environmental conditions on production of CGTase

The various optimum conditions for CGTase production and the influence of environmental conditions like agitation, incubation temperature, pH and inoculum density, age of inoculum, inoculum size and inoculum medium on CGTase production were investigated using the conventional ‘ one variable at a time approach’.

3.3.4.1 Effect of Agitation on production of CGTase

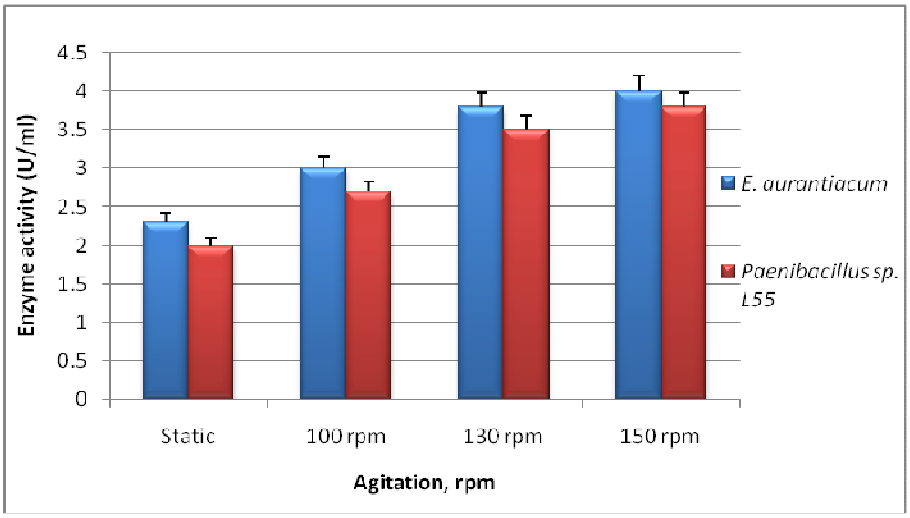


Fig. 3.8 : Effect of Agitation on production of CGTase

Effect of agitation on CGTase production was studied. Both the organisms show high CGTase production at 150 rpm (Fig. 3.8). Cells may rupture and sporulate in 50 ml medium placed in 250 ml flask if incubated beyond 200 rpm. Wong *et al.* (2008) and Rauf *et al*. (2008) have mentioned that agitation is a significant factor for CGTase production.

3.3.4.2 Effect of incubation temperature on production of CGTase

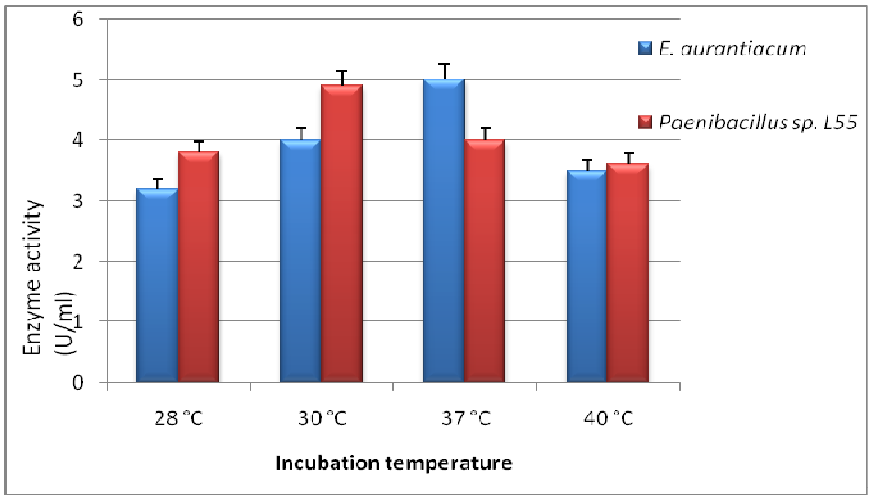


Fig. 3.9 : Effect of incubation temperature on production of CGTase

The effect of temperature on CGTase production was studied by incubating the cultures at different temperatures viz. Room temperature (28±2°C), 30°C, 37°C and 40°C for 24 h. It was observed that the optimum temperature for production of CGTase by *Paenibacillus* sp. L55 is 30°C while the optimum temperature for CGTase production by *E. aurantiacum* was 37°C (Fig. 3.9). There are no previous reports on production of CGTase by *Exiguobacterium spp*. However reports on CGTase production by *Paenibacillus* have mentioned the optimum temperature for CGTase production as 30 - 37°C (Zheng *et al*., 2011; Zhou *et al*., 2012).

3.3.4.3 Effect of pH on production of CGTase

Effect of initial pH of medium on production of CGTase was studied. The optimum pH for production of CGTase by *E. aurantiacum* was 10 and optimum pH for production of CGTase by *Paenibacillus* sp L55 was 9.0 (Fig. 3.10).

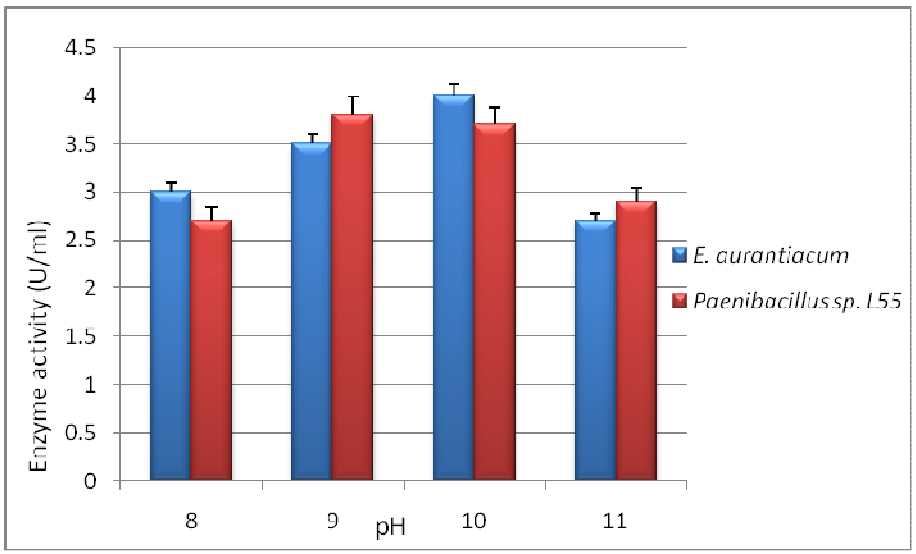


Fig. 3.10: Effect of pH on production of CGTase

Bonilha *et al*. (2006) have reported the optimum pH for production of CGTase by *Bacillus alkalophilis* as 9 and for *B. licheniformis* as 10. Rosso *et al*. (2002) have reported optimum pH for CGTase production by *B. circulans* DF 9R as 8.3. Similar reports were obtained for *B. firmus* (optimum pH 9) for CGTase production in fermentor (Savergave *et al*., 2008).

3.3.4.4 Effect of Inoculum density on production of CGTase

The effect of initial inoculum density on production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55 was studied by inoculating three different inoculum densities in production medium. The optimum inoculum density for both the organisms was 1.0.

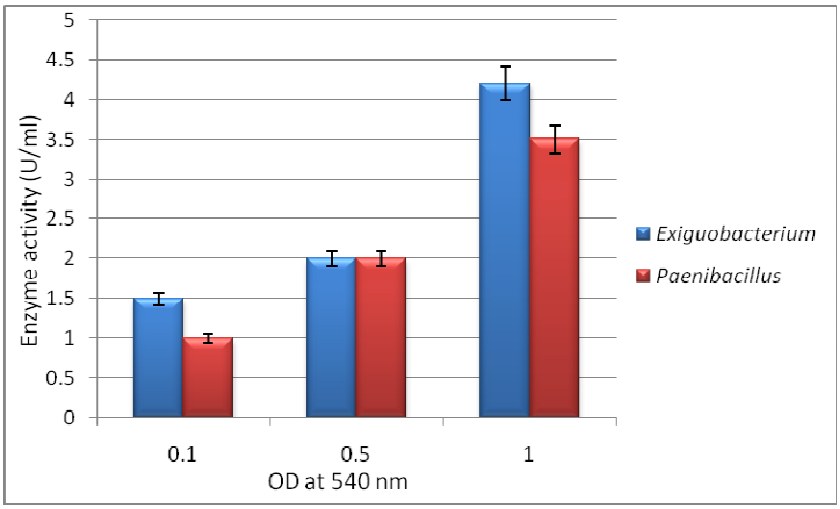


Fig. 3.11 : Effect of Inoculum density on production of CGTase

It can be observed from Fig. 3.11 that CGTase production increases with increase in optical density. There was increased interference by the light orange colored pigment produced by *E .aurantiacum* with optical density > 1.0. As the inoculum density directly affects CGTase production, its effect on production of CGTase by both cultures was optimised further using inoculum containing 10^6, 10^7 and 10^8 CFU/ml.

3.3.4.5 Effect of Inoculum size, age and medium on CGTase production

The effect of inoculum size, age and inoculum medium on production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55 was studied. It was observed that as the size of inoculum increased, the enzyme activity increased proportionately. The optimum inoculum size was 10 % (v/v) inoculum in fermentation medium (Fig.3.12).

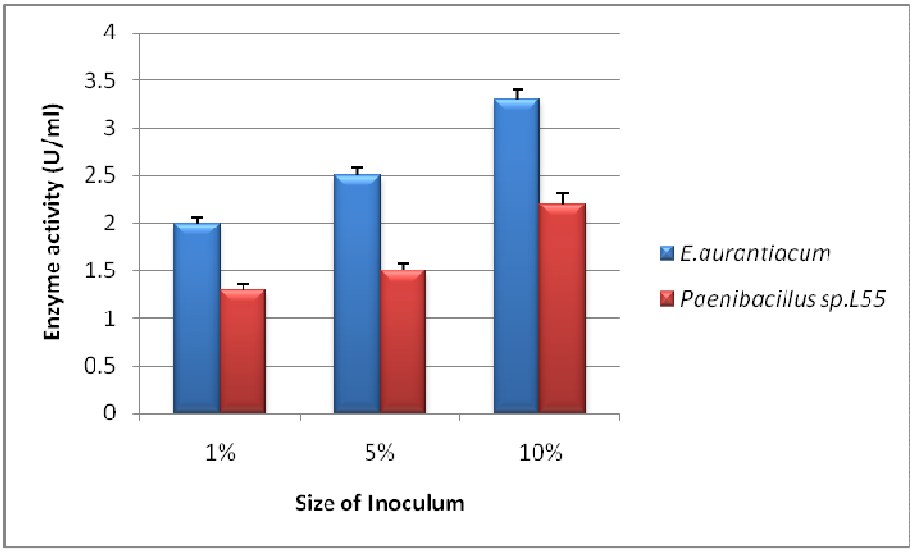


Fig. 3.12 : Effect of Inoculum size on production of CGTase

The amount of initial biomass controls the kinetics of growth and metabolic functions leading to the overall biomass and extracellular CGTase production. Ravinder *et al*. (2012) have reported optimum inoculum size for CGTase production as 3.5 %. Most of the fermentations employ inoculum size as 10 % and increase in the size may lead to feed back repression in some cases (Bonilha *et al.*, 2006).

The effect age of inoculum on production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55 was studied. It was observed that there was no significant difference in CGTase production when the age of inoculum was varied (Fig. 3.13).

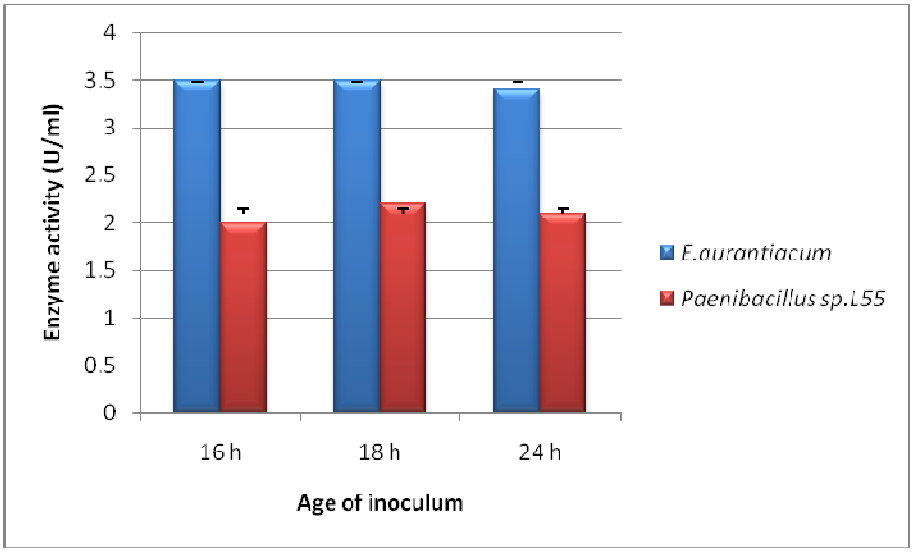


Fig. 3.13 : Effect of age of inoculum on production of CGTase

Effect of nutrient medium used for preparation of inoculum on CGTase production was studied. It was observed that maximum CGTase activity was obtained when nutrient medium was used as a medium for preparation of inoculum (Fig. 3.14). Starch induces production of CGTase and when the enzyme is produced it causes feed back repression (Noi *et al.*, 2008). Hence starch medium and horikoshi medium (containing starch) is not ideal for use as a medium for preparation of inoculum.

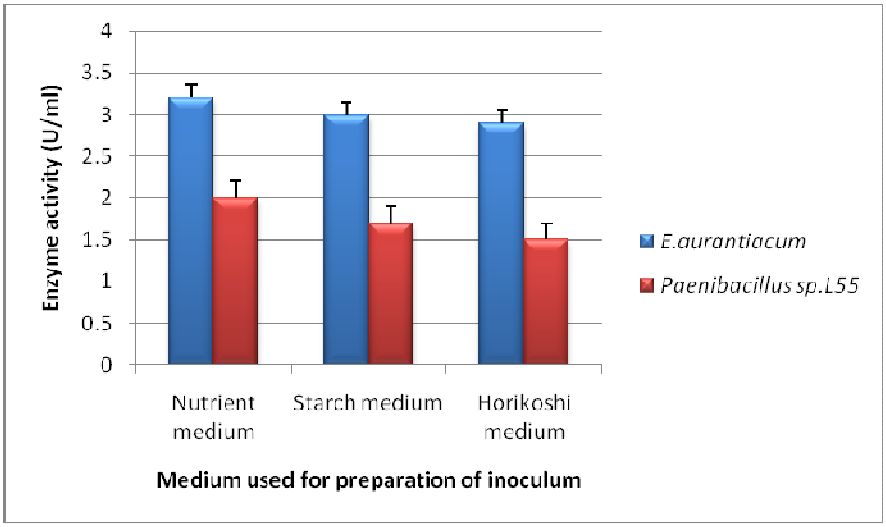


Fig. 3.14 : Effect of medium used for preparation of inoculum on production of CGTase

3.3.5 Optimisation of CGTase production by varying 'two factors at a time'

Four important factors which affect fermentation were selected and multilevel testing of 2 factors at a time was studied. Effect of medium volume: flask volume ratio and inoculum density on CGTase production and each other was studied .The enzyme activity was estimated and analysed by 2 way ANOVA using Minitab® software.

3.3.5.1 Combined effect of inoculum density and medium volume: flask volume ratio on CGTase production

The effect of initial inoculum density and the medium volume: flask volume ratio on CGTase production by *E. aurantiacum* and *Paenibacillus* sp L55 was investigated.

The optimum medium: flask volume was found to be 1:5 which is 50 ml medium in 250 ml volume Erlenmeyer flask. The optimum inoculum density of the culture is crucial for production of CGTase. It was observed that there was a significant increase in yield of CGTase production by *E. aurantiacum* with increase in the inoculum density (Table 3.3).

Table 3.3 : Combined effect of inoculum density and medium volume: flask volume ratio on CGTase production by *E. aurantiacum*

Inoculum density	**10^6 CFU/ml**	**10^7 CFU/ml**	**10^8 CFU/ml**
Medium: flask volume ratio	**Enzyme activity (U/ml)**		
1:5 (50 ml)	4.7 U/ml	5.5 U/ml	**7.25** U/ml
2:5 (100 ml)	4.3 U/ml	4.8 U/ml	6.15 U/ml
1:10 (25 ml)	5.0 U/ml	5.5 U/ml	6.75 U/ml

Table 3.4 : Two way ANOVA of optimisation of medium: flask ratio and inoculum density for CGTase production by *E. aurantiacum*

Source	DF	SS	MS	P	F	Fcrit
Inoculum density	2	6.66500	3.33250	**0.001 p < 0.1 Significant**	**68.95 Significant**	19.00
Medium: Flask Ratio	2	0.98667	0.49333	0.027 Not significant	10.21 Not significant	19.00
Error	4	0.19333	0.04833			
Total	8	7.84500				

The influence of inoculum density on production of CGTase was studied by statistical analysis of data using two way ANOVA at $p< 0.01$ and F- test. It was observed that inoculum density of the culture significantly affects the production of CGTase (Table 3.4). The optimum inoculum density for production of CGTase was 10^8 CFU/ml. Production of CGTase is adversely affected when the inoculum density is increased beyond this, due to pigment production and feed back inhibition in the inoculum medium.

There are a very few reports on optimisation of inoculum density for CGTase production. There are many reports on optimisation of pH, temperature and carbon source for CGTase production but very less data is available on optimisation of inoculum density (Noi *et al*., 2008; Rosso *et al*., 2002).

Similar results were obtained for *Paenibacillus* sp. L55. There was increase in enzyme activity with increase in inoculum density. The optimum medium volume: flask volume ratio was found to be 1:10 and 1:5 (Table 3.5).

Table 3.5 : Combined effect of inoculum density and medium volume: flask volume ratio on CGTase production by *Paenibacillus* sp. L55

Inoculum density	**10^6 CFU/ml**	**10^7 CFU/ml**	**10^8 CFU/ml**
Medium: flask volume ratio	**Enzyme activity (U/ml)**		
1:5 (50 ml)	3.0 U/ml	3.5 U/ml	**6.75** U/ml
2:5 (100 ml)	4.7 U/ml	5.5 U/ml	6.15 U/ml
1:10 (25 ml)	4.0 U/ml	5.5 U/ml	**6.75** U/ml

Table 3.6 : Two way ANOVA of optimisation of Medium: Flask ratio and inoculum density for CGTase production by *Paenibacillus* sp. L55

Source	**DF**	**SS**	**MS**	**P**	**F**	**Fcrit**
Inoculum density	2	10.6956	5.34778	0.032	9.26	19.00
Medium: Flask Ratio	2	2.0372	1.01861	0.282	1.76	19.00
Error	4	2.3111	0.57778			
Total	8	15.0439				

Generally, the medium: flask volume ratio in shake flask is not considered for optimisation (Rosso *et al*., 2002, Noi *et al*., 2008). It was observed that there was not much difference in enzyme activity of CGTase when the medium volume was varied. The statistical analysis confirmed that this parameter is not significant for production of CGTase by *Paenibacillus* sp L55 as seen from F value.

Though there was a sequential increase in CGTase production with increase in inoculum density, it was observed that the effect of inoculum density was statistically insignificant as evidenced by F value.

3.3.5.2 Combined Effect of temperature and time of incubation (incubation period) on CGTase production

The production of CGTase by *E. aurantiacum* was studied at different incubation periods viz., 12, 16, 24 h. The results are presented in Table 3.7. It was observed that there was a sequential increase in production of CGTase from 12 h to 24 h. The CGTase production varied from 1.1 U/ml to 3 U/ml at 12 h when temperature was varied from 28°C to 40°C. However the CGTase activity ranged from 2.6 to 3.6 U/ml at 16 h. The optimum temperature for CGTase production was 37°C. Both the parameters were statistically insignificant (Table 3.8) as seen from F value.

Table 3.7 : Combined Effect of Temperature and incubation period (time of incubation) on CGTase production by *E. aurantiacum.*

Time of incubation	**12 h**	**16 h**	**24 h**
Temperature	**Enzyme activity (U/ml)**		
R.T (25-28°C)	1.1 U/ml	2.65 U/ml	3.0 U/ml
30 °C	3.0 U/ml	2.65 U/ml	3.5 U/ml
37 °C	1.35 U/ml	3.6 U/ml	**6.25** U/ml
40 °C	3.0 U/ml	2.65 U/ml	2.65 U/ml

Table 3.8 : Two way ANOVA of optimisation of temperature and incubation period (time of fermentation) for CGTase production by *E. aurantiacum.*

Source	**DF**	**SS**	**MS**	**P**	**F**	**Fcrit**
Temperature	3	3.4417	1.14722	0.531	0.81	
Time of incubation	2	6.0613	3.03063	0.198	0.198	19.00
Error	6	8.4621	1.41035			
Total	11	17.9650				

Effect of temperature and incubation period on CGTase production by *Paenibacillus* L55 was investigated. The results are presented in Table 3.9. The enzyme activity varied from 1.35 to 3.55 U/ml at 12 h and was almost constant at 3.5 U/ml at all the four incubation temperatures at 16 h. The yield of enzyme was maximum at 24 h. It is observed that optimum temperature for production of CGTase is 30^{o}C. The optimum temperature for growth of *Paenibacillus* sp L55 is also 30^{o}C. As CGTase is a growth related product, the optimum temperature for growth of the bacteria and for enzyme production is the same. Statistical analysis shows the insignificance of these two parameters for CGTase production (Table 3.10) as evidenced from F values.

Table 3.9 : Combined effect of temperature and incubation period (time of fermentation) on CGTase production by *Paenibacillus* sp L55

Time of incubation	**12 h**	**16 h**	**24 h**
Temperature	**Enzyme activity (U/ml)**		
R.T (25-28^{o}C)	2.6 U/ml	3.55 U/ml	3.85 U/ml
30^{o}C	3.55 U/ml	3.2 U/ml	**4.75** U/ml
37^{o}C	1.35 U/ml	3.55 U/ml	3.85 U/ml
40^{o}C	2.6 U/ml	1.35 U/ml	2.6 U/ml

Table 3.10 : Two way ANOVA of optimisation of Temperature and incubation period (time of fermentation) for CGTase production by *Paenibacillus* sp L55

Source	**DF**	**SS**	**MS**	**P**	**F**	**F *crit***
Temperature	3	4.3850	1.46167	0.172	2.32	9
Time of incubation	2	3.2054	1.60271	0.156	2.57	19
Error	6	3.7363	0.62271			
Total	11	11.3267				

The effect of four important factors on CGTase production was investigated by 'two parameters at a time approach'. The enzyme activity was estimated and analysed by 2 way ANOVA using Minitab®. Inoculum density was found as statistically significant factors for CGTase production by *E. aurantiacum*. All other parameters were statistically insignificant for both the organisms.

3.3.6 Effect different carbon and nitrogen sources on CGTase production

The effect of carbon source on production of CGTase by *E. aurantiacum* and *Paenibacillus* sp L55 was studied. The organism could utilise a large number of sources of carbohydrates. The optimum carbohydrate utilised by *E. aurantiacum* and *Paenibacillus* sp L55 for CGTase production was soluble starch as represented in Fig. 3.15.

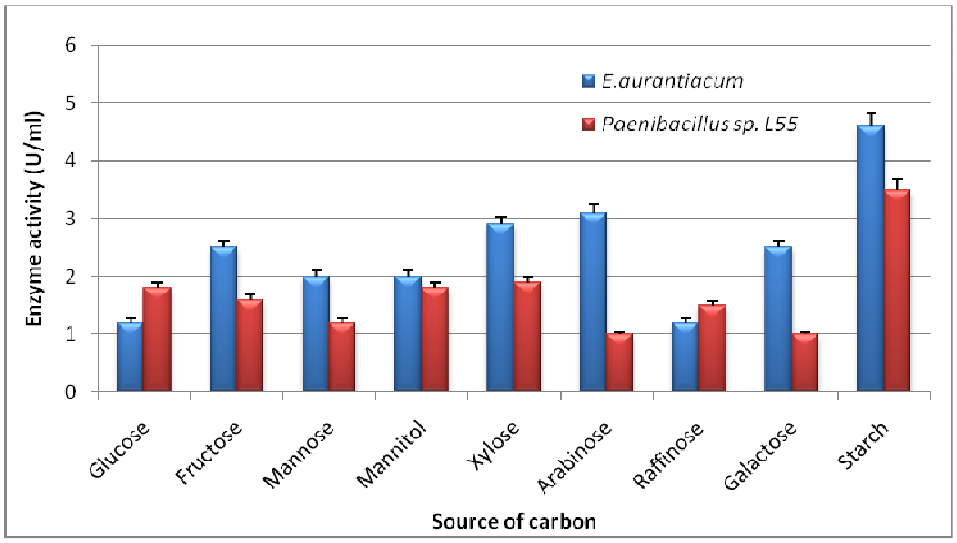


Fig. 3.15 : Effect of different carbon sources on productionof CGTase by *E. aurantiacum* and *Paenibacillus* sp.L55

Starch was the most optimum source for production of CGTase. Similarly, starch was the optimum source for production of CGTase by *Paenibacillus* sp L55. Mahat *et al*. (2004) have reported that starch and yeast extract concentration has significant contribution in the production of CGTase from alkalophilic *Bacillus* sp. TS1-1. Rauf *et al.* (2008) have also reported that starch and pH has significant effect on CGTase production.

The effect of nitrogen source on the growth of CGTase producers was investigated. It was observed that both *E. aurantiacum* and *Paenibacillus* sp L55 could use a wide variety of nitrogen sources. The growth was lesser in inorganic carbon sources like ammonium sulphate, ammonium nitrate, ammonium phosphate and ammonium choride. *E. aurantiacum* demonstrated maximum growth in the presence of peptone followed by yeast extract and ammonium sulphate (Fig. 3.16).

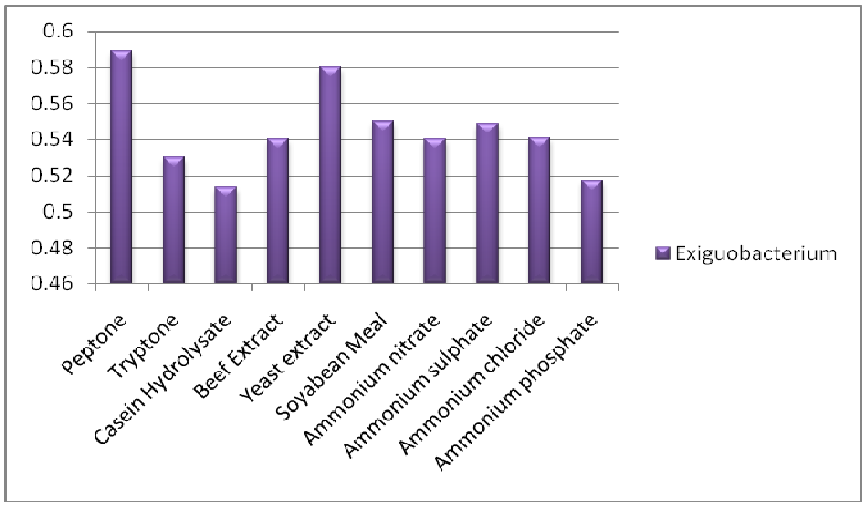


Fig. 3.16 : Effect of different nitrogen sources on CGTase production by *E. aurantiacum*

Similarly, the effect of nitrogen source on growth of *Paenibacillus* sp L55 was studied. The optimum nitogen source was found to be peptone followed by soyabean meal, beef extract and ammonium nitrate (Fig. 3.17).

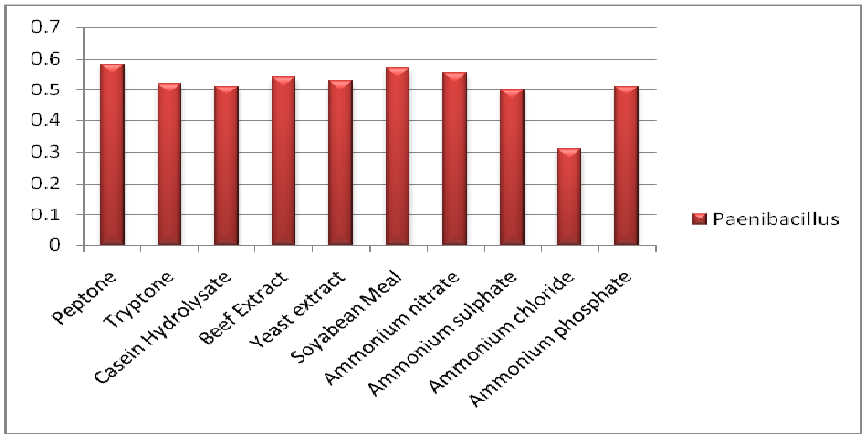


Fig. 3.17 : Effect of different nitrogen sources on CGTase production by *Paenibacillus* sp L55

3.3.7 Optimisation of CGTase production using Taguchi Design of Experiments (DOE)

Taguchi DOE was carried out to study the effect of various factors and their effect on CGTase production in combination. The medium components and initial medium pH were optimised at three levels using L9 array.

There was a significant variation in production of CGTase by *E. aurantiacum.* The production is highly dependant on medium composition and pH of the medium. Trial 6 showed the highest CGTase production i.e. 6.4 U/ml followed by Trial 4 with 5.7 U/ml respectively, while lowest CGTase production was observed in Trial 8 with 3.9 U/ml (Fig. 3.18)

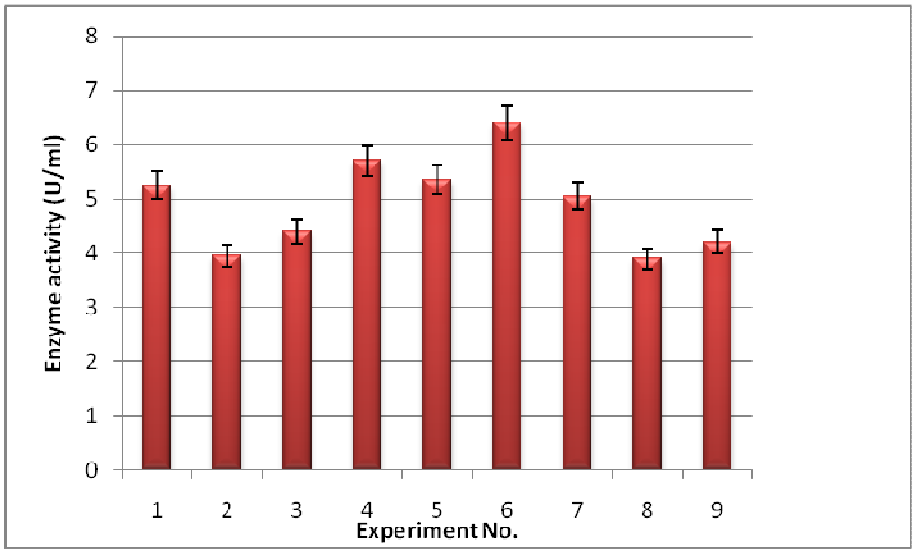


Fig. 3.18 : Comparative data of production of CGTase by *E.aurantiacum* in 9 experimental sets according to Taguchi DOE's L9 orthogonal array (Tables 3.1, 3.2)

The effect of individual factors at assigned levels on CGTase production by *E.aurantiacum* were studied. While studying the effect of concentration of starch on CGTase production, it was observed that enzyme activity was high at Level 2 i.e. 10 g/L starch. While studying the effect of concentration of peptone on CGTase production, it was observed that enzyme activity was high at Level 1 i.e. 5 g/L peptone and was lowest at level 2 i.e. 10 g/L peptone. The effect of yeast extract on CGTase production was studied and it was observed that , the enzyme

activity was highest at level 1 i.e. 1 g/L yeast extract. Effect of pH of production medium at assigned levels on CGTase production was studied. It was observed that pH 9 was the optimum pH for enzyme production. The average effect of the individual factors at assigned levels on CGTase production is presented in Table 3.11.

Table 3.11 : Individual performances of the factors at the assigned levels (%) and resulting influence of each factor on CGTase production by *E. aurantiacum*

Factors	L1	L2	L3	L2-L1	L3-L1	L3-L2
Starch	13.068	15.269	12.784	1.28	-1.433	-0.150
Peptone	14.529	12.773	13.819	-0.933	0.0600	-0.333
Yeast Extract	14.116	13.171	13.834	-0.567	0.317	-0.250
pH	13.812	14.04	13.269	-0.20	-0.467	-0.256

Table 3.11 indicates the influence of individual factors (Starch, Peptone, Yeast Extract and medium pH) on CGTase production at assigned levels (Level 1, 2 and 3, Table 3.1). It can be seen that starch has the highest effect on CGTase production at level 2 followed by peptone at level 1 and yeast extract at level 1. The difference between average value of each factor at lower and higher level depicts the relative influence of each factor on CGTase production at their individual capacity (L3- L2, L2-L1 and L3-L1). Larger the difference (in numeric values), more stronger is the influence of the factor on CGTase production. The sign of the difference (+/-) indicates whether the change from one level to other could increase or decrease the result (Tupe *et. al.*, 2007). It is clearly evident that concentration of starch had a pronounced effect on CGTase production followed by yeast extract, peptone and pH.

The percentage contribution of factors on production of CGTase by *E.aurantiacum* is illustrated in Fig. 3.19.

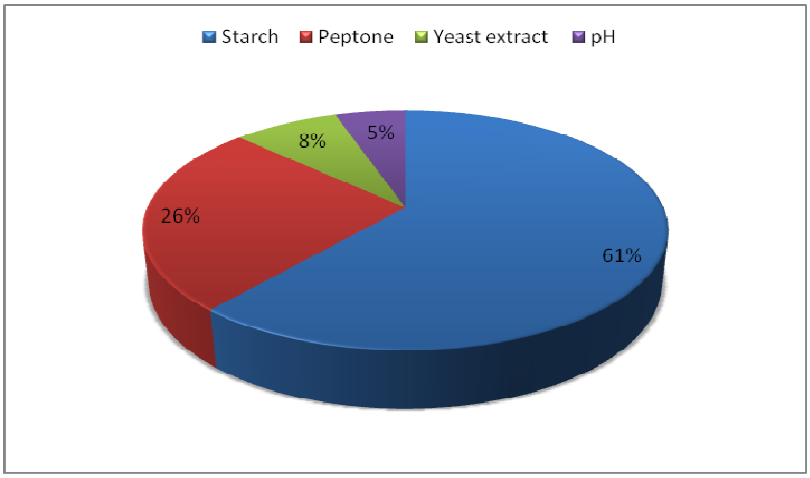


Fig 3.19 : Percentage contribution of factors on production of CGTase by *E.aurantiacum*

ANOVA with the percentage of contribution of each factor with interactions was calculated based on the ratio of pure sum to total sum of squares. The analysis revealed that the contribution of pH in CGTase production by *E. aurantiacum* is least; however the contribution of starch and peptone is the highest. The effect of starch was the most significant with 61 % contribution in CGTase production.

Table 3.12 : Statistical analysis of pooled ANOVA for optimisation of CGTase production by *E.aurantiacum* using Taguchi DOE

Factor	Degree of freedom	Sum of squares	Mean squares	F value	Interpretation
Starch	2	11.10	5.55	9.44	Significant
Peptone	2	4.68	2.34	3.98	Significant
Yeast Extract	2	1.41	0.71	1.20	-
pH	2	0.94	0.47	0.80	-
Error	0	-			
Total	8	18.13			
(Error)	4	0.58798345			

Grand total sum of squares=1709.11
Sum of squares due to mean =1690.977
Total sum of squares =18.13

From the F ratios given in ANOVA table, it is seen that the effect of the two factors starch and peptone is large and statistically significant. The effect of yeast extract on CGTase production was small. The effect of pH is smaller than the error of the additive model.

For predicting the performance under optimum conditions , factors with confidence intervals < 90 % were pooled and the ANOVA after pooling is given in Table 3.12. The optimum conentration of factors at assigned levels and the predicted condtition for CGTase production are summarised in Table 3.13.

Table 3.13 : Prediction table for CGTase production by *E.aurantiacum*

Factors	**Levels**	**Concentration**	**Prediction**	
Starch	2	10 g/L	predicted S/N	16.8
Peptone	1	5 g/L	CGTase activity	6.4 U/ml
Yeast Extract	1	1 g/L		
pH	3	10		

On the basis of signal : noise ratio of 16.8, the predicted optimum medium composition for CGTase production by *E. aurantiacum* was found to be starch 10 g/L, peptone 5 g/L, yeast extract 1 g/ L and medium pH as 10 which would result in the yield of 6.4 U/ml of CGTase .

Validation experiments were performed to confirm the predicted results which yielded 6.15 U/ml CGTase activity.

The effect of various factors and their effect on CGTase production by *Paenibacillus* sp L55 were studied using Taguchi DOE. The medium components and initial medium pH were optimised at three levels using L9 array.

There was a significant variation in production of CGTase by *Paenibacillus* sp L55. The production is highly dependant on medium composition. Trial 6 showed the highest CGTase production i.e. 5.35 U/ml followed by Trial 3 and trial 1 with 5.15 U/ml and 5.05 U/ml respectively, while lowest CGTase production was observed in Trial 8 with 3.65 U/ml (Fig. 3.20)

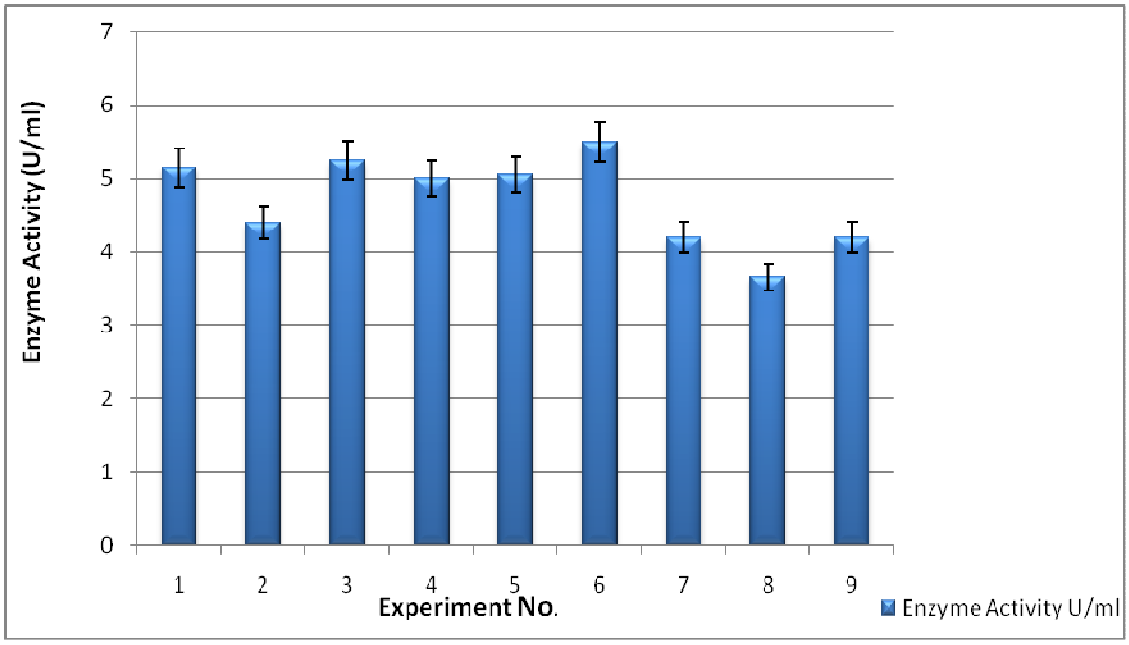


Fig. 3.20 : Comparative data of production of CGTase by *Paenibacillus* sp L55 in 9 experimental sets according to Taguchi DOE L9 orthogonal array (Tables 3.1, 3.2)

The effect of individual factors at assigned levels on CGTase production by *Paenibacillus sp* L55 was studied. While studying the effect of concentration of starch on CGTase production, it was observed that enzyme activity was high at Level 2 i.e. 10 g/L starch. While studying the effect of concentration of peptone on CGTase production, it was observed that enzyme activity was high at Level 3 i.e. 25 g/L peptone and was lowest at level 2 i.e. 10 g/L peptone. The effect of yeast extract on CGTase production was studied and it was observed that , the enzyme activity was highest at level 1 i.e. 1 g/L yeast extract. Effect of pH of production medium at assigned levels on CGTase production was studied and it was observed that pH 9 was the optimum pH for enzyme production. The average effect of the individual factors at assigned levels on CGTase production is presented in Table 3.14.

Table 3.14 : Individual performances of the factors at the assigned levels (%) and resulting influence of each factor on CGTase production by *Paenibacillus* sp L55

Factors	L1	L2	L3	L2-L1	L3-L1	L3-L2
Starch	13.836	14.284	12.059	0.25	-1.167	-0.917
Peptone	13.56	12.727	13.892	-0.417	0.617	0.2
Yeast Extract	13.43	13.104	13.645	-0.233	0.3	0.067
pH	13.589	13.38	13.209	-0.1	-0.067	-0.167

The influence of individual factors (Starch, Peptone, Yeast Extract and medium pH) on CGTase production by *Paenibacillus* sp L55 at assigned levels (Level 1, 2 and 3, Table 3.1) can be observed from Table 3.14. It is found that starch has the highest effect on CGTase production at level 2 followed by peptone at level 3 and yeast extract at level 1. As mentioned previously, the difference between average value of each factor at higher and lower level (L3- L2, L2-L1 and L3-L1) shows the relative influence of each factor on CGTase production at their individual capacity. Larger the difference (in numeric values), more stronger is the influence of the factor on CGTase production (Tupe *et. al.*, 2007). It is clearly evident that concentration of starch had a pronounced effect on CGTase production followed by peptone, yeast extract and pH.

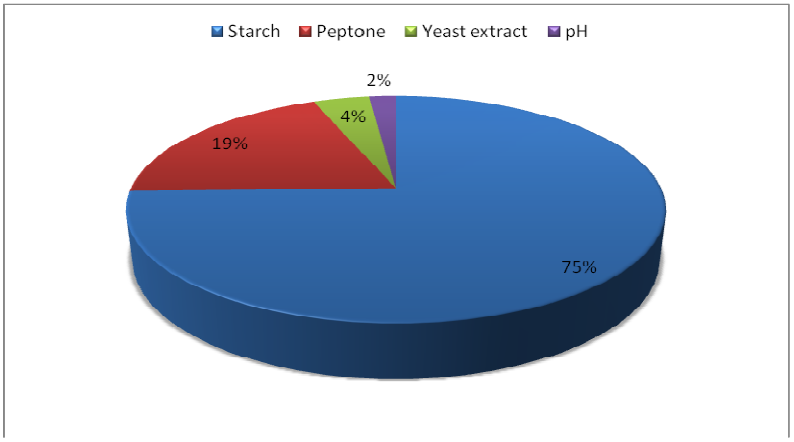


Fig 3.21 : Percentage contribution of factors on CGTase production by *Paenibacillus* sp L55

The statistical analysis revealed that the contribution of starch is highest in CGTase production by *Paenibacillus* sp L55, however the contribution of peptone and yeast extract is least with 4 % and 2 % contribution respectively. The effect of starch was the most significant with 75 % contribution in CGTase production (Fig.3.21).

Table 3.15 : Statistical analysis of pooled ANOVA for optimisation of CGTase production by *Paenibacillus* sp L55 using Taguchi DOE

Factor	Degree of freedom	Sum of squares	Mean squares	F value	Interpretation
Starch	2	11.10	5.55	5.55	Significant
Peptone	2	4.68	2.34	2.34	Significant
Yeast Extract	2	1.41	0.71	0.71	Significant
pH	2	0.94	0.47	0.47	Insignificant
Error	0				
Total	8	18.13			
(Error)	4	0.58798345			

Grand total sum of squares = 1625.479065
Sum of squares due to mean = (no. of experiments) x m^2 = 1614.344
Total sum of squares = 11.13520684

Analysis of variance (ANOVA) is used to analyze the results of the OA experiment and to determine extent of variation contributed by each factor. From the calculated ratios (*F*), it can be referred that all factors and interactions considered in the experimental design are statistically significant effective at 95% confidence limit (Table 3.15). By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process of enzyme production can be characterized. The characteristics can be controlled such that a lower or a higher value in a particular influencing factor produces the preferred result. Thus, the levels of factors to produce the best results can be predicted and the result can be confirmed using validation experiments. The data obtained in the trials show that CGTase production varied from 3.65 to 5.5 U/ml demonstrating the significance of

parameters in CGTase production. The key nutrient showing maximum percentage of contribution to variance is starch with significant effect while effect of pH was insignificant and even smaller than the error of the additive model.

Table 3.16 : Prediction table for CGTase production by *Paenibacillus* sp L55

Factors	Levels	Concentration	Prediction	
Starch	2	10 g/L	predicted S/N	14.8
Peptone	3	25 g/L	CGTase activity	5.5 U/ml
Yeast Extract	1	1 g/L		
pH	2	9		

On the basis of signal : noise ratio of 14.8 (Table 3.16), the predicted optimum medium composition for CGTase production by *Paenibacillus* sp L55 was found to be starch 10 g/L, peptone 25 g/L, yeast extract 1 g/ L and medium pH as 9 which would result in the yield of 5.5 U/ml of CGTase .

Validation experiments were performed to confirm the predicted results which yielded 5.15 U/ml CGTase activity.

The predicted optimised conditions were validated and it was observed that the enzyme activity was > 93 % of the predicted enzyme activity. Taguchi methodology was thus successfully employed to enhance the production of CGTase from *Paenibacillus* sp L55.

3.3.8 Production under optimised conditions

Production of CGTase was carried out under all optimum conditions. The yield of CGTase from *Exiguobacterium* increased to 6.15 U/ml while *Paenibacillus* yield increased to 5.15 U/ml using Taguchi DOE. Thus under optimised conditions there was almost two fold increase in enzyme activity by both the cultures.

3.4 Discussion

Microbial process efficiency determines its industrial potential. To have efficient productivity, optimization of process parameters is necessary. Production of CGTase can be optimized by manipulating physio-environmental factors such as the nutrient concentration and composition of the production medium. Media optimization using statistical experimental design has been reported by Casas *et al*. (1997), Lee and Chen (1997), Pujari and Chandra (2000), Dey *et al*. (2001), Liu *et al*. (2001), Cockshott and Sullivan (2001), Hujanen *et al*. (2001), Chen *et al*.(2002) and Li *et al*.(2002) in optimizing either biomass growth, enzymes, certain extracellular proteins and bioactive metabolites. Typically modes of CGTase production are conducted initially using shake flask culture and submerged fermentation utilizing selective media. Optimisation of various types of medium composition mainly concentration of carbon and nitrogen source, inoculum size, pH and temperature for production of CGTase have been studied and reported (Bonilha *et al*., 2006, Gawande *et al.,* 1998, Stefanova *et al*., 1999).

The orthogonal array design technique is a traditional method that has been successfully applied to improve the culture media for fermentation processes and it provides the relationship among various factors, and the order of significant factors for the optimum results (Bakhtiari *et al*., 2006; Krishna *et al*., 2005; Sreenivas *et al*., 2004). In the present study, L9 (3^4) design was applied to screen the significant factors according to the preliminary experiments. The four factors, viz., concentration of Starch, Peptone, Yeast extract and initial pH of medium and their relevant levels were optimised by Taguchi DOE. In the course of optimization experiments, the fermentation temperature, initial pH, rotation speed and fermentation period were set at 37°C, pH 10, 150 rpm and 24 hr, respectively. These parameters have been optimised by conventional and statistical approach as mentioned above. Huang *et al*. (2007) have also reported the use of one-factor-at-a-time method and orthogonal array design in optimization of biomass and exopolymer from mushrooms. The sole aim of this unique methodology of using a combination of conventional and statistically designed experiments was to elevate enzyme production which was achieved.

Gawande and Patkar (2001) have reported dextrin as optimum carbon source for CGTase producer *Klebsiella pneumoniae* AS-22 with the optimum medium composition containing yeast extract and peptone.The carbon source plays an important role in induction of CGTase. Khairizal *et al.* (2004) have reported the optimum carbon source as sago starch. Starch is the most preferred source for CGTase production as the use of simple sugars gives low yield of CGTase (Noi *et al*., 2008). Jing- Bong *et al.* (1999) and Sreenivasan *et al.* (2004) have reported better yields with soluble starch and tapioca starch respectively. Addition of ammonium salts decrease CGTase production, whereas some reports have mentioned that organic nitrogen sources are required for production of CGTase and growth of the producers (Gawande and Patkar, 2001). Rosso *et al.* (2002) have reported contradictory findings that prove that CGTase production and growth decrease when a mixture of organic nitrogen sources and ammonium salts are used

The optimum carbon source and nitrogen source for enzyme production by *E. aurantiacum* and *Paenibacillus* sp L55 was soluble starch and peptone. Menocci *et al.* (2008) have also reported that potato starch is the best and most significant carbon source for CGTase production using *Bacillus* sp. Bonilha *et al.* (2006) have also reported maximum specific activity of CGTase in presence of starch. It has been reported that some starches may contain an inducer for CGTase production. The difference in CGTase activity obtained with different starches may be due to the differences in their physical structures and chemical properties. Noi *et al.* (2008) have reported significant contribution of sago starch for CGTase production using *Bacillus* sp. However Gawande and Patkar (2001) have reported the advantage of dextrin as carbon source over starch.

CGTase production has been previously reported from organisms like *Paenibacillus azotofixans*, *Paenibacillus macerans* and *Paenibacillus* sp RB01 (Zheng *et al*., 2012; Zhou *et al.,* 2011), but this is the first report of production of CGTase from alkaliphilic *Paenibacillus* sp L55 from soda lake of Lonar, India.

3.5 Conclusions

E. aurantiacum and *Paenibacillus* sp L55 were selected for CGTase production on the basis of CGTase activity and HPLC analysis of β- Cyclodextrin produced. The optimum production medium was predicted for CGTase production by Taguchi method. The optimal culture condition obtained for the CGTase production from the proposed methodology resulted in almost two fold increase in enzyme activity of CGTase produced by *E. aurantiacum* and 96 % increase in enzyme activity as compared to predicted value. The optimal nutritional and culture conditions for CGTase production for *Paenibacillus* sp L55 were also predicted by Taguchi Design of Experiments and the yield was enhanced from 3 U/ml to 5.15 U/ml. Taguchi experimental design was thus used in combination with conventional and statistical methods successfully to optimise fermentation parameters of CGTase production and enhance activity of the enzyme. Based on the enzyme activity and growth rate, *E. aurantiacum* was selected for further studies.

Chapter 4

Scale up production of Cyclodextrin glycosyl transferase from *Exiguobacterium aurantiacum*

4.1 Introduction

4.2 Materials and methods

4.2.1 Microorganism

4.2.2 Inoculum development

4.2.3 Production medium

4.2.4 Design of the fermentor

4.2.5 Calculation of time required for sterilization of medium in 3 L fermentor

4.2.6 Operation of 3 L fermentor

4.2.7 Analysis for monitoring of production of CGTase by submerged aerobic batch fermentation (3 L)

4.2.8 Optimisation of fermentation conditions

4.2.9 Growth kinetics in 3 L fermentor

4.2.10 Kinetics of growth linked product formation (CGTase) in fermentor and calculations of $q_{p,}$ $Y_{p/x}$ and Y

4.2.11 Prediction of Volumetric Mass Transfer Coefficent (K_La) by empirical method

4.2.12 Scale up production of CGTase using 14 L SS fermentor

4.2.12.1 Operation of 14 L SS fermentor

4.2.11.2 Production of CGTase in 14 L fermentor

4.2.13 Downstream processing

4.3 Results

4.3.1 Calculation of sterilisation time of medium in 3 L fermentor using Autoclave

4.3.2 Production of CGTase using different aspect ratios

4.3.3 Production of CGTase at different rates of agitation

4.3.4 Production of CGTase at different rates of aeration

4.3.5 Production of CGTase in 3 L fermentor under optimised conditions

4.3.6 Growth kinetics of *E.auratiacum* in 3 L fermentor

4.3.7 Kinetics of growth linked product formation (CGTase) in 3 L Fermentor and calculations of $q_{p,}$ $Y_{p/x}$, Y, K_La, N_P and $N_{FR.}$

4.3.8 Scale up production of CGTase using 14 L SS fermentor

4.3.9 Downstream processing

4.4 Discussion

4.5 Conclusions

4.1 Introduction

The basic production process of bioactive metabolites, biopharmaceuticals and enzymes is fermentation. Fermentation can be performed in shake flask of 250 ml capacity and also in industrial fermentors of up to several thousand litres capacity. Fermentations are operated either in batch mode, fed batch mode or continuous mode. The enzyme fermentations can be economical on a large scale because of short fermentation cycles and inexpensive media. The factors which influence fermentation are inoculum, media composition, environmental parameters such as nutrient availability, pH, temperature, dissolved oxygen, dissolved carbon dioxide, aeration and agitation. The most important factor to be considered during the operation of a fermentor is the provision of adequate mixing of its contents. The main objectives of mixing in fermentation are to disperse the air bubbles, to suspend the microorganisms (or animal and plant tissues), and to enhance heat and mass transfer in the medium.

Since most nutrients are highly soluble in water, very little mixing is required during fermentation just to mix the medium as microorganisms consume nutrients. However, dissolved oxygen in the medium is an exception because its solubility in a fermentation medium is very low, while its demand for the growth of aerobic microorganisms is high.

Production of CGTase in fermentors

Mahat *et al*. (2004) have reported the production of CGTase from *Bacillus* sp. TS 1-1 in shake flask. Major reports on production of CGTase are in shake flask with a medium volume of 50 to 250 ml volume (Higuti *et al.,* 2004; Gawande and Patkar, 1998; Rosso *et al*., 2002; Noi *et al*., 2008). There are a very few reports on optimisation of CGTase production at fermentor level. Frietas *et al*. (2004) have reported the production of CGTase from alkaliphilic *Bacillus* cCG strainII strain isolated from waste water of a flour industry in a 5 L fermentor.

Optimisation of aeration, agitation and aspect ratio are important as they play a crucial role in growth of the bacteria and in turn CGTase production. The availability of oxygen, determined by Oxygen Transfer Rate (OTR), is also governed by the volumetric oxygen transfer coefficient (K_La) and the concentration

of DO in the growth medium. The fixing of K_La values has been commonly used criterion for scale up of aerobic fermentation processes. The K_La values ensure a mass transfer capability of the process that can cope up with oxygen demand of the culture and often serve to determine the efficiency of bioreactors and mixing devices as well as an important scale-up factor from small cultivation to large scale production. However, very little attention has been paid to study the role of aeration rate, agitation speed and K_La values in the production of extracellular CGTase production.

Optimisation of medium components and process parameters for CGTase production by *E. aurantiacum* was done previously in 250 ml shake flask by combinatorial approach and Taguchi method. As the dimensions of a lab scale fermentor and a 250 ml flask are different, the aeration, agitation and medium: flask ratio (optimised in flask) expressed as Aspect ratio in fermentor (H/D) were optimized in a Biotron 3 L fermentor.

The present chapter describes optimisation of CGTase production in 3 L fermentor and subsequent scale up of the production to a 14 L stainless steel fermentor.

4.2 Materials and Methods

4.2.1 Microorganism

Exiguobacterium aurantiacum MCM B- 1021 was employed for the scale up production of CGTase. The culture was maintained on nutrient medium with pH 10 (adjusted using 10 % sodium carbonate). The culture was preserved at – 20°C as glycerol stocks.

4.2.2 Inoculum development

E. aurantiacum was inoculated in nutrient medium pH 10 (adjusted using 10 % sodium carbonate) containing 1 % (w/v) soluble starch and incubated at 37°C for 16 h. After incubation, the cells were centrifuged at 10,000 g for 20 min; the cell pellet obtained was washed with sterile saline and centrifuged again at 10,000 g for 20 min. The cell pellet obtained was resuspended in sterile saline and cell density was measured spectrophotometrically (Schimadzu, Japan) at 540 nm. The optical

density was adjusted to 1.0 $\pm$ 0.05 which corresponded to 10^8 CFU/ml as enumerated by standard plate count using spread plate method.

4.2.3 Production medium

The production medium used for production of CGTase from *E. aurantiacum* was the medium optimised by Taguchi DOE as described in section 3.3.7 containing (g/L) starch 10, peptone 5, yeast extract 1 , sodium chloride 5 and magnesium sulphate 0.2. pH of the medium was adjusted to 10 with 10 % (w/v) sodium carbonate. The production medium was sterilised by autoclaving the medium in 3 L fermentor (Biotron BIOG-Micom jar fermentation system , Korea) at 121 ℃ at pressure of 103 kPa (15 p.s.i) for 20 min. The production medium in 14 Stainless Steel (SS) fermentor was sterilised *in situ* for 30 min at 121 ° at pressure of 200 kPa (2 bar or 29 p.s.i).

4.2.4 Design of the fermentor

The production of CGTase was carried out in the 3 L glass fermentor and then scaled up in a 14 L SS fermentor (Biotech Engineering Pvt. Ltd., Pune). The technical details of both the fermentors are outlined in Table 4.1.

Table 4.1 : Technical details of the fermentors used for CGTase production

S.No	Specifications	3 L fermentor	14 L fermentor
1	Vessel material	glass vessel with stainless steel lid	stainless steel
2	Fermentor inner diameter (D), mm	190	2500
3	Height of vessel (H), mm	505	2500
4	Number of baffles	2	2
5	Number of impellers	2	2
6	Total blades of impeller	6	6
7	Type of impeller	fixed blade disc	fixed blade disc

For aeration in 3 L fermentor, the air was sterilised by passing through a presterilised autoclavable 0.45 µ membrane filter (Millipore) and introduced in the medium via a ring orifice porous sparger. The rate of aeration was expressed as volume of air per volume medium (vvm) and controlled by a rotameter controlled by a needle valve. The agitation in the medium was achieved using a 60 watt top driven impellor. The fermentor was equipped with pH probe and temperature probe (Fig. 4.1).

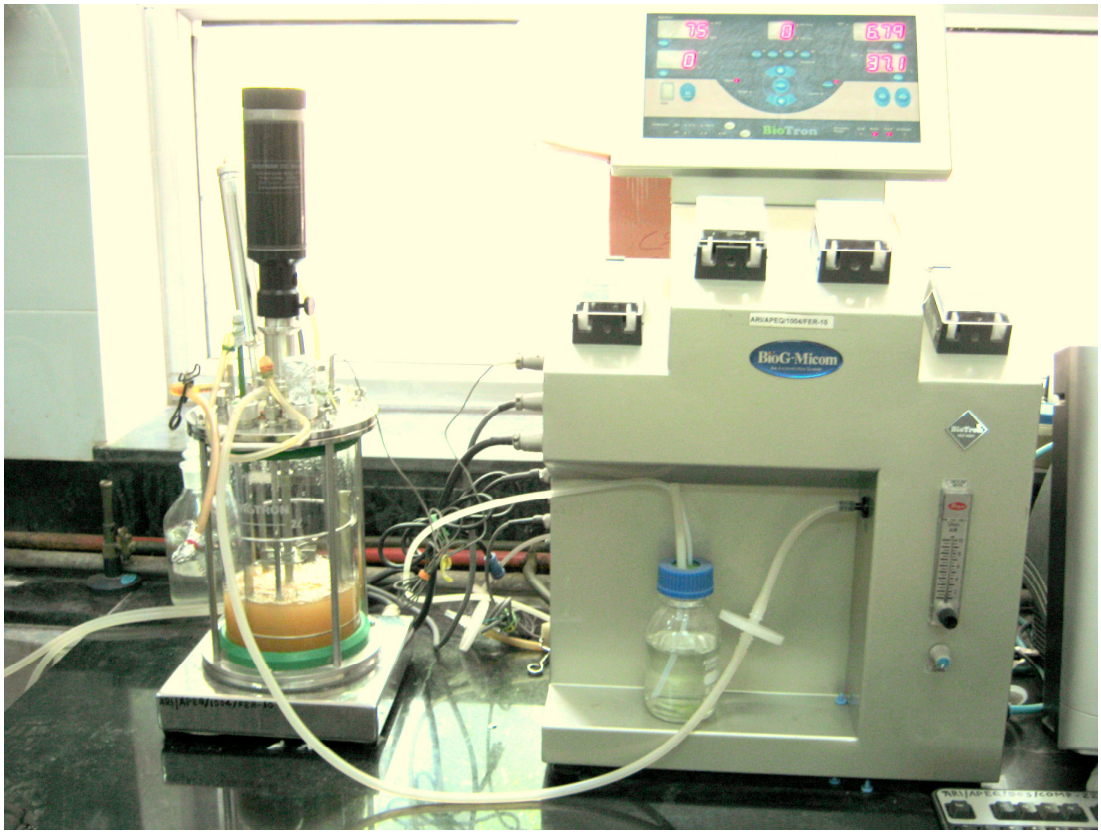


Fig. 4.1 : 3 L Glass fermentation system (Biotron BIOG-Micom, Korea) used for production of CGTase

For aeration in the 14 L SS feremntor, air was passed through a hydrophobic 0.2µ PTFE filter and a 0.5 µ ceramic filter with a SS 316 housing. The flow rate was expressed as Litres of oxygen Per litre Medium (LPM) and controlled via a rotameter. Inlet and outlet gas flow rates were controlled using diaphragm valves. Agitation was achieved by four fixed blade disc impellers driven by a 1118 watt motor. Temperature was maintained by circulation of steam or cold water through

heating/cooling jackets. The pH, temperature and Dissolved Oxygen (DO) was measured using respective probes. The monitoring of process parameters (pH, tempertaure and DO) and process control in the 14 L fermenter was computerised using a Supervisory Set point Control system (SSC) called as the Supervisory Control And Data Acquisition (SCADA) software (Fig. 4.2).

Fig. 4.2 : The 14 L SS fermentor used for CGTase production

4.2.5 Calculation of time required for sterilisation of medium in 3 L fermentor using autoclave (Equitron)

In order to calculate the time required for batch sterilisation of medium at 121°C in 3 L fermentor, the number of organisms in the fermentation medium, before (No) and after (Nt) sterilisation was enumerated by spread plate technique.

The ∇ Factor (Del factor) or Nabla factor was calculated using following equation:

∇ = ln (No / Nt) where No is the number of organisms before sterilisation and Nt is number of organisms after sterilisation. The time required for sterilization (∇ Holding) was calculated using following equation:

$$\nabla \text{ Holding} = \nabla \text{ overall} - \nabla \text{ Heating} + \nabla \text{ Cooling}$$

4.2.6 Operation of 3 L fermentor

The production of CGTase was carried out as a 24 h submerged aerobic batch fermentation mode. The 3 L fermentor used in the study was an autoclavable bench top glass fermentor. Production medium was added to the fermentor and sterilised by steam sterilization as mentioned in section 4.2.4. After cooling of the sterilized medium, appropriate amount of sterile 10 % sodium carbonate was added to maintain pH as 10. The pH probe was calibrated using standard buffers of pH 4, 7.5 and 9.2 (Himedia). The medium was saturated with oxygen by sparging sterile air and the medium was incubated over night for sterility checking. Sterile 10 % (v/v) Silicone oil (Himedia) was added as antifoam agent at 0.005 % final concentration in medium. The inoculum of 16 h old *E. aurantiacum* (10 % v/v) having concentration of 10^8 CFU/ml was aseptically added and 0 h sample were withdrawn for analysis. The samples from the fermentor was withdrawn after 6 h interval till 24 h and analysed as described in section 4.2.6. The samples collected were examined under a microscope to check for bacterial or other types of contamination.

4.2.7 Analysis for monitoring of production of CGTase by submerged aerobic batch fermentation (3 L)

Each sample withdrawn from the fermentor for production was analysed as follows:

1 Cell growth by optical density was measured spectrophotometrically at 540 nm and ascertained by total viable count by spread plate method.

2 The biomass was expressed as dry cell weight (DCW, g/L) measured gravimetrically by centrifugation of medium at 10,000 g for 20 min, drying

the pellet in the hot air oven and measuring the weight on a digital balance (Sartorius, Japan).

3 CGTase activity was estimated by the phenolphthalein assay as described by Goel and Nene (1995) (Section 2.2.3). One unit of CGTase activity is defined as an amount of enzyme required to produce 1 µg of β-CD /ml/min.

4 The pH was measured *ex situ* using an equilibrated pH meter (Equitron).

5 Microscopic examination was performed using Gram staining to check sterility of the medium in the fermentor.

4.2.8 Optimisation of fermentation conditions

The effect of aspect ratio, rate of aeration and rate of agitation on production of CGTase by *E. aurantiacum* in 3 L fermentor was studied. The aspect ratio was varied from 0.45 to 1.04 (0.45, 0.625. 1.04). The rate of agitation was varied from 75 rpm to 150 rpm (75, 100,150). The aeration rate was varied from 0.5 vvm to 1.5 vvm (0.5, 1.0, 1.5 vvm). The other conditions of the fermentor were set as initial pH of medium as 10.0 and temperature as 37°C. A positive pressure was maintained to avoid external contamination.

4.2.9 Growth kinetics in 3 L fermentor

The growth of *E .aurantiacum* in 3 L fermentor under optimised conditions was studied. Samples were withdrawn every 6 h from the fermentor and the total viable count was obtained using spread plate method. The residual substrate concentration i.e. concentration of starch was estimated by colorimetric iodine assay (Smith *et al*., 1948). The maximum specific growth rate was obtained by plotting a log of biomass concentration versus time. The substrate utilisation constant was calculated using following equation (Stanbury *et al*., 1995):

$$\mu = \mu_{max}\, s / K_s + s$$

4.2.10 Kinetics of growth linked product formation (CGTase) in Fermentor and calculations of q_p, $Y_{p/x}$ and Y.

The kinetics of product formation i.e. production of CGTase by *E. aurantiacum* in 3 L fermentor was studied. CGTase is a growth-linked primary metabolite which is synthesized by growing cells in exponential or late log phase. The specific rate of

product formation ($\mathbf{q_p}$) i.e. rate of production of CGTase in mg per gram biomass of *E. aurantiacum* was calculated by following equations

$$\mathbf{dp/dt = q_p\, x} \qquad (1)$$

where *p* is the concentration of product (i.e amount of CGTase enzyme protein in mg) and *qp* is the specific rate of product formation (mg product/ g biomass/ h).

The product yield $\mathbf{Y_{p/x}}$, i.e CGTase activity (U) per gram of biomass (dry cell weight) can be calculated by the following equation:

$$\mathbf{dp/dx = Y_{p/x}} \quad (2)$$

Specific rate of product formation q_px was calculated by estimation of amount of CGTase enzyme in the form of mg protein per gram biomass (dry cell weight) per hour of the fermentation and can be expressed as follows.

$$\mathbf{q_px = Y_{p/x.}\, x} \quad (3)$$

The Yield factor is the measure of efficiency of conversion of substrate into biomass. In this study, yield factor pertains to the conversion of soluble starch to biomass of the microbial cells; i.e. *E. aurantiacum*. The yield factor can be calculated as follows:

$$\mathbf{X = Y_{x/s}\,(S_R - s)} \quad (4)$$

where X is the concentration of biomass produced,

$\mathbf{Y_{x/s}}$ is the yield factor (g biomass produced substrate consumed),

S_R is the initial substrate concentration, and

s is the residual substrate concentration

4.2.11 Prediction of Volumetric Mass Transfer Coefficent (K_La) by empirical method

The 3 L fermentor used in this study lacked the presence of an oxygen electrode. We hence predicted the volumetric mass transfer coefficient (K_La) theoretically using empirical mathematical relationship between K_La, superficial air velocity

(aeration) and power consumption as described by Cooper *et al.* (1944) and Van Riets constants (1983) k, x and y as follows:

$$\mathbf{K_L a = k\ (P_g / V)^x\ V_s^y}$$

Where P_g = Power absorption in system, V = Liquid volume in vessel and

Vs = Superficial air velocity.

Rushton *et al.* (1950) have described the relationship between power consumption and power absorption during agitation of non-gassed Newtonian liquids which could be represented by a dimensionless group termed the power number by the expression:

$$N_p = P / (\rho N^3 D^5)$$

where *Np* is the power number, *P* is the external power from the agitator, *p* is the liquid density, *N* is the impeller rotational speed, *D* is the impeller diameter.

The Froude number, relates inertial force to gravitational force and was calculated by following equation :

$$\boldsymbol{NFr = (pND^2)/g}$$

where *N*Fr is the Froude number and

g is the gravitational force.

4.2.12 Scale up production of CGTase using 14 L SS fermentor

Scale up production of CGTase was carried out in 14 L SS fermentor (Biochem Engineering Ltd, Pune) to obtain higher yield of enzyme.

4.2.11.1 Operation of 14 L SS fermentor

The Fermentor was equipped with digitally controlled sterilizable pH electrode (F365-B120-DH, Broadly James Corporation, USA), temperature probe, polarographic DO electrode (Model –D400-B070-PT-D9) and two six-blade fixed blade disc impellor of 0.1 m diameter with distance of 0.055 m between each other ,fixed on the agitator shaft above porous ring orifice air sparger of 0.1 m diameter. The pH electrode was calibrated using

standard buffers (Himedia) at pH 7 and 9 prior to sterilisation of the fermentor. The working volume used was 8 L and the foam was controlled manually by adding 3 ml of Silicone oil to 8 L of the broth prior to fermentation. A cycle of steam sterilisation was performed for 90 min prior to addition of the medium. The fermentor and airline was sterilized *in situ* by steam sterilization at 121°C for 30 min. The medium was sterilized by steam sterilisation with a heating cycle of 20 min, holding cycle of 30 min at 121 °C at 2 bar pressure and cooling cycle of 20 min.

4.2.11.2 Production of CGTase in 14 L fermentor

The medium used for production was the medium optimised by Taguchi Design of experiments. Four flasks containing 200 ml medium were inoculated with 16 h old culture of *E. aurantiacum* and incubated at 37 °C at 150 rpm for 16 hours. The culture broth from four flasks was pooled and used to inoculate 8 L production medium containing (g/L) soluble starch 10, peptone 5, yeast extract 1 and $MgSO_4.7\ H_20$ 0.02. The initial pH of the medium was adjusted to 10 by addition of sterile 10 % Na_2CO_3 after sterilization of the production medium. Fermentation was carried out at 37°C for 12 h with 8 L working volume with air flow rate of 2 vvm (volume of air per unit volume of medium per minute) and agitation was 250 rpm. Fermentation was carried out in batch mode till stationary phase approached and aliquots at 6 h intervals were withdrawn and analysed for the following:

1 Optical density, dry cell weight, pH and enzyme activity as mentioned in section 4.2.6

2 Protein content was measured using Biuret reagent, the total protein content and nitrogen content (N) was calculated by Jones method (Jones *et al*., 1941).

3 Starch utilisation was estimated by colorimetric iodine assay (Smith *et al*., 1948), the carbon content (C) was calculated as described by Zain *et al*. (2007) and the C: N ratio was calculated.

4.2.13 Downstream processing

After the fermentation, the broth was centrifuged in a continuous centrifuge (Penwalt, India Ltd. Model – AS 16 Y) at 15,000 rpm for 10 minutes at 28° C. The supernatant (crude enzyme) was precipitated with 80 % enzyme grade ammonium sulphate at 4°C overnight and the precipitate obtained was dialysed against 50 mM Tris Cl buffer pH 8.5 to give partially purified CGTase enzyme. The enzyme was stored at 4°C until further use.

4.3 Results and Discussions

4.3.1 Calculation of time required for sterilisation of medium in 3 L fermentor using autoclave

The Del factor is a sterilization design criterion and used to measure fractional reduction in viable count of bacteria produced by a exposure heat over a time period. The del factor was calculated as follows:

The total number of viable organism before sterilisation = No = 10^{11} CFU/ml.

The total number of viable organisms after sterilization = Nt = 10^{-3} CFU/ml.

Therefore ∇ overall = ln (No/Nt) = ln(10^{11}/ 10^{-3}) = 41.44

Thus, ∇ holding = ∇overall - ∇heating - ∇cooling

∇heating = 12.2 X 20 (time in minutes required for heating of autoclave) / 21(temperature change in heating cycle) = 11 .6

∇cooling = 12.5 X 15 X (time in minutes required for cooling of autoclave) X 21 = 8.71

∇ holding = 41.44 – 11.6 – 8.7 = 20 min.

Thus for obtaining sterilized medium in 3 L fermentor the medium should be steam sterilized by holding the medium at 121°C at 15 psi for a minimum of 20 min.

Optimisation of medium components and process parameters for CGTase production by *E. aurantiacum* was done previously in 250 ml shake flask. As the dimensions of a lab scale fermentor and a 250 ml flask are different, the aeration, agitation and medium: flask ratio (optimised in flask) expressed as aspect ratio in fermentor (H/D) were optimised in a Biotron 3 L fermentor.

4.3.2 Production of CGTase using different aspect ratios

The aspect ratio is defined as height of medium in fermentor to the diameter of the fermentor (H/D). The H/D ratio is a measure of the medium volume in the fermentor. The H/D ratios used in this study are summarised in Table 4.2.

Table 4.2 : The Aspect ratio optimised for production of CGTase by *E. aurantiacum*

Aspect Ratio	Volume of fermentation medium	Height of medium (H)
0.45	600 ml	40 mm
0.625	1000 ml	75 mm
1.04	1500 ml	120 mm

The growth of *E. aurantiacum* in 3 L fermentor with aspect ratio 0.45 corresponding to a total medium volume of 600 ml was studied. It was observed that the culture showed no lag phase and immediately entered exponential phase. The exponential phase lasted upto 6 h and thereafter the absorbance of the culture remained constant. There was a sharp drop in pH from 9.5 to 5.9 (Fig. 4.3). Maximum CGTase activity was observed between 2- 3 U/ml at 12 h after which the enzyme activity decreases rapidly (Fig. 4.3).

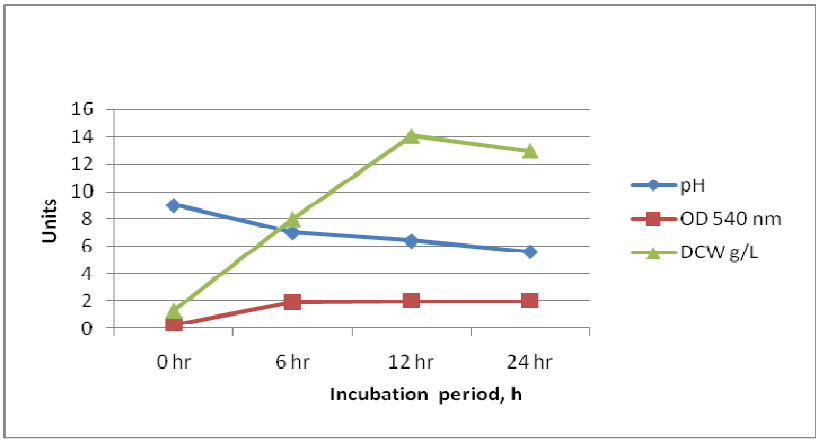


Fig. 4.3 : Growth of the culture in 3 L fermentor operated at aspect ratio of 0.45 (600 ml working volume)

It was observed that this aspect ratio of 0.45 (working volume 600 ml in 3 L fermentor) was not suitable for CGTase production as only one impeller was immersed in the medium resulting in insufficient agitation (Fig. 4.3).

The growth of *E. aurantiacum* in 3 L fermentor with aspect ratio of 0.625 corresponding to a total medium volume of 1000 ml is illustrated in Fig. 4.4.

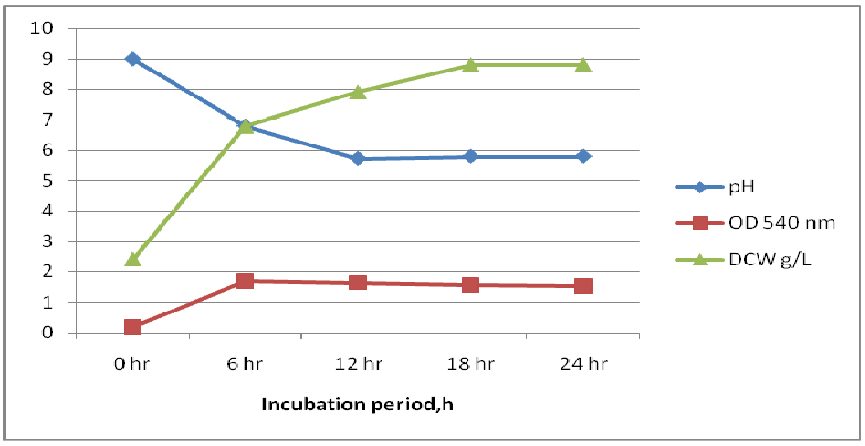


Fig. 4.4 : Growth of the culture in 3 L fermentor operated at aspect ratio of 0.625 (1000 ml working volume)

It was observed that the culture showed no lag phase and immediately entered exponential phase. The exponential phase lasted upto 6 h and thereafter the absorbance of the culture decreased. The pH decreased from 9 to 5.8 (Fig. 4.4). The CGTase activity increased rapidly with maximum activity of 5 U/ml at 12 h after which the activity decreased (Fig. 4.4).

The effect of aspect ratio 1.05 on CGTase production is presented in Fig. 4.5.

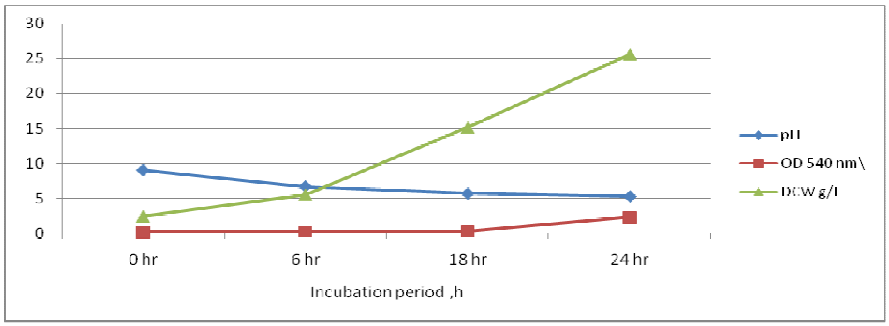


Fig. 4.5 : Growth of the culture in 3 L fermentor operated at aspect ratio 1.05 (1500 ml working volume)

This ratio corresponed to 1.5 L of medium in the fermentor. It was observed that the growth of the orgainsm was slow. There was a prolonged lag phase and the culture entered exponential phase only after 18 h. The pH ameliorated from 10 to 5 units within 24 h (Fig. 4.5). Maximum CGTase production was obtained after 24 h.

The effect of aspect ratio of 0.45, 0.625 and 1.04 on CGTase production is illustrated in Fig. 4.6.

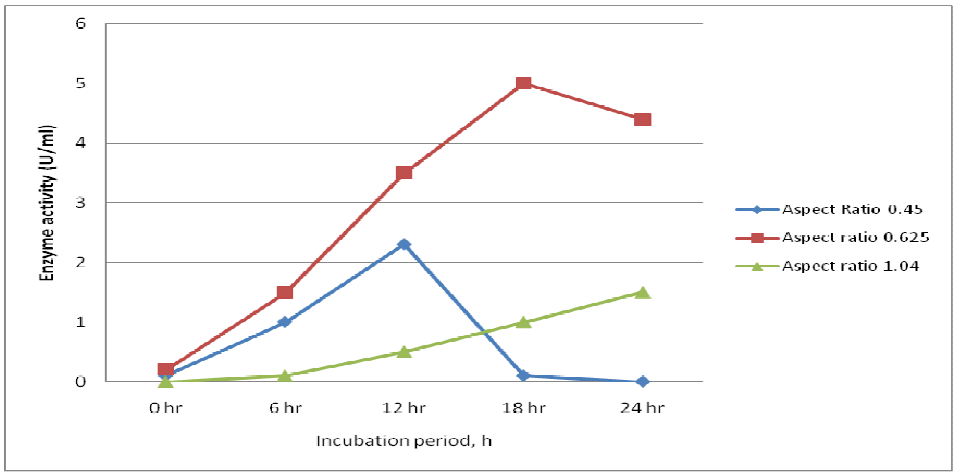


Fig. 4.6 : Effect of aspect ratios (0.45, 0.625, 1.04) on production of CGTase

When the working volume is kept minimum at 600 ml , the enzyme activity rises sharply and decreases sharply after 12 hr. Decrease in enzyme activity is due to decrease in pH which hampers the yield of the CGTase. It is evident from the above graph that maximum enzyme activity is obtained in 1000 ml working

volume. When the working volume is increased to 1500 ml, the enzyme activity rises steadily, however it is still lesser i.e. 1.5 U/ml after 24 hrs as compared to 5.0 U /ml obtained for 1000 ml working volume. This shows that aeration is highly essential for CGTase production and as the volume decreases (600 ml), amount of oxygen dissolved increases and the culture approaches the stationary phase very fast. However the production of CGTase is lesser which may be because of hastening of the log phase. There are very few reports on optimisation of aspect ratio for production of CGTase. The common aspect ratio used is 1.0 with a medium volume corresponding to atleast 2/3rd of total volume of fermentor. An aspect ratio of 0.6 i.e. working volume of 1000 ml in 3 L fermentor yields optimum CGTase production (Fig. 4.6).

4.3.3 Production of CGTase at different rates of agitation

Effect of agitation on CGTase production in 3 L fermentor by *E .aurantiacum* was studied. The temperature, pH, aeration and aspect ratio was maintained as 37°C, 10, 1vvm and 0.625 respectively. It was observed that increasing agitation also increases enzyme activity and microbial growth; however enzyme activity decreased after 100 rpm. This may be because of drop in pH which resulted due to increase in microbial biomass production. Effect of agitation on CGTase production was studied in shake flask during preliminary optimisation of process parameters by conventional method. It was observed then that a higher agitation rate (more than 250 rpm) caused froth formation in the flask, exhilarated growth, increased pigment production, rapid drop of pH and change in morphology of the organism visible under Light microscope. The morphology varied from cocci, coccobacilli and ruptured pleomorphic forms. This may be due to the shear caused by agitation. We have therefore resorted to choose agitation rates lesser than 200 rpm to avoid the above mentioned consequences. This result corroborated the result obtained by Techapun *et al.* (2003).

The effect of agitation set at 75 rpm on growth and enzyme production by *E. aurantiacum* is depicted in Fig. 4.7.

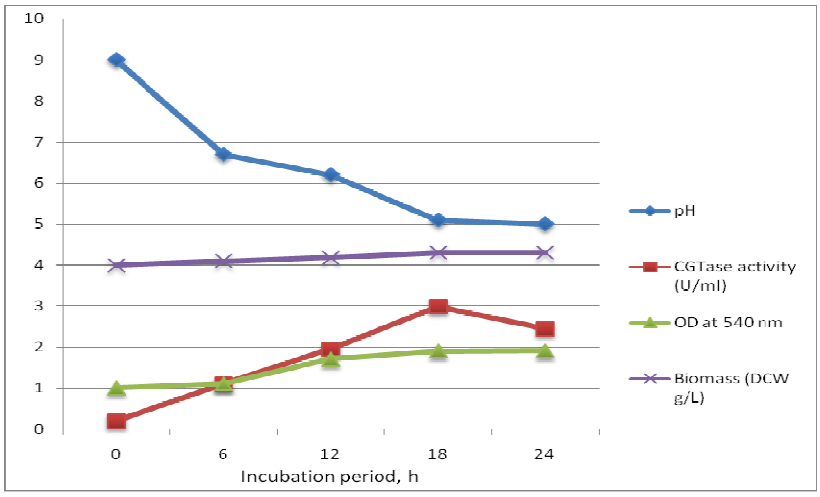


Fig. 4.7 : Effect of agitation set at 75 rpm on growth and production of CGTase by *E. aurantiacum*

It was observed that maximum CGTase activity of 3 U/ml was obtained at 18 h with maximum biomass production. The enzyme activity decreased after 18 h. The growth of the organism was comparatively slow with lesser enzyme activity as the pH dropped from 9 to 5 in 24 h at (Fig. 4.7). This rate of low agitation (75 rpm) is not suitable for CGTase production as it results in low yield of product.

The effect of agitation set at 100 rpm on growth and enzyme production by *E. auratiacum* is illustrated in Fig. 4.8.

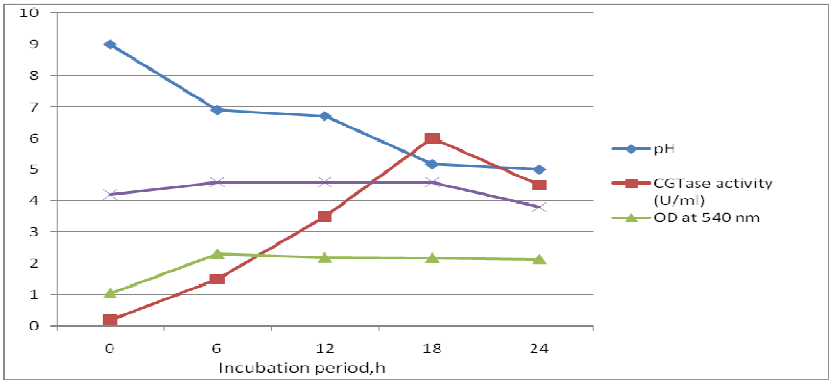


Fig. 4.8 : Effect of agitation set at 100 rpm on growth and production of CGTase by *E. aurantiacum*

It was observed that maximum CGTase activity of 6 U/ml was obtained at 18 h with maximum biomass production. The enzyme activity decreased to 4.5 U/ml after 18 h. The pH dropped from 9 to 5 in 24 h (Fig. 4.8), however higher enzyme activity (6 U/ml) was obtained at agitation rate of 100 rpm.

The effect of agitation set at 150 rpm on growth and enzyme production by *E. aurantiacum* is presented in Fig. 4.9.

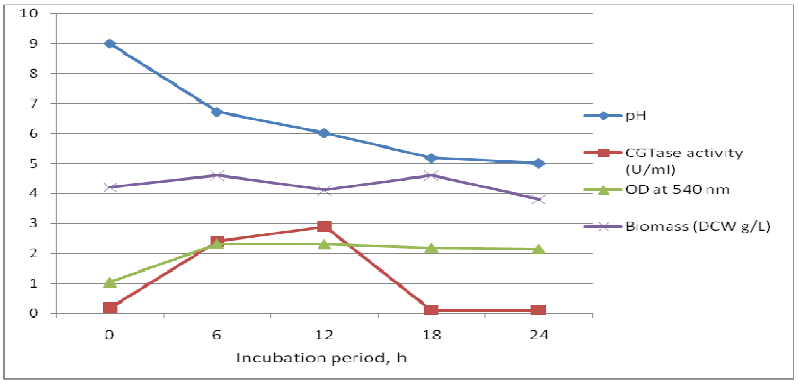


Fig. 4.9 : Effect of agitation set at 150 rpm on growth and production of CGTase by *E .aurantiacum*

It was observed that maximum CGTase activity of 3.1 U/ml was obtained rapidly at 12 h with maximum biomass production after which the enzyme activity decreased subsequently. The growth of the organism was faster however the log phase lasted only upto 6 h after which the culture entered into the stationary phase. The pH dropped from 9 to 5 in 24 h (Fig. 4.9). Though the enzyme activity reached its peak rapidly in 6 h, the yield of enzyme was lesser as compared to yield attained at 100 rpm.

The effect of agitation is summarised in Fig. 4.10.

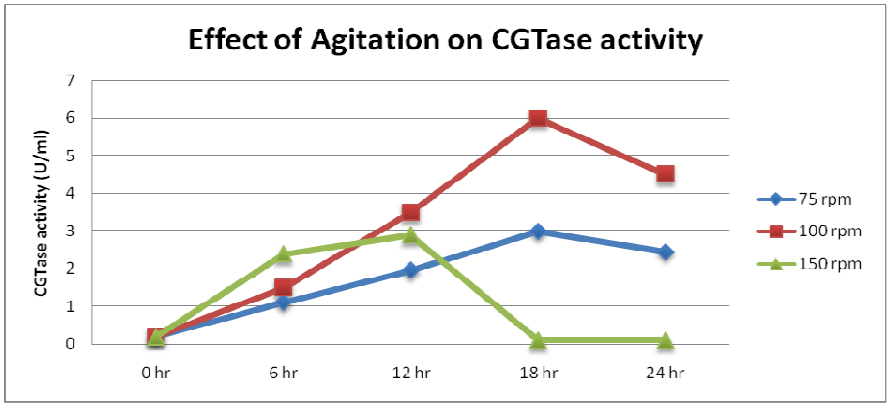


Fig. 4.10 : Effect of agitation on production of CGTase

Agitation demonstrated significant effect on enzyme activity. The enzyme activity was maximum at agitation of 100 rpm followed by 75 rpm and 150 rpm. The fastest production of enzyme was observed at 100 rpm. This may be due to rapid growth of the culture.

The optimum agitation of 100 rpm was selected for all further experiments in 3 L fermentor. Oxygen has diverse effects on enzyme production in aerobic fermentation processes by influencing metabolic pathways of bacteria. The dissolved oxygen (DO) concentration is a rate limiting factor in aerobic processes due to poor solubility of the oxygen in medium or due to high Oxygen Uptake Rate (OUR) of fast growing microorganisms. *E. auratiacum* is a highly aerobic and a fast growing organism. The Oxygen transfer rate (OTR) is thus an important parameter for CGTase production. This depends on agitation and aeration rate in bioreactors. Aeration and agitation are thus instrumental parameters responsible for productivity of the fermentation.

4.3.4 Production of CGTase at different aeration

The production of CGTase depends on growth of the bacteria which is governed by supply of oxygen. The availability of oxygen depends on the OTR and is controlled by the concentration of DO in the growth medium. The effect of aeration cannot be studied at shake flask level due to lack of spargers.

A typical ring sparger was used for introduction of sterile air in the fermentor. Air was regulated using rotatmeter and expressed as volumes of air per volume medium (vvm).

The effect of aeration on CGTase production and growth was observed by varying the aeration from 0.5 vvm to 1.5 vvm. Higher aeration was avoided as it may lead to faster growth of bacteria and insufficient CGTase production as observed previously in Fig. 4.10.

The effect of rate of aeration set as 0.5 vvm on growth and CGTase production is presented in Fig. 4.11.

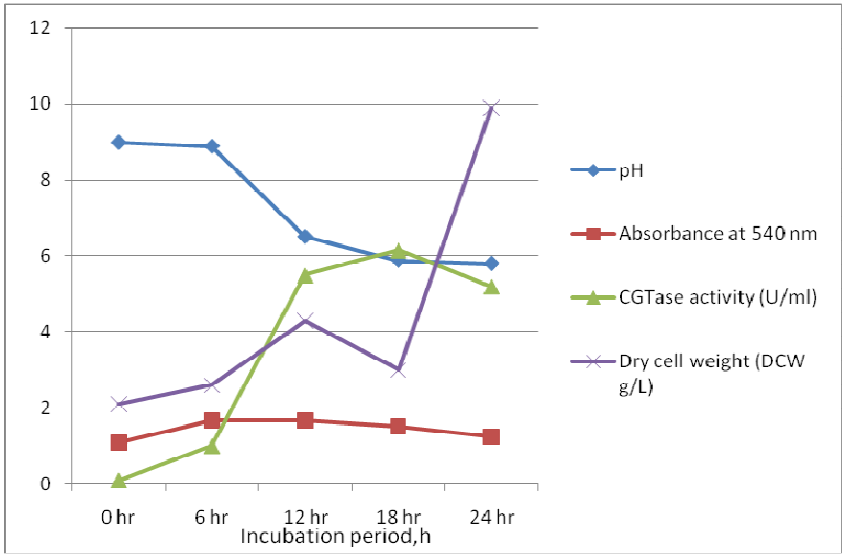


Fig. 4.11 : Effect of aeration set at 0.5 vvm rpm on growth and production of CGTase of *E. aurantiacum*

It was observed that maximum CGTase activity was observed at 18 h as 6 U/ml. The growth of the culture remained constant after 6 h. There was a distinct drop in pH from 9.5 to 5.9 (Fig. 4.11).

In most fermentor, the aeration is maintained at 1 vvm to 2 vvm. The effect of rate of aeration set as 1 vvm on growth and CGTase production is presented in Fig. 4.12.

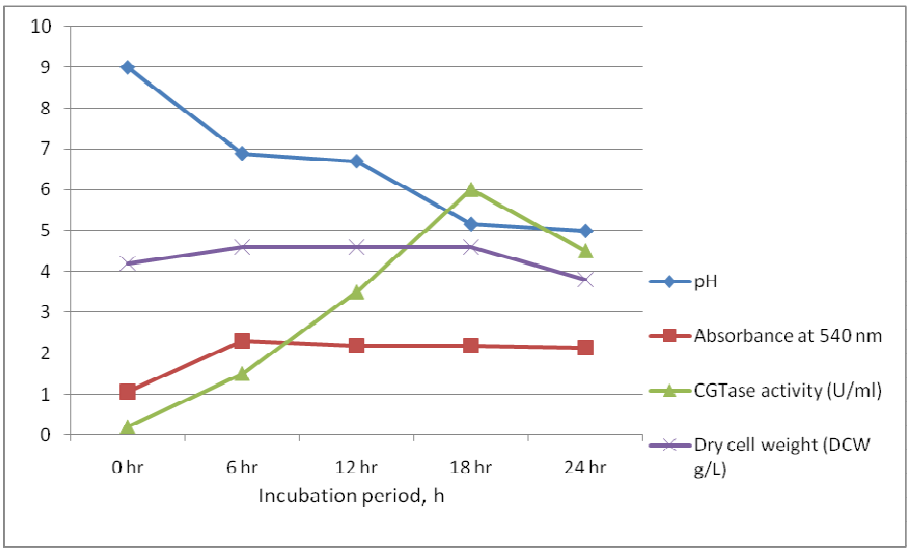


Fig. 4.12 : Effect of aeration set at 1 vvm rpm on growth and CGTase production of *E. aurantiacum*

Maximum CGTase activity was obtained at 18 h as 6.1 U/ml. The growth of the culture remained constant after 6 h. There was a distinct drop in pH from 9.0 to 5.0 (Fig. 4.12). There was very less variation in activity of CGTase when the organism was aerated at 0.5 vvm (6 U/ml) and 1 vvm (6.1 U/ml).

The effect of rate of aeration set as 1.5 vvm on growth and CGTase production is depicted in Fig. 4.13.

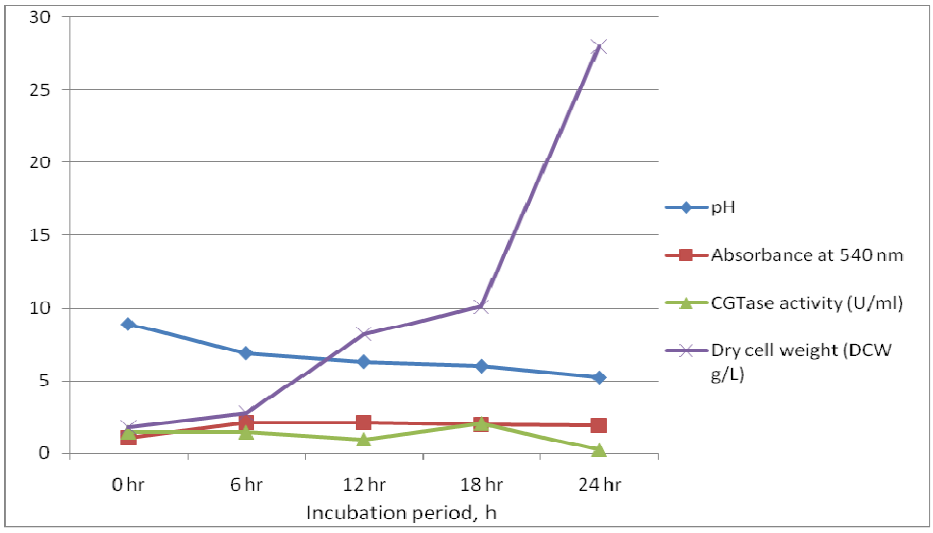


Fig. 4.13 : Effect of aeration set at 1.5 vvm rpm on growth and CGTase production of *E. aurantiacum*

Maximum CGTase activity was achieved at 18 h as 3.1 U/ml. As observed above, the growth of the culture remained constant after 6 h. There was a distinct drop in pH from 9.0 to 5.0 (Fig. 4.13).

Effect of aeration on CGTase production by *E. aurantiacum* was studied. It was observed that as aeration increased, the microbial growth increased proportionately. This shows that higher aeration rates favour growth of the organism. A higher rate of 2 vvm can also be experimented for the same. However, the enzyme activity did not rise proportionately with increased aeration. Enzyme activity was found to be 6 – 6.15 U/ml in 0.5 and 1 vvm. As the aeration was increased to 1.5 vvm, the enzyme activity dropped rapidly (Fig. 4.14).

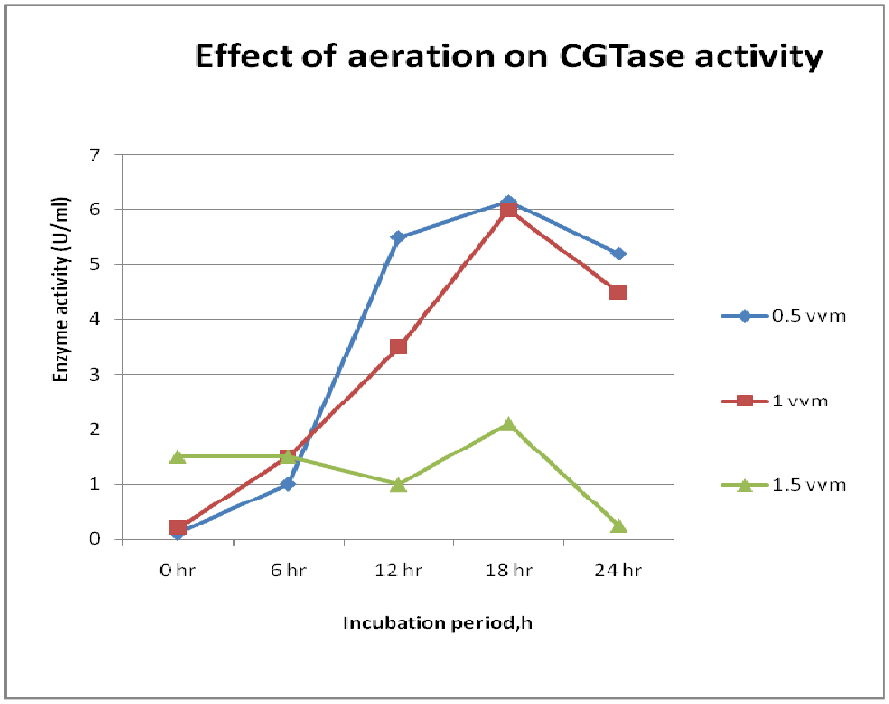


Fig. 4.14 : Effect of aeration on CGTase production by *E. aurantiacum*

The increase in aeration results in increase in microbial biomass but does not lead to a proportionate increase in enzyme activity. These results are in agreement with the results obtained by Techapun *et al.* (2003). Changes in pH were also studied. It was observed that the pH dropped rapidly with time. Since the organism is an alkaliphile, it may be possible that the activity of the enzyme is decreased with increase in acidic pH. The aeration of 0.5 to 1 vvm was found to be optimum in a 3 L fermentor (Fig. 4.14).

The residual carbon source and nitrogenous substrates in the broth after fermentation were estimated during optimisation of various parameters.

The changes in carbon: nitrogen content and effect on protein content produced during the optimisation of aspect ratio, aeration and agitation are summarized in Fig. 4.15.

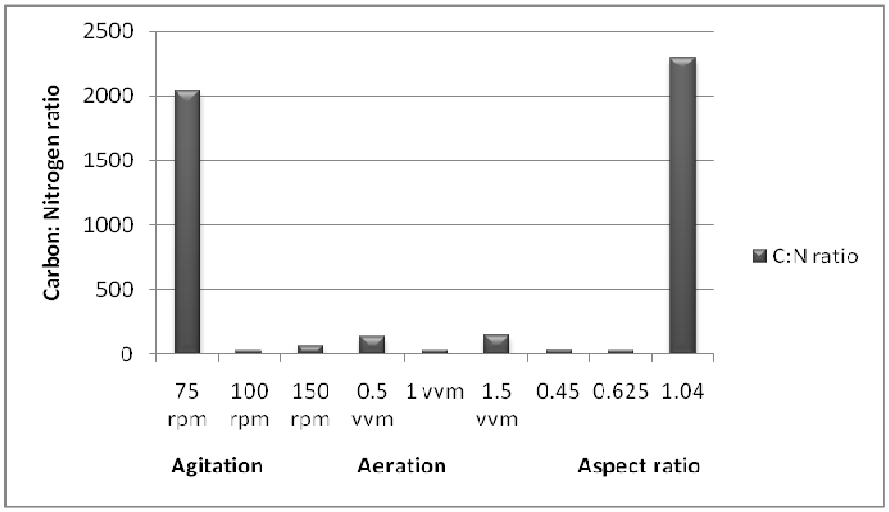


Fig. 4.15 : C: N ratio during optimisation of aspect ratio, aeration and agitation for production of CGTase in 3 L fermentor

The main purpose of this study was to remove the repressive effects of rapidly utilized starch, to reduce the viscosity of the medium, to reduce the effect of toxic medium constituents or simply to extend the product formation stage of the process for as long as possible. The residual starch was estimated and expressed as carbon content and the residual protein content was expressed as nitrogen content. As the product (CGTase) was a protein, it increased the nitrogen content after fermentation. Thus in spite of utilization of peptone and yeast extract, the nitrogen content was higher. There are very few reports on the study of effect of Carbon to Nitrogen (C/N) ratio on production of CGTase. Production of CGTase in batch processes show many limitations caused by substrate suppression, catabolite repression and limiting of some essential nutrients. Therefore, studying C/N ratio is critical to overcome these limitations in batch fermentation. When C/N ratio was reduced, the CGTase activity increased. These phenomena show that though both carbon and nitrogen source are critical factors for CGTase production, lower C/N ratio showed higher activity. It was observed that the least C: N ratio of 30 $\pm$ 0.5

was observed at aspect ratio 0.625, aeration 1vvm and agitation 100 rpm (Fig. 4.15). These optimum conditions were used for production of CGTase in the fermentor.

4.3.5 Production of CGTase in 3 L fermentor under optimised conditions

A fermentor with all the above optimised parameters was run using working volume as 1000 ml, aeration 1 vvm and agitation 100 rpm and is presented in Fig.4.16.

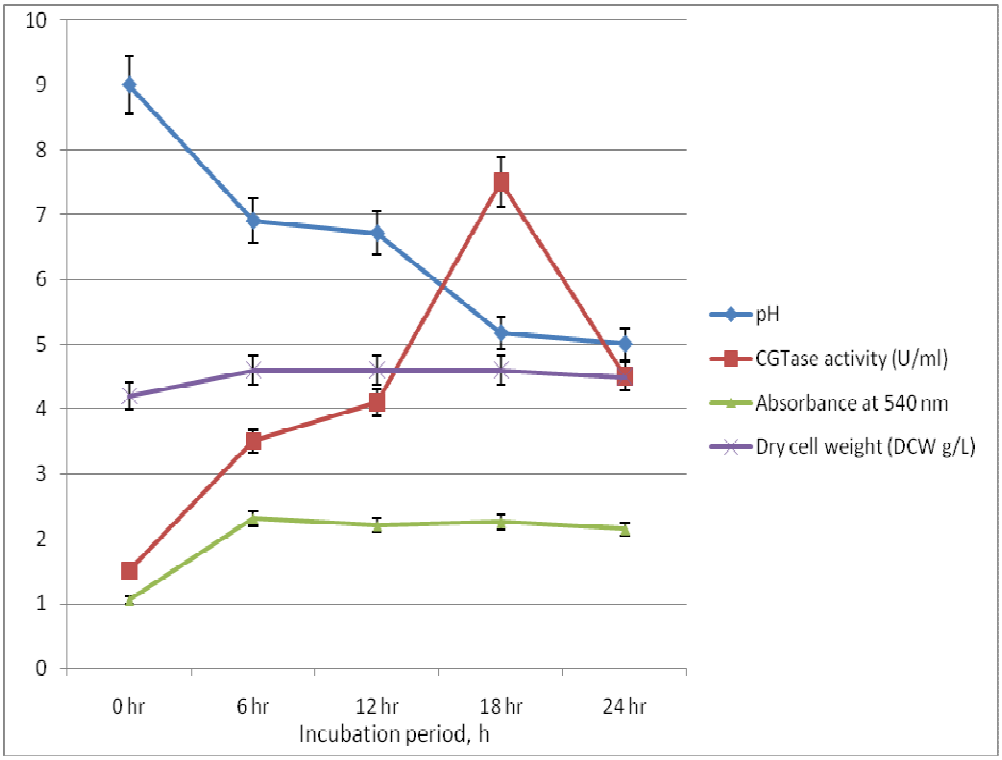


Fig. 4.16 : Time course of growth, change in pH and production of CGTase in a 3 L fermentor under optimised conditions

The enzyme activity obtained was 7.7 U /ml as compared to 6.15 U/ml which was obtained in shake flask fermentation using Taguchi Design of Experiments under optimised conditions. This showed that the yield of CGTase increased in the fermentor.

4.3.6 Growth kinetics of *E. auratiacum* in 3 L fermentor under optimum conditions

The typical microbial growth kinetics is as illustrated in Fig. 4.17.

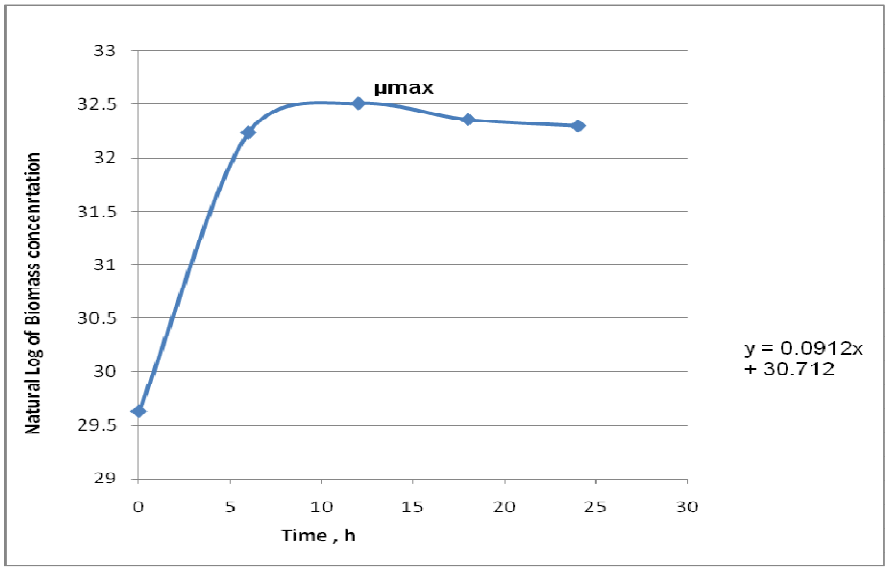


Fig. 4.17 : Microbial growth kinetics of *E. aurantiacum* in 3 L fermentor

The slope obtained in plot of Natural log (ln) of biomass concentration versus time was used to calculate specific growth rate (μ) (Fig. 4.17) using Monods equation, i.e. $\mu = \mu_{max}\ s / K_s + s$. $K_s = 2.71$

The growth rate and growth linked constants are summarized in Table 4.3.

Table 4.3 : Growth rate and growth linked constants calculated for production of CGTase by *E. aurantiacum* in 3 L fermentor under optimum conditions.

μ_{max} h^{-1}	K_s mg/dm^3	q_p	$Y_{p/x}$	$Y_{x/s}$	K_La h^{-1}	N_P	N_{FR}	Qp
2.8	2.71	7.4	875	0.795	0.276	2500	0.0005	32

Agitation speed 100 rpm; Temperature 37°C; pH 10 . qp: mg protein / g dry cell wt / h; $Y_{p/x:}$ enzyme units/ g dry cell wt; $Y_{x/s:}$ g dry cell wt/ g starch consumed; K_La : 0.138(1 vvm), 0.276(2 vvm) by empirical calculation ; $N_{P:}$ power number, N_{FR} : Froude number and Qp : enzyme units produced/L medium/h.

The growth rate of *E. aurantiacum* in 3 L fermentor was 0.09 h^{-1}, the maximum specific growth rate was 2.8 h^{-1} and substrate utilisation constant for soluble potato starch was 2.71. *E. coli* has a Ks (mg/dm^3) of 0.068 for glucose, *Saccharomyces* spp. has a Ks value of 25 for glucose while *Pseudomonas* spp. has a K_s value of 0.7 (Stanbury *et al.*, 1995). This K_s value of *E. aurantiacum* is higher than K_s of *E coli* for glucose which shows strong affinity of the culture for substrate.

4.3.8 Scale up production of CGTase using 14 L SS fermentor

The procedure involves stopping the supply of air to the fermentation which results in a linear decline in the dissolved oxygen concentration due to the respiration of the culture, as shown in Fig. 4.18. The slope of the line AB in Fig. 4.19 is a measure of the respiration rate of the culture. At point B the aeration is resumed and the dissolved oxygen concentration increases until it reaches steady state concentration C. Over the period, BC, the observed increase in dissolved oxygen concentration is the difference between the transfer of oxygen into solution and the uptake of oxygen by the respiring culture as expressed by the equation:

$$dCL/dt = K_La\ (C^* - CL)\ xQ_{O2}$$

where x is the concentration of biomass , Cl = DO concentration, C^* = Saturated DO concentration and Q_{O2} is the specific respiration rate (mmoles of oxygen g-l biomass h- I)

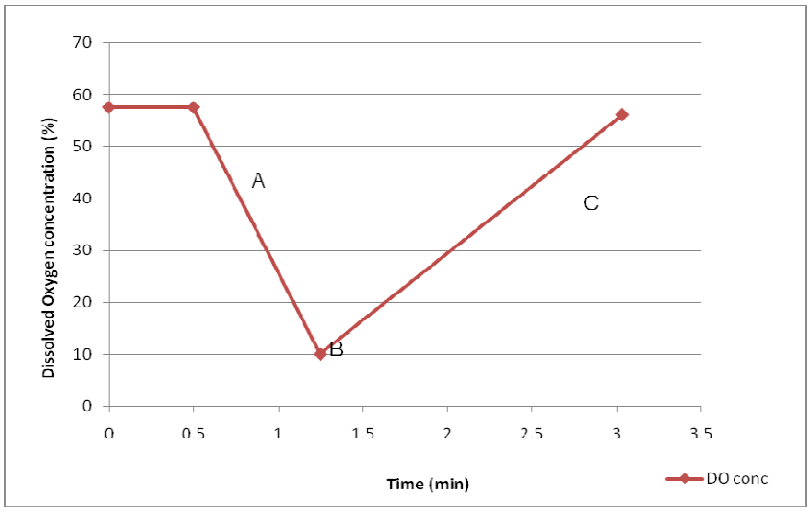


Fig. 4.18 : Dynamic gassing out for the determination of K_La values. Aeration was terminated at point A and recommenced at point B.

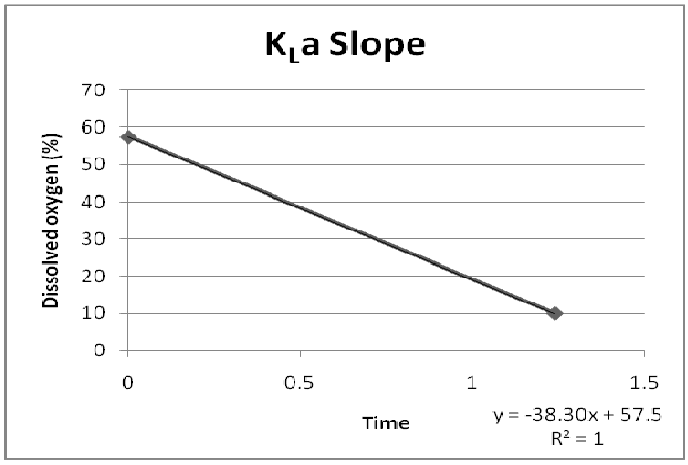


Fig. 4.19 : Line AB for calculation of slope, y = mx + c (m= slope = - 38.306). The term Q_{O2} is given by the slope of the line AB in Fig. 4.19.

The specific respiration rate is thus equal to slope of AB = 38.306 mmoles oxygen per gm biomass per hour. When aeration is switched off at time t = 0, OTR becomes 0 and falls according to the following equation, dCl/dt = - OUR (Rate of fall is equal to O_2 uptake rate).Then aeration is recommenced and Cl returns to original steady state value. k_La can be calculated using folloeing equation :

$$dCl/dt = OTR - OUR \quad or \quad dCl/dt = k_La\,(C^* - CL) - OUR$$

Integrating and taking natural logarithm of above equation for K_La, a plot of ln (C* - CL) versus time in minutes yields a straight line , the slope of which is k_La as presented in Fig. 4.20.

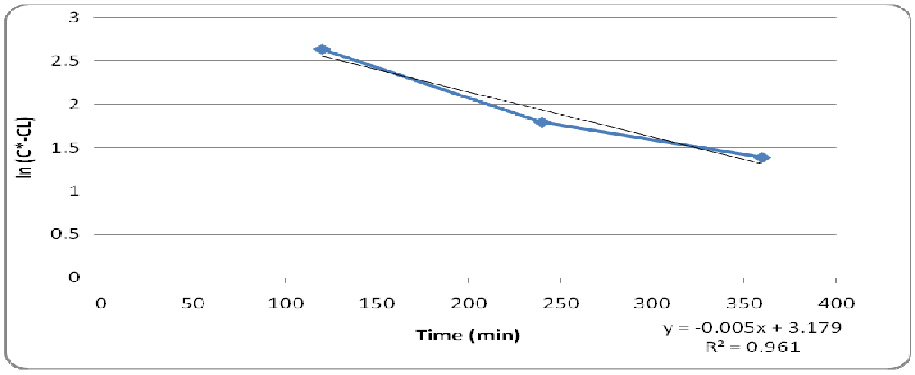


Fig. 4.20 : Plot of ln(C*- Cl) versus time for calculation of k_La by dynamic gassing out technique. Y = mx + c, Y= - 0.0052 x + 3.17, M= slope = -0.0052.

Thus , $K_La = 0.0052 \text{ X } 60 = 0.312\ h^{-1}$

The volumetric mass transfer coefficient for *E.aurantiucum* during production of CGTase in 14 L fermentor under optimum conditions was calculated as 0.312 h^{-1} using the dynamic gassing out technique. This technique is convenient in that the equations may be applied using dissolved oxygen tension rather than concentration because it is the rate of transfer and uptake that are being monitored so that the percentage saturation readings generated by the electrode may be used directly. It is also noted that the natural log term is dimensionless, which eliminates the need to calibrate the system for $[O_2]$. Any pair of points may be used to calculate k_La. Alternatively, the value for k_La may be determined from the slope of a line plotted from several pairs of data, as shown in Figure 4.20. This method has the significant advantage of smoothing the data and reducing measurement errors. We had predicted k_La in 3 L fermentor using Van Riets contant as 0.276 h^{-1} which is close to the value obtained for Kla in 14 L fermentor by Dynamic gassing out method (0.31 h^{-1}).

After initial optimisation in 3 L fermentor, the production was scaled up in 14 L SS stirred tank, in-situ sterilizable fermentor. The time course of growth and CGTase production in 14 L fermentor is presented in fig.4.21.

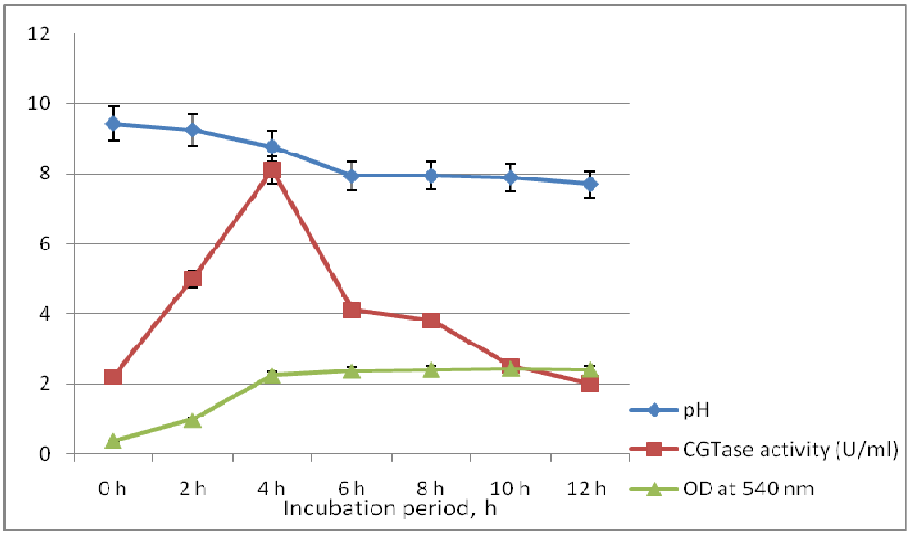


Fig. 4.21 : Time course of growth and CGTase production in 14 L fermentor

The organism shows rapid growth without a lag phase. This may be due to inoculation with a 16 h old culture. Initial amount of CGTase level at 0 h in fermentor is due to the presence of enzyme in inoculum medium which is same as

production medium. CGTase is known to be an extracellular enzyme and is produced in late log phase or early stationary phase. The maximum enzyme activity of *E .aurantiacum* reached after 4-5 h of growth when the organism enters the stationary phase. The enzyme activity remains constant until pH decreases after which there is loss in activity. Harvesting of cells for CGTase was thus performed after 4 h prior to the commencement of stationary phase. Rosso *et al.* (2002) have also reported a typical late – log production/secretion profile not associated with growth for *Bacillus circulans.* However some of the reports have indicated growth associated and/or biphasic profiles.

4.4 Discussion

Studies on scale up production of bioactive molecules are carried out to evaluate efficiency of the microbial strain and efficacy of the process for industrial production and eventually enhance the yield by optimizing process parameters. Production of enzyme in bioreactor is greatly influenced by aeration, agitation and geometry of the bioreactor.

Large scale production of enzymes like proteases, amylases and xylanases has been studied extensively. However there are only a few reports on scale up production of CGTase. Studies on CGTase production in 3-5 L fermentor are very limited (Gawande and Patkar, 1998; Savergeve *et al*., 2008). Most of the optimisation procedures are reported for shake flask (Mahat *et al*., 2004; Rosso *et al*., 2002; Bonilha *et al.,* 2006; Abdel Naby *et al*., 2011).

In the present investigation the production of CGTase using *E. aurantiacum* was scaled up to 3 L and then to 14 L SS fermentor. As the dimensions of a lab scale fermentor and a 250 ml flask are different, the aeration, agitation and medium: flask volume ratio (optimised in flask) expressed as aspect ratio in fermentor (H/D) were optimised in a Biotron 3 L fermentor. Optimum conditions in 3 L fermentor were a volume of 1000 ml, agitation of 100 rpm , aeration of 0.5 – 1 vvm and K_La as 0.312 h^{-1}. These results are in accordance to Savergave *et al*. (2008) and Gawande and Patkar (2001).

The production of CGTase is influenced by the growth rate of the microbial strain. The growth kinetics of *E. aurantiacum* was investigated using 3 L fermentor. The maximum specific growth rate of *E. aurantiacum* was found to be 2.8 h^{-1}. The Yp/x obtained is much higher as compared to the values obtained by Wong *et al*. (2008) and Ibrahim *et al*. (2010).

Production of CGTase was carried out in a 14 L fermentor with agitation at 200 rpm and aeration as 2 vvm with working volume 8 L in batch mode. Savergave *et al.* (2008) have produced CGTase from *Klebsiella* spp in fed batch mode with 10 L working volume in 14 L fermentor with a total yield of 6.8 U/ml after 33 h. In the present studies, maximum enzyme activity of 8.2 U/ml was obtained after 4-5 h of growth when *E. aurantiacum* reached late log phase- early stationary phase. *E. aurantiacum* as compared with other reports demonstrated rapid production of CGTase. This organism can thus be used as a potential industrial CGTase producer.

Rosso *et al*. (2002) have also reported a typical late – log production/secretion profile not associated with growth for *Bacillus circulans* in shake flask fermentation. However some of the reports have indicated growth associated and/or biphasic profiles .Most bacteria have been reported to produce CGTase during late stationary phase. Bacilleae show prolonged lag phase due to which the onset of CGTase production is only after 20 -24 h. *E. aurantiacum* produced CGTase in early stationary/ late log phase after 4 h of fermentation in the 14 L fermentor. An active log phase inoculum reduced the lag phase during production. *E. aurantiacum* synthesized CGTase during exponential phase.

It is believed that the enzyme is attached to the cell wall and then is released in the broth. Kabaivanova (1999) have reported that sporulation in *Bacillus* spp. triggers CGTase production. The literature suggests that CGTase production reaches peak within 16 to 20 hrs of incubation period (Jamuna *et al*., 1993). Makela *et al*. (1988) observed CGTase activity peak after a 40 hr lag period. Thatai *et al.* (1999) have produced 7.5 U/ml CGTase after 24 hr. In the present investigation, CGTase activity of 8.2 U/ml was obtained in 4-5 h in 14 L fermentor. To the best of our knowledge, this is the first report of scale up production of CGTase in a short fermentation period (4-5 h) using 14 L fermentor.

4.5 Conclusions

This chapter describes the production of CGTase by *E. aurantiacum* in 3 L and 14 L fermentor. Various parameters like aspect ratio, aeration and agitation were studied. The yield of CGTase was enhanced from 6.15 U/ml in 250 ml shake flask to 7.7 U/ml in 3 L and upto 8.2 U/ml in 14 L fermentor. The CGTase production was attained in 4 hours in 14 L fermentor which demonstrates that the production process is of a remarkably short time indicating its industrial potential. An optimised and rapid fermentative process for production of CGTase by *E. aurantiacum* was thus developed successfully using starch based medium.

Chapter 5

Purification and Characterisation of cyclodextrin glycosyl transferase produced by *Exiguobacterium aurantiacum*

5.3 Results

5.3.1 Production of enzyme

5.3.2 Ammonium sulphate precipitation and dialysis

5.3.3 Starch adsorption chromatography

5.3.4 Ion Exchange chromatography

5.3.5 Effect of pH on purified CGTase activity

5.3.6 Effect of pH on the stability of purified CGTase

5.3.7 Effect of temperature on purified CGTase activity

5.3.8 Effect of Temperature on the Stability of Purified CGTase

5.3.9 Effects of metal ions, inhibitors and detergents on purified CGTase

5.3.10 Study of kinetic parameters of purified CGTase

5.3.11 Determination of molecular weight of CGTase

5.3.12 Activity staining and phenolphthalein staining of gel

5.3.13 MALDI – TOF MS analysis

5.4 Discussion

5.5 Conclusions

5.1 Introduction

Purification of the enzyme becomes necessary for understanding properties of the enzyme and recommending its applications. There is a need to concentrate the enzyme before purification by chromatographic methods. The methods used for concentration include ultrafiltration, ammonium sulfate precipitation, solvent precipitation (ethanol, acetone) or reverse osmosis. Many scientists use a combination of various methods to concentrate the CGTase present in the supernatant of the culture fluid. Some researchers have reported the application of ultrafiltration, ammonium sulphate precipitation and starch adsorption purification of CGTase (Tachibana *et al.,* 1999 and Yagi *et al.,*1986). Purification of CGTase can be achieved by high resolution chromatographic methods to increase the purity of the enzyme. Different methods are used for purification of CGTase. Most of the methods are three step methods, i.e., ammonium sulphate precipitation, dialysis and affinity chromatography / adsorption chromatography / Ion exchange chromatography or Gel permeation chromatography.

Affinity chromatography is a method of separating biochemical mixtures and is based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand.

Many researchers have used affinity chromatography to purify their CGTases in line with other chromatographic methods. Affinity chromatography is commonly used in the purification of CGTase (Tachibana *et al.,* 1999; Larsen *et al,.* 1998; Volkova *et al.,* 2000; Bovetto *et al.,* 1992; Mori *et al.,* 1994 and Gawande and Patkar, 2001. Gawande & Patkar (2001) utilised an affinity matrix with gelatinized corn starch and purified a CGTase from *Klebsiella pneumoniae* with a 68% yield.

Some other researchers, such as Ferrarotti *et al.,* (1996) have even chosen affinity chromatography as their main chromatographic method to play a vital role in purifying their CGTases. The molecular weight of the enzyme can be estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE).

SDS- PAGE is a technique that separates proteins according to their electrophoretic mobility. SDS is a strong detergent which denatures proteins and renders a net negative charge to them. The electrophoretic mobilities of these

proteins will be a linear function of the logarithms of their molecular weights. Proteins are separated according to their mass and their molecular weight can be calculated using standard molecular weight markers. The bands of the desired protein is then eluted from the gel and then treated and analyzed using Matrix Assisted Laser Desorption Ionization –Time of Flight Mass Spectrophotometry (MALDI – TOF MS).

MALDI is a soft ionization technique used in mass spectrometry which allows the analysis of biomolecules that tend to be fragile and fragment when ionized by more conventional ionization methods. The MALDI is a two step process in which first, desorption is triggered by a UV laser beam. The matrix material consists of crystallized molecules of 3, 5-dimethoxy-4-hydroxycinnamic acid or α-cyano-4-hydroxycinnamic acid which heavily absorbs UV laser such as nitrogen lasers (337 nm). The matrix is desorbed, then ionized which in turn ionizes the peptide. The mass spectrophotometer *then* measures the mass-to-charge ratio (m/z) of charged peptide which generates a characteristic mass spectrum called peptide mass fingerprint. Computer programs like Mascot (Matrix Science Ltd., London) analyze the peptide mass fingerprint and predict the protein sequence based on sequence homology with existing peptides in database.

In this study, CGTase produced by *E .aurantiacum* was purified using ammonium sulphate precipitation, dialysis, starch adsorption chromatography and ion exchange chromatography. The molecular weight was estimated using SDS-PAGE and the partial sequence was obtained using MALDI – TOF MS and Mascot Peptide mass fingerprinting software.

5.2 Materials and Methods

5.2.1 Production of CGTase in 3 L fermentor under ptimized conditions

The medium used for production was the medium ptimized by Taguchi Design of experiments. One 250 ml flask containing 100 ml nutrient broth pH 10 was inoculated with 16 h old culture of *E. aurantiacum* and incubated at 37°C at 150 rpm for 16 hours. The flask culture was used as seed medium to inoculate 900 ml production medium containing (g/L) soluble starch 10, peptone 5, yeast extract 1

and $MgSO_4$.$7H_20$ 0.02 . The pH of the medium was adjusted to 10 using sterile 10 % Na_2CO_3. Fermentation was carried out at 37°C for 24 h with 1 L working volume with air flow rate of 0.5 vvm (volume of air per unit volume of medium per minute) and agitation was 100 rpm. Fermentation was carried out in batch mode till stationary phase approached. After fermentation, the cells were centrifuged at 10,000 g for 20 min at 4°C. Cell free supernatant was used as crude enzyme.

5.2.2 Enzyme assay and protein estimation

The CGTase activity was estimated using the modified method of Goel and Nene (1995) as described in section 2.2.3. The protein content was determined using bovine serum albumin as standard by the method described by Bradford (1976).

5.2.3 Ammonium sulphate precipitation and Dialysis of CGTase

Ammonium sulphate was added to five flasks containing 100 ml of the crude enzyme to obtain 40, 50, 60, 70& 80% saturation and incubated overnight at 4^oC. After 24 h, the aliquots were centrifuged at 10,000 g for 15 min at 4^0C and the weight of each precipitate was recorded. A graph of weight of the precipitate against percentage concentration of ammonium sulphate was plotted and the optimum ammonium sulphate concentration for CGTase was estimated from the graph. The precipitate was dissolved in 50 mM Tris buffer pH 8.5. It was added in the pre treated dialysis bag and dialysed against Tris buffer. The buffer was changed every 2- 3 hrs. Dialysis was performed for a minimum period of 6 hours or overnight at 25^oC. The enzyme activity and specific activity of the dialysate was estimated. The dialysate was stored at 4^oC until further use. The dialysis bag was pre treated by boiling in a solution of 2% sodium bicarbonate containing 1mM EDTA.

5.2.4 Starch adsorption chromatography

Starch adsorption column was prepared by gently heating 10 % (w/v) soluble starch in 50 mM Tris buffer. The gel was poured in an alcohol rinsed glass tube and allowed to set overnight. The column was washed and equilibrated with wash buffer containing Tris Cl buffer pH 7.6 with 1mM $CaCl_2$. The absorbance of the wash was measured at 280 nm (A*280*) till a constant baseline or readings less than

0.002 was obtained. Two ml of dialysate was then loaded in the equilibrated starch adsorption column. The wash buffer was added and 1 ml fractions were collected. After obtaining the baseline, the elution buffer containing 50 mM Tris Cl pH 7.6 and 1 mm β-cyclodextrin was added. 1 ml fractions were collected and the absorbance at 280 nm and enzyme activity of the fractions was estimated. The fractions showing enzyme activity were pooled and stored as adsorption purified enzyme at 4^{o}C until further use.

5.2.5 Ion Exchange chromatography

For purification of CGTase using ion exchange chromatography, 500 µl of adsorption purified enzyme was loaded in a previously equilibrated DEAE-Sepharose (Sigma) column. The column was washed with 50 mM Tris Cl buffer pH 7.6 with 0.1 M NaCl. The enzyme was eluted with 50 mM Tris Cl buffer pH 7.6 with 0.2 M NaCl. One ml fractions were collected, their absorbance was measured at 280 nm and their enzyme activity was estimated. The fractions showing enzyme activity were pooled and stored as ion exchange purified enzyme at 4^{o}C until further use.

The enzyme activity, protein content and specific activity of the crude enzyme, dialysate, starch adsorption purified enzyme and Ion exchange purified enzyme was calculated.

The adsorption chromatography and ion exchange chromatography purified enzyme was used for characterisation of CGTase.

5.2.6 Effect of pH on purified CGTase activity

The optimum pH for the purified enzyme was determined by replacing 50 mM Tris Cl buffer pH 8.0 in the CGTase assay with the following buffers: 50 mM Phosphate buffer (pH 5 – 7) and 50 mM Tris buffer (pH 8 – 12). The reaction was carried out by incubating 1 % starch and 0.1 ml enzyme in respective buffer for 20 min at 60°C. The enzyme activity was estimated as described by Goel and Nene (1995). A graph of enzyme activity versus pH profile was plotted.

5.2.7 Effect of pH on the stability of purified CGTase

The pH stability of the enzyme was measured by incubating 0.1 ml of purified CGTase enzyme with 1 ml of 50 mM Phosphate buffer (pH 5 – 7) and 50mM Tris buffer (pH 8 – 12) at 60°C, without substrate for 30 minutes. The residual activity of the enzyme was assayed as described in section 2.2.3 by adding 0.1 ml of the enzyme mixture to 1 ml of 1 % soluble starch incubated at 60^{o}C for 20 minutes.

5.2.8 Effect of temperature on purified CGTase activity

The optimum temperature for the purified recombinant CGTase enzyme was determined by incubating the reaction mixture of CGTase assay in 50 mM Tris buffer pH 8.0, at different temperatures, ranging from 10^{o}C – 100^{o}C for 20 minutes. The CGTase activity was assayed as mentioned in section 2.2.3.

5.2.9 Effect of temperature on the stability of purified CGTase

The temperature stability of the enzyme was measured by incubating 0.1 ml of purified CGTase enzyme in 50 mM Tris buffer pH 8.0 without substrate at different temperatures (10^{o}C – 100^{o}C) for 30 minutes. Standard CGTase assay was performed to determine the residual activity. The graph of residual CGTase activity versus temperature was plotted.

5.2.10 Effects of metal ions and inhibitors on purified CGTase activity

To study the effect of metal ions and inhibitors on CGTase activity, 0.1 ml of purified CGTase was added in 50 mM Tris buffer pH 8.0, containing different metals at 5 mM (final concentration), detergents at 10 % concentration and inhibitors at 1 mM (final concentration) and incubated for 10 minutes at 25^{o}C. The CGTase assay was performed to determine the residual activity of the enzyme.

5.2.11 Study of Kinetic Parameters of enzyme

The Michales constant (K_m) and reaction rate (V_{max}) values for the pure enzyme were determined by incubating 0.1 ml of purified CGTase enzyme in 1 ml of 50 mM Tris buffer at various concentrations of soluble starch solution, ranging from 2 mg/ml to 20 mg/ml at 60^{o}C for 10 minutes. The values of K_m and V_{max} were then

determined using Sigma plot enzyme kinetics software. The turnover number (K_{cat}) was calculated using V_{max} value.

5.2.12 Stability of CGTase at 4°C (shelf life studies)

The purified enzyme was stored at 4°C and the residual activity was estimated every month.

5.2.13 Molecular weight determination of CGTase using SDS-PAGE

The enzyme for Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS- PAGE) was concentrated using acetone precipitation method. This method irreversibly denatures and concentrates proteins for SDS –PAGE (Smith et al, 1985). For acetone precipitation of the enzyme, 1.2 ml of ice cold chilled acetone was added to 0.2 ml of purified CGTase, vortexed for 5 min and incubated overnight in ice at 4°C. After incubation, the sample was centrifuged at 10,000 g for 15 min at 4°C to obtain the enzyme precipitate. The sample for SDS PAGE was prepared by adding 30 µ SDS- PAGE sample buffer to the acetone precipitate and boiling in boiling water bath for 10 min.

The SDS-PAGE was performed as using the vertical electrophoresis unit described by Laemmli (1970). The plates were wiped with alcohol and the cassette was assembled and sealed with 1 % agarose. The resolving gel mix (Table 5.1) was added in cassette and overlayed with water saturated isobutanol. After 40 minutes of polymerisation, the stacking gel mix (Table 5.1) was overlayed over the resolving gel and the comb was inserted. After 20 minutes, the comb was removed and the wells were washed with distilled water. For electrophoresis, 20 µl of sample was loaded in the well and electrophoresed in Tris Glycine electrophoresis buffer at 100 volts for 1 h. After electrophoresis, the gel was removed from the cassette and stained with Commassie brilliant blue R- 250 for 1 -2 hr and destained using destaining solution till completely decolorised.

The gel was observed using Syngene gel documentation system UK and molecular weight was estimated using molecular weight standards of 6500 – 97400 Da (Bangalore Genei) and G-BOX Syngene software. The bands corresponding to CGTase were excised from the gel and used for MALDI- TOF MS analysis.

Table 5.1 : Composition of Stacking and Resolving gel used for SDS – PAGE

S. No	Component	Resolving gel mix Volume (ml) / 10 ml gel mould (12 %)	Stacking gel Mix Volume/ 4 ml gel mould
1	Water	3.3	2.7
2	Acrylamide mix (30 % T, 2.6 % C)	4.0	0.67
3	1.5 M Tris Cl Resolving buffer pH 8.8	2.5	-
4	1.0 M Stacking gel buffer pH 6.8	-	0.5
5	10 % SDS	0.1	0.04
6	10 % Ammonium persulphate	0.1	0.04
7	TEMED	0.004	0.004

5.2.14 Activity staining of CGTase

For activity staining, native PAGE was performed with 10% polyacrylamide according to the method described by Davis (1964). After electrophoresis, the gel was washed in 0.02 M acetate buffer pH 5.5 containing 3 % soluble starch and then incubated at 40°C for 2 hr. After enzymatic reaction the excess starch was washed off from the gel surface using acetate buffer and the gel was stained with a solution containing 3 % potassium iodide and 1.3 % iodine (Jeang *et al.,* 1999).

5.2.15 Detection of CGTase in phenolphthalein indicator gel

For detection of CGTase by the phenolphthalein indicator method, the enzyme was separated using 10 % native PAGE as described above. After electrophoresis, the gel was removed and indicator gel was uniformly poured over the polyacrylamide gel and was allowed to solidify**.** The indicator gel was prepared by mixing 0.24 g soluble starch, 0.14 g agar (prepared in 0.2 M phosphate buffer pH 8) and 0.5 ml of 0.4% phenolphthalein at 50°C. After incubation at 37°C for ten minutes, the indicator gel was flooded with a 0.1% (w/v) sodium carbonate solution and observed for CGTase activity (Pakzad *et al.,* 2005).

5.2.16 Analysis of CGTase using MALDI –MS

The band corresponding to CGTase was excised from the gel and transferred to a 0.5 µl microfuge tube. It was washed with 50 mM NH_4HCO_3/acetonitrile 1+1 (v/v) for 15 min till the gel particles shrink. The gel pieces are retreated with 50 mM NH_4HCO_3 and equal volume of acetonitrile (HPLC grade) and incubated for 15 min. After appropriate incubation, the acetonitrile is removed and the gel particles are dried. The dried gel particles are reduced for 45 min at 56°C by addition of freshly prepared 10 mM dithiotreitol (in 50 mM NH_4HCO_3). After incubation, the excess liquid is removed and replaced with freshly prepared 55 mM iodoacetamide (in 50 mM NH_4HCO_3). After incubation for 30 min at room temperature in the dark, the gel particles are washed with 50 mM NH_4HCO_3 and acetonitrile (1+1; v/v).

Freshly prepared trypsin solution (Sigma, Proteomics grade) (in 25 mM NH_4HCO_3) was added to the reduced and alkylated gel and incubated at 37°C for 30 minutes. The excess enzyme solution was removed and 25 mM NH_4HCO_3 (approx. 2-3µl) was added to keep the gel moist. The peptides from the gel were extracted by addition of extraction buffer (50/50 trifluoroacetic acid and 0.1% acetonitrile) in the microfuge tube containing the treated gel.

After extraction, the sample was mixed with crystallized 3, 5- dimethoxy-4-hydroxy cinammic acid and spotted on a MALDI plate. The solvents vaporize leaving recrystallised matrix. The matrix and analyte (CGTase from the gel) were co-crystallised on the spot. The plate was analyzed on a MALDI- TOF MS analyser (Bruker Daltonics GmBh) according to manufacturers instructions. The peaks and the peptide mass generated by the mass spectrophotometer was analyzed using Mascot search engine (Matrix Science) that uses mass spectrometry data to identify proteins from primary sequence databases. The primary sequence was also analysed using BLASTp (Basic Local Assignment Search Tool for proteins, NCBI).

5.3 Results

5.3.1 Enzyme production

The CGTase production increased with incubation period (Fig. 5.1). It was optimum at 18 h and activity decreased after stationary phase. The optical density increased with time and was maximum at 6h.

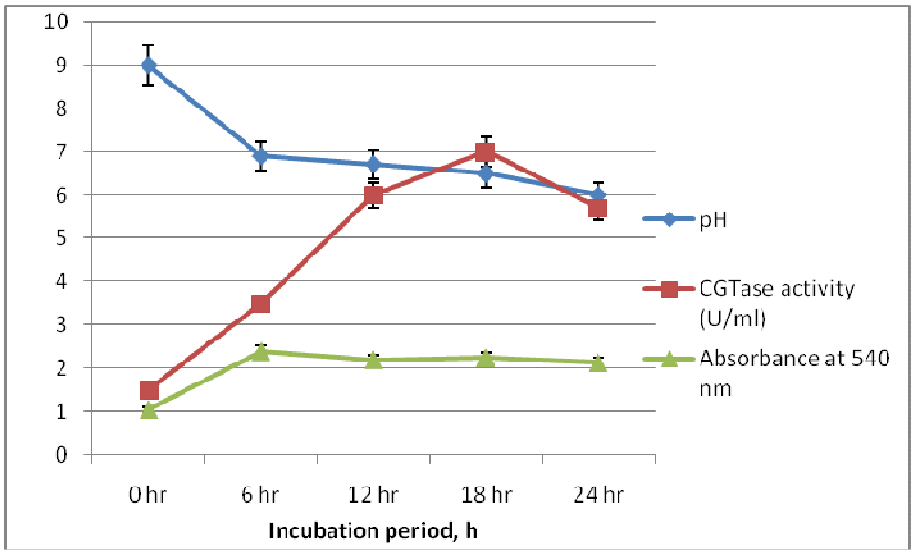


Fig. 5.1 : Time course of CGTase production by *E. aurantiacum* using soluble starch as a substrate

After appropriate incubation, the broth was centrifuged and the cell free supernatant was used for ammonium sulphate precipitation.

5.3.2 Ammonium sulphate precipitation and dialysis

The optimum concentration of ammonium sulphate required for maximum yield of CGTase was found to be 70 % based on the weight of the precipitate (Fig. 5.2). Some researchers recommend addition of 80 % concentration of ammonium sulphate for precipitation of CGTase (Tachibana *et al.*, 1999).

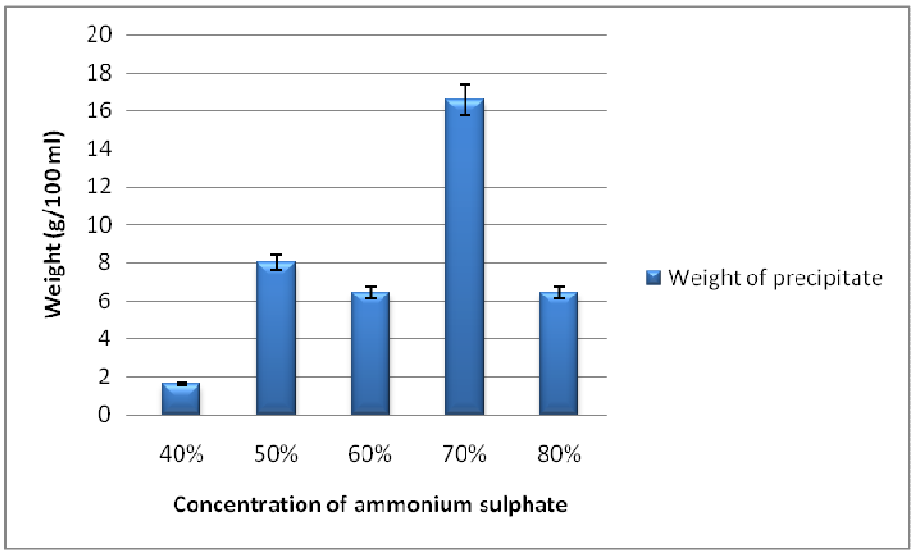


Fig. 5.2 : Optimization of ammonium sulphate concentration for precipitation of CGTase

When the concentration of ammonium sulphate increases, the solubility of CGTase decreases and the enzyme precipitates due to a process called salting out. The salts and other contaminants were eliminated from the enzyme precipitate using a dialysis membrane. The dialysate was loaded in a starch adsorption column.

5.3.3 Starch adsorption chromatography

The enzyme was purified by starch adsorption chromatography. It was observed that the enzyme started to elute at the fourth fraction with enzyme activity of 8 U/ml and reached its peak at the tenth fraction with 24 U/ml. The concentration then decreased subsequently to 0.5 U/ml at the fourteenth fraction. All the ten fractions (No. 4 to 14) which demonstrated CGTase activity were pooled and stored at 4°C as adsorption purified CGTase. The absorbance at 280 nm increased reaching a peak at fraction no. 9 and 10 corresponding to the eluants containing maximum enzyme activity (Fig. 5.3).

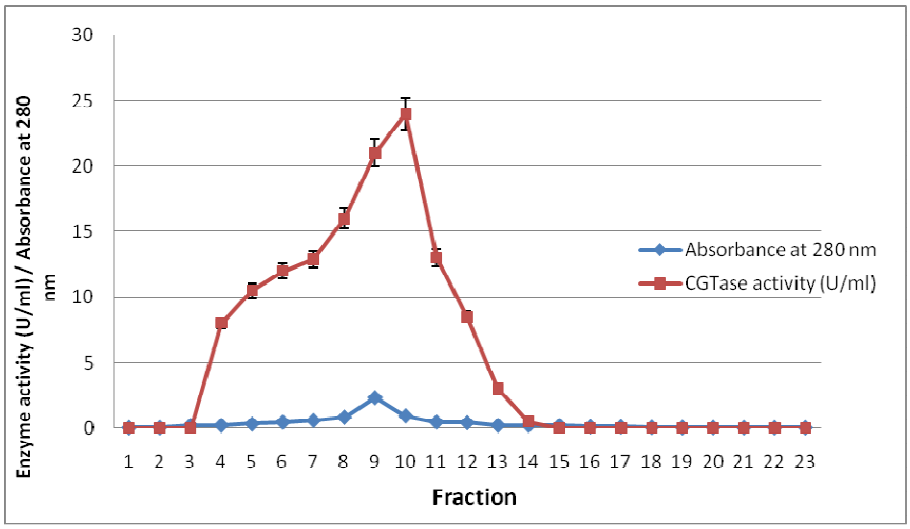


Fig. 5.3 : Chromatographic profile of CGTase produced by *E. aurantiacum* on Starch adsorption column

CGTase is generally purified by two steps purification procedures which were also used by Volkova *et al.* (2000) to obtain pure CGTase from *Bacillus* sp. 1070 by using β-CD Sepharose 4B chromatography and immobilized metal ion affinity chromatography. CGTase binds to cyclodextrin present in column. The presence of C-terminal raw starch binding motif on the CGTase plays an important role for binding the CGTase to CD affinity that is used in this column (Wind *et al.*, 1995). Other researchers also have suggested that raw starch binding domain in domain E were capable of binding strongly to cyclodextrins (Penninga *et al.,* 1996). Gawande and Patkar (1995) reported purification of a novel raw starch degrading cyclomaltodextrin glucanotransferase (CGTase; E.C. 2.4.1.19) produced by *Bacillus firmus* by ultrafiltration, affinity and gel filtration chromatography.

5.3.4 Ion Exchange chromatography

The enzyme purified by starch adsorption column was applied to a DEAE-Sepharose anion exchange column in a 5 ml syringe with 7 cm X 1.5 cm dimension filled with 4 ml of resin at a flow rate of 1ml/min. There was a complete loss of CGTase activity when the enzyme was purified via DEAE-sepharose. Two distinct peaks corresponding to protein fractions were observed at fraction 25 and 37. But both the fractions demonstrated absence of CGTase activity (Fig.5.4).

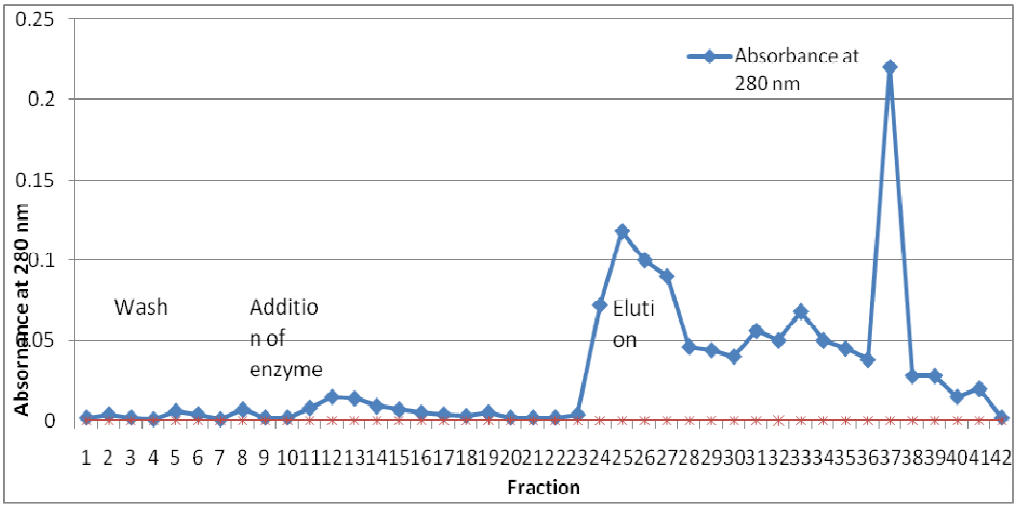


Fig. 5.4 : Chromatographic profile of CGTase produced by *E. aurantiacum* on DEAE-Sepharose column

Tachibana *et al.* (1999) have purified CGTase using ion exchange chromatography, hydrophobic interaction chromatography and affinity chromatography. Wind *et al*. (1998) purified amylase with CD producing activity from *Thermoanaerobacterium thermosulfurigenes* in a single step using affinity chromatography. Savergave *et al.* (2008) have also reported DEAE-sepharose as a efficient exchange column for CGTase. The enzyme activity and yield of the enzyme obtained by the two step purification method is presented in Table 5.2.

Table 5.2 : Purification of CGTase produced by *E. aurantiacum*

Fraction	**Protein mg/ml**	**Total Units**	**Specific Activity U/mg**	**Yield %**	**Fold Purification**
Crude enzyme	30	75	0.05	100	-
Dialysate	19	87	0.45	86.2	9
Starch Adsorption	17	144	1.263	52.08	25

CGTase was successfully purified using Starch Adsorption chromatography with 25 fold purification. Ion exchange chromatography was also used but there was no increase in enzyme activity. After repeated elutions, the enzyme was recovered from the pooled fractions, but there was loss in enzyme activity. The enzyme activity of affinity purified fraction was 24 U/ml. The affinity purified enzyme was

thus used as purified enzyme for the further studies on characterisation of CGTase. A two step method for CGTase purification is demonstrated in the present studies.

5.3.5 Effect of pH on purified CGTase activity

The effect of pH on activity of purified CGTase is illustrated in Fig. 5.5. The optimum pH for purified CGTase of *E. aurantiacum* was found to be 9 which is suitable for cyclisation and hence production of cyclodextrins.

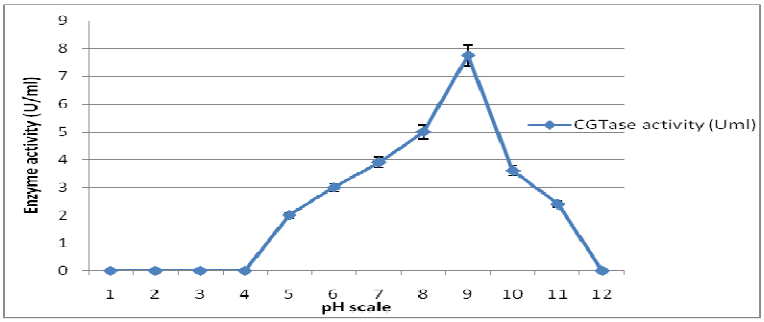


Fig. 5.5 : Effect of pH on activity of CGTase

Most of the reported purified CGTase exhibited optimum pH ranging from 5.0 to 8.0. Purified CGTase from *Bacillus stearothermophilus* (Ahn *et al*., 1990 and Tachibana *et al*., 1999) exhibited an optimum pH of 5.0 - 5.5 while Bovetto *et al.* (1992) found that the optimum pH for *Bacillus circulans* E 192 CGTase was pH 5.5. CGTase from recombinant *Brevibacillus brevis* CD162 (Myung *et al*., 1998) has an optimum pH of 8.0.

5.3.6 Effect of pH on the stability of purified CGTase

The effect of pH on stability of CGTase was studied (Fig. 5.6).

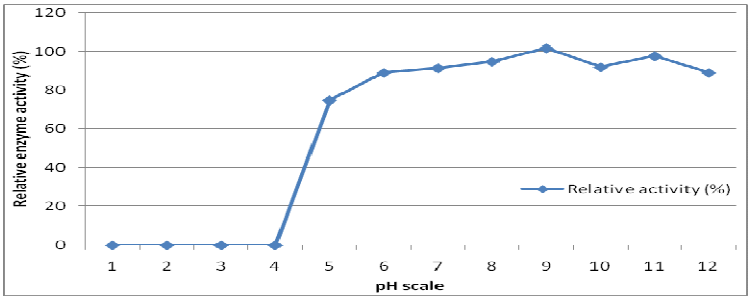


Fig. 5.6 : Effect of pH on stability of CGTase

E. aurantiacum CGTase was stable from pH 5 – 12, but demonstrated greater stability at alkaline pH scale). As alkaliphiles are preferred sources of CGTase, the enzyme is generally stable at alkaline pH.

5.3.7 Effect of temperature on purified CGTase activity

The results of effect of temperature on the activity of purified CGTase are presented in Fig. 5.7.

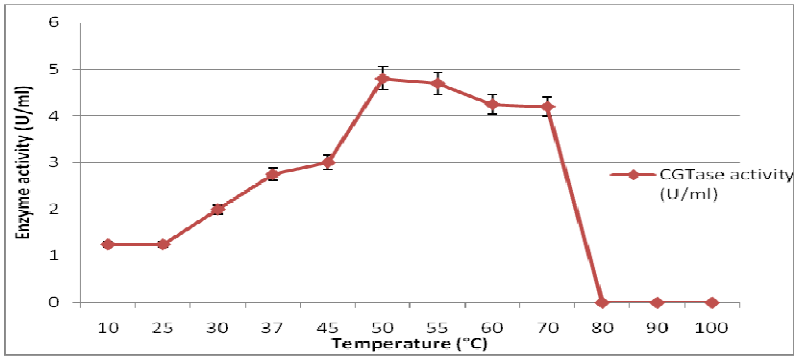


Fig. 5.7 : Effect of temperature of CGTase activity

Purified CGTase from *E. aurantiacum* had temperature optimum of 50 °C (Fig. 5.7). The optimum temperature of CGTase activity for most mesophiles and alkaliphiles is generally in the range of 30 – 40 °C. Likewise, CGTases from thermophiles show a higher optimum temperature for enzyme activity. *E. aurantiacum* is a mesophile with optimum growth temperature of 37 °C but the enzyme CGTase it produces is moderately thermostable. CGTase from *Thermoanaerobacterium thermosulfurigenes* EM1 (Wind *et al.*, 1998) and *Thermococcus kodakaraensis* KODI (Rashid *et al*., 2002) exhibited higher range of optimum temperatures of 90ºC to 100ºC, 80ºC to 85ºC and 80ºC respectively.

5.3.8 Effect of Temperature on the stability of purified CGTase

The results of effect of temperature on stability of purified CGTase are illustrated in Fig. 5.8. The enzyme was found to be stable in temperature range of 50 to 60°C.

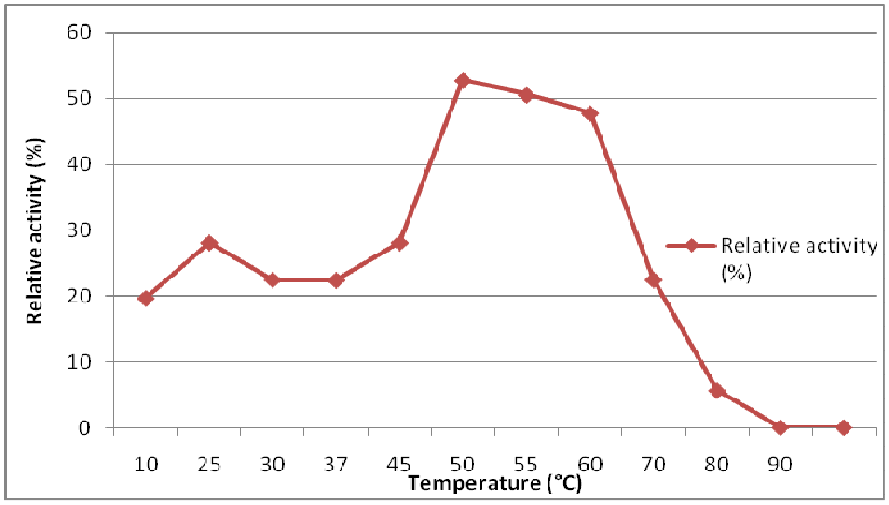


Fig. 5.8 : Effect of temperature on stability of CGTase

Generally CGTases from mesophiles like *Klebsiella pneumoniae* AS-22 (Gawande and Patkar, 2001) have optimum temperature range of 35-50°C. CGTase from *Bacillus clarkii* 7364 (Takada *et al*., 2003) and *Brevibacillus brevis* CD162 (Myung *et al*., 1998) which were stable up to 40ºC, 45ºC and 50ºC respectively. The stability studied revealed that *E. aurantiacum* was stable at 10°C upto six months.

5.3.9 Effects of metal ions, inhibitors and detergents on CGTase

The effect of metal ions, inhibitors and detergents on purified CGTase activity was studied and the results are presented in Fig. 5.9.

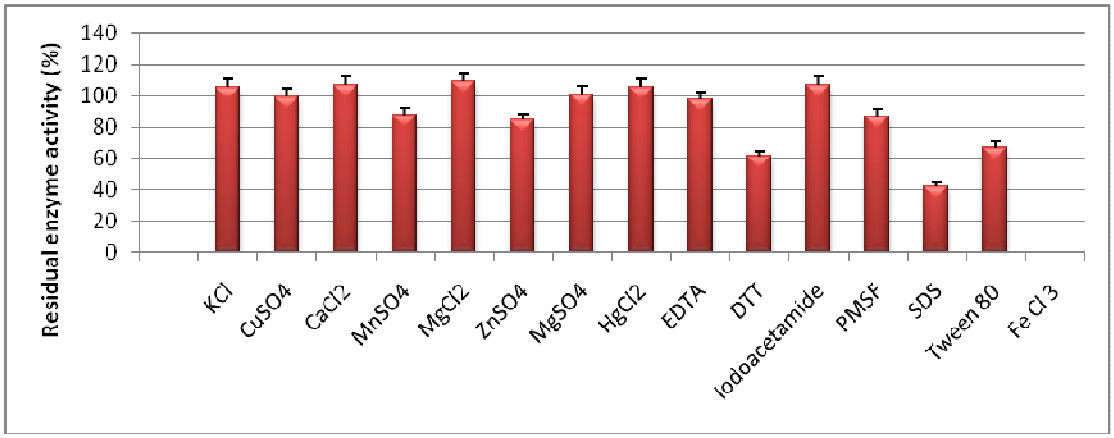


Fig. 5.9 : Effect of inhibitors and metal ions on CGTase activity

This enzyme was shown to be metal independent since it retains more than 90 % of activity with EDTA. This demonstrates that it is not a metallo- enzyme as no metal ions significantly enhances its activity. Additionally K^{+}, Mg^{+2} and Ca^{+2}, do not lower the CGTase activity either. But strong inhibition of CGTase activity was observed by Fe^{+3} metal ions with complete loss of activity which suggests the presence of histidine and tyrosine residues in the active center.

Cu^{+2} and Zn^{+2} neither decreased nor enhanced CGTase activity of *E. aurantiacum*. The majority of the CGTases are inhibited by Cu^{+2} and Zn^{+2} (Tonkova, 1998) including the recombinant CGTase of *Bacillus* sp. TS1-1. CGTase from *Brevibacterium* sp. No. 9605 (Mori *et al*., 1994), *Bacillus halophilus* INMIA-3849 (Abelian *et al.*, 2002), *Bacillus firmus* (Yim *et al.*, 1997), *Bacillus* sp. AL-6 (Fujita *et al*., 1990) and *Bacillus agaradhaerens* (Martins and Hatti-Kaul, 2002) were strongly inhibited by Zn^{2+}.

It is interesting to find out that K^{+} and Ca^{2+} in the form of KCl and $CaCl_2$ help to promote the CGTase activity. Previous studies show that CGTase from *Brevibacterium* sp. 9605 (Mori *et al.*, 1994), *Bacillus autolyticus* 11149 (Tomita *et al.*, 1993) and *Paenibacillus* sp. F8 (Larsen *et al.*, 1998) exhibited more stable activity in the presence of Ca^{2+}.

In the presence of Hg^{+2} ions CGTase retains 100% of its activity, that probably proves the absence of sulfur-containing amino acids in the active center of the enzyme. The detergents tween 80 and SDS caused partial loss of activity. Iodoacetamide binds covalently with the thiol group of cysteine so the protein cannot form disulfide bonds rendering the enzyme inactive. Iodoacetamide does not inhibit CGTase activity of *E. aurantiacum* suggesting absence of cysteine in active site. However DTT caused about 60 % decrease in activity suggesting presence of sulphide linkages in the enzyme which may be responsible for thermostability. Phenyl MethylSulfonyl Fluoride (PMSF) inhibits serine proteases specifically. There was decrease in activity but not a complete loss in activity due to PMSF, which suggest that serine may not be present in active site of *E. aurantiacum* CGTase.

5.3.10 Study of kinetic parameters of purified CGTase

The Michaelis-Menten constant (K_m) and the maximum forward velocity of the reaction (V_{max}) were determined by Sigmaplot® Enzyme Kinetics Module using the Lineweaver Burke plot (Fig.5.10).

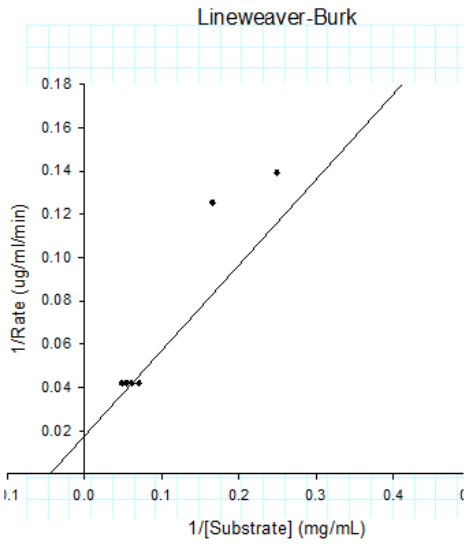


Fig. 5.10 : Lineweaver Burke plot for *E.aurantiacum* CGTase

The K_m and V_{max} value calculated by Sigmaplot enzyme kinetics module for *E. aurantiacum* CGTase was 0.022 mg/ml and 57 µg of β- cyclodextrin/ml/min which shows greater affinity for substrate. The Kcat value (turnover number) was calculated using V_{max} as 316.7 s^{-1}. CGTase from *Bacillus circulans* E 192 (Bovetto *et al.*, 1992) had a K_m of 5.7 mg/ml and CGTase from *Bacillus firmus* (Gawande *et al*., 1998) with its K_m equal to 1.21 mg/ml. Gawande and Patkar (2001) reported that the K_m value of *Klebsiella pneumoniae* AS-22 CGTase was 1.35 mg/ml, while Martins and Hatti-Kaul (2002) have estimated K_m of CGTase from *Bacillus agaradhaerens* to be 21.2 mg/ml.

5.3.11 Determination of molecular weight of CGTase

The CGTase purified by chromatography was precipitated using acetone. The concentrated precipitate was freeze dried and used for sample preparation. CGTase produced by *E. aurantiacum* was purified using a 12 % SDS- PAGE stained by

Coomassie brilliant blue (Fig.5.15). The gel was analyzed and molecular weight determined by using molecular weight standards of 6500 – 97400 Da (Bangalore Genei) and G-BOX Syngene software. A single band of purified CGTase (Fig.5.11 a, well 5) was observed suggesting the homogeneity of the purified enzyme.

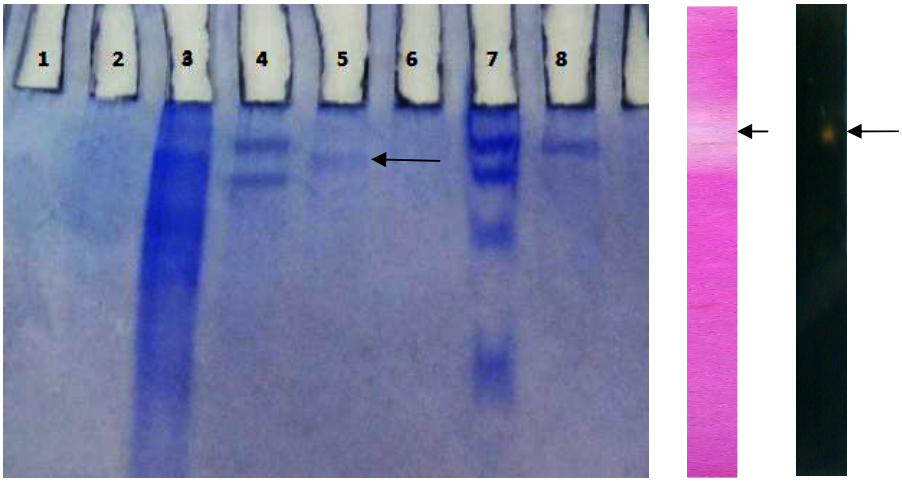


Fig: 5.11 a) Well 3: Crude CGTase, Well 4 : Dialysate, Well 5: Affinity purified CGTase (arrow), Well 7: Molecular weight markers, Well 8: Ion exchange purified fraction ; b) Activity staining of CGTase using iodine (arrow) and c) Staining of CGTase using phenolphthalein indicator (arrow).

Two separate bands were observed in the dialysate in well 4 (Fig. 5.11). A single band was obtained in well 5 which contained the starch adsorption purified CGTase demonstrating purity of the enzyme. A similar band is also observed in well 4 containing dialysate which confirms the presence of CGTase in dialysate. This band was eluted from the gel and further processed for MALDI-MS analysis. The band obtained in well 8 depicts the protein purified by ion exchange chromatography. There is complete loss in CGTase activity after the starch adsorption purified enzyme was subjected to ion exchange chromatography. A single band of approximately 90 kDa was purified by ion exchange. This band was also present in the dialysate. Moreover, it is known that most of the CGTase-secreting bacterial strains produce other amylolytic enzymes, such as alpha amylase or glucoamylase (MW = 90- 95 k Da) and these starch hydrolyzing enzyme may have been purified by ion exchange chromatography.

A single band in well 5 clearly confirms the presence of purified CGTase from *E. auratiacum* .The molecular weight was estimated to be 77.84 k Da by graph of Log molecular weight versus distance travelled (Rf) generated by analysis of SDS-PAGE by Syngene software. Most of the purified CGTases from various *Bacillus* species have Molecular Weight (MW) in the range of 68 kDa to 88 kDa. MW of CGTase from *Bacillus* sp. KC201 is 75kDa (Kitamo *et al*., 1992), of CGTase from *Brevibacillus brevis* CD162 is 75kDa (Myung *et al*., 1998) and of CGTase from *Bacillus* sp. HA3-3-2, is 68 kDa (Nomoto *et al.*, 1986). The MW of CGTase from *E. aurantiacum* is comparable to the MW of CGTases produced by other bacilli.

5.3.12 Activity staining and phenolphthalein staining of gel

The purified CGTase was subjected to non denaturing native gel electrophoresis and stained using iodine and phenolphthalein. The zymogram of CGTase demonstrated its starch hydrolytic activity which was clearly observed in iodine gel (Fig. 5.11, b) whereas a faint colorless band was observed in pink background of phenolphthalein gel (Fig. 5.11, c). The phenolphthalein indicator gel method differentiates CGTase from other starch hydrolytic enzymes.

5.3.13 MALDI – TOF MS analysis

The CGTase band was picked up from the 1-D SDS- PAGE gel, extracted, mixed with crystallized 3,5- dimethoxy-4-hydroxy cinammic acid and spotted on a MALDI plate. The plate was analyzed on a MALDI- TOF MS analyser (Bruker Daltonics GmBh) according to manufacturer's instructions and the peaks generated are shown in Fig.5.12.

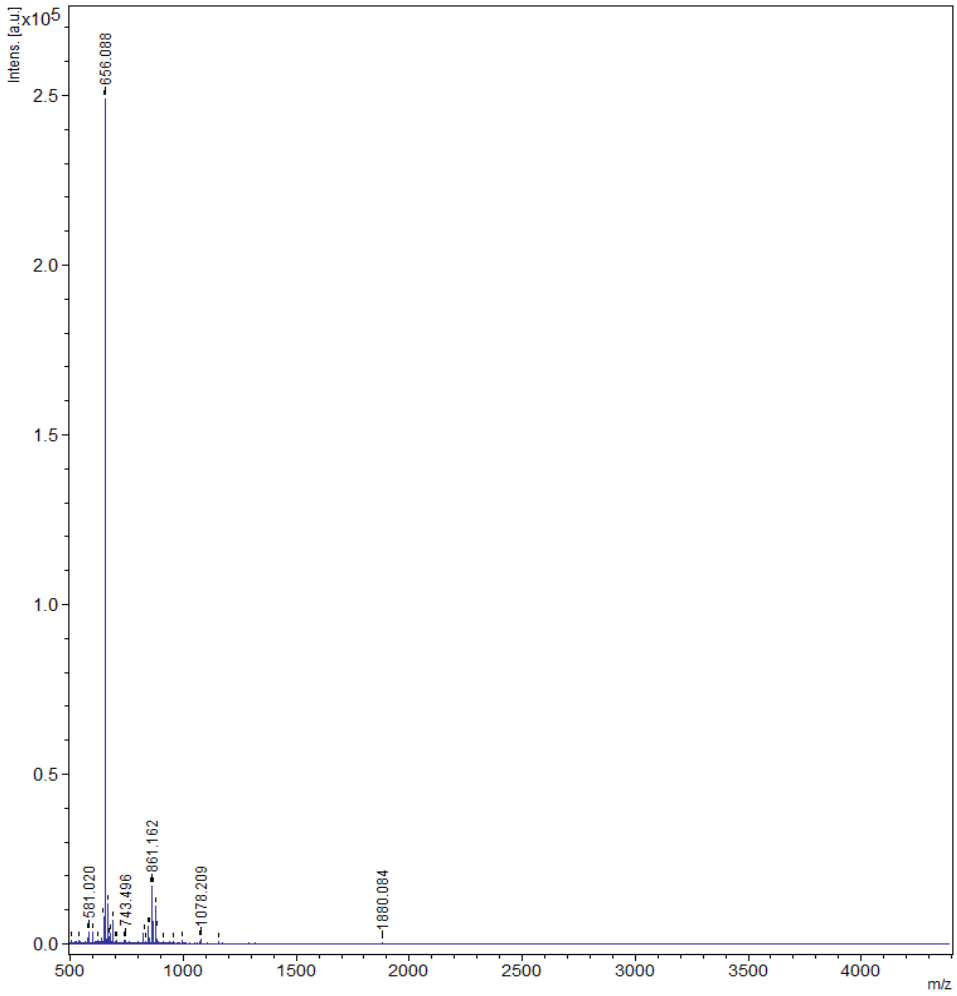


Fig. 5.12 : Graph of Atomic units vs m/z generated by MALDI- TOF MS analysis of purified CGTase

The peaks generated (Fig. 5.13) and the peptide mass was analyzed using Mascot search engine (Matrix Science) that uses mass spectrometry data to identify proteins from primary sequence databases. The primary sequence was also analysed using BLASTp (NCBI).

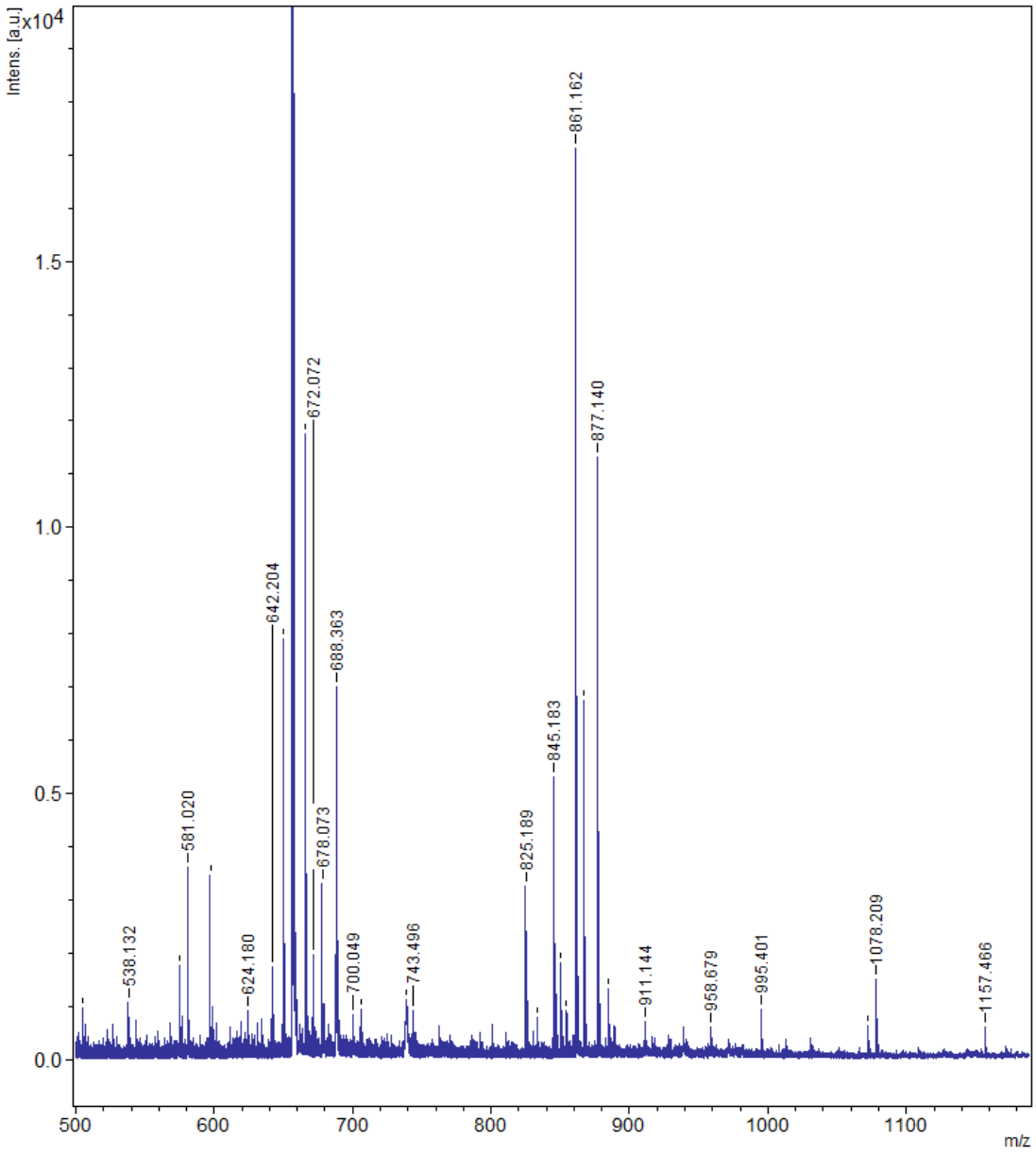


Fig. 5.13 : Spectrum analysis of Peak intensity (a.u) vs m/z. (**Bruker Daltonik GmbH BioTools Version 3.0**)

The MASCOT analysis revealed that the CGTase enzyme did not demonstrate significant homology to any of the known and existing CGTase sequences deposites in Swissprot and Uniprot (Fig. 5.14). A total of 33 peaks were generated out of which three peptide fragments matched peptide fragments of glycosylase OS-*Colwellia psychrerythraea* (strain 34H / ATCC BAA-681) GN=mutM PE=3 SV=3 FPG_COLP3 with Intensity Coverage of 0.5 % (1710 cnts) and Sequence Coverage MS of 12.9%.

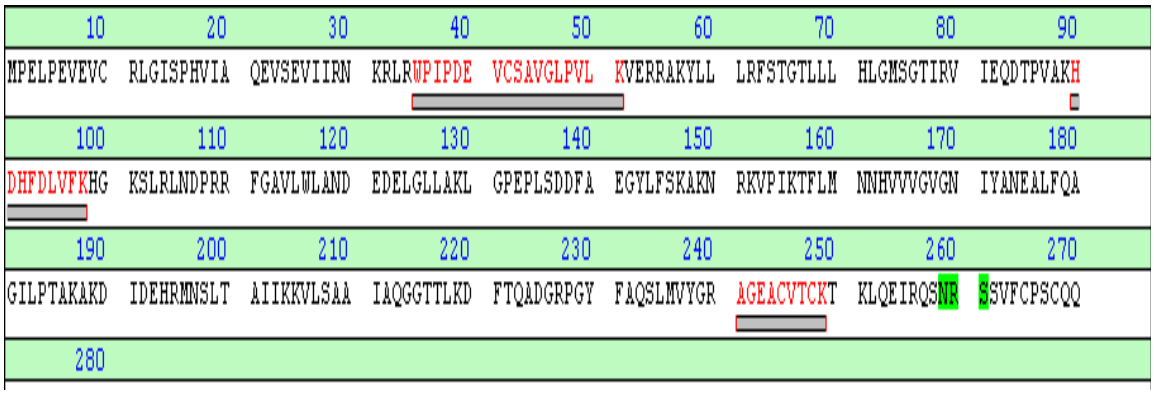


Fig. 5.14 : Comparison of *E. aurantiacum* CGTase (Red) and *Colwellia glycoslase* (Black) using MASCOT

It is observed from the above figure that all the three peptides end in Lysine and trypsin had cleaved CGTase at lysine (K). The presence of acidic amino acids is seen in peptide 1 (Asp 39, Glu 40, Asp 91 and Glu 253). Proline which is responsible to give rigidity to the enzyme is observed (Pro 38, Pro 48) and a single amino acid of tryprophan (Try 35) and histidine (His 90) was also observed. The isoelectric point of CGTase from *E. aurantiacum* is predicted as 9.1.

5.4 Discussion

The most important parameter in study of any enzyme is to obtain it in pure form suitable for appropriate applications and to characterize it for better understanding of its properties. There are many approaches for purification of CGTase from bacteria. A simple two step process involving ammonium sulphate precipitation and dialysis, followed by starch adsorption chromatography with 52 % yield is reported as also described by Higuti *et al.* (2004) and Ibrahim *et al.* (2012). Many researchers have used three steps procedure to purify their CGTase, such as Gawande *et al.* (1998) on *Bacillus firmus* (Ultrafiltration + starch affinity chromatography + gel filtration) and Sohn *et al.* (1997) on *Bacillus firmus* (ammonium sulfate + DEAE-Sephadex A-50 column + Sephadex G-100 column). Other researchers have attempted to use more steps to purify their CGTase, viz. Tachibana *et al.* (1999) used ammonium sulfate + Resource Q column + phenyl-Superose column + α-CD affinity column for purification of CGTase from *Thermococcus sp.* Mori *et al.* (1994) purified CGTase produced by

Brevibacterium sp. No. 9605 using ultrafiltration + butyl-Toyopearrl 650M column + γ-CD Sepharose column + Toyopearl HW-55S column.

Matioli *et al.* (1998) purified CGTase from alkaliphilic *Bacillus* to upto 157-fold by biospecific affinity chromatography. Pongsawasdi and Yagisawa (1988) purified a CGTase from *Bacillus circulans* with a 30% recovery by adsorption on corn starch followed by size-exclusion chromatography. Martins & Hatti-Kaul (2002) purified a CGTase from *Bacillus agaradhaerens* with a yield of 50% by adsorption to corn starch in the presence of 1 M ammonium sulphate followed by elution with β-CD solution.

Rosso *et al.* (2002) isolated and purified a CGTase from *B.circulans* using a-cyclodextrin-derivatised Sepharose 4B affinity chromatography (Ferrarotti *et al.*, 1996). Many of the researchers use gel permeation chromatography including Sephadex G-150, Sephadex 75 HR, Biogel A-500, Biogel P-100, Biogel P-150, Sephacryl S-100, Sephacryl S-200 in their purification steps (Nakamura and Horikoshi., 1976; Larsen *et al.* 1998; Pongsawasdi and Yagisawa, 1988; Gawande and Patkar, 2001). Purification of *E. aurantiacum* CGTase using DEAE- Sepharose resulted in loss in enzyme activity and the single protein band in the SDS –PAGE suggest purification of some other peptide instead of CGTase. The characterization of CGTase revealed that the pH optimum was 9 and the enzyme was stable over a wide range of pH (5- 11). This property is useful in application of the enzyme in detergent industry. There are a few CGTase enzymes that showed higher optimum pH such as CGTase from *Brevibacterium* sp. 9605 (Mori *et al*., 1994) and *Bacillus clarkii* 7364 (Takada *et al*., 2003). Both exhibited an optimum pH of 10. CGTase from *E. aurantiacum* demonstrates pH optimum of 9 which is suitable for cyclisation and hence production of cyclodextrins. pH plays a significant role in preserving biological activity and stability of CGTase because changes in pH may alter the three dimensional structure of the enzyme, as well as changing the native ionic form of the active site. Thus, it is vital to ensure that the pH of the buffer is always monitored to maintain the optimal stability of the enzyme. CGTase from other organisms such as *Bacillus agaradhaerens* (Martins and Hatti-Kaul, 2002) and *Bacillus firmus* (Sohn *et al.*, 1997) exhibited stability over a wide range of pH, from pH 5.0 - 11.4 and pH 5.5 - 9.0, respectively. CGTase from *Klebsiella pneumoniae* AS-22 was stable between pH 6.0-9.0 (Gawande and Patkar, 2001).

E. aurantiacum CGTase showed significant activity in a wide temperature range, 30°C–70°C, showing maximal enzyme activity at 50°C. Temperature optima in the range of 55–65°C have been previously reported for CGTases from various alkaliphiles (Alves-Prado *et al*., 2008; Martins and Hatti- Kaul, 2002 and Ibrahim *et al.*, 2012). This shows that CGTase of *E. aurantiacum* is an Intermediate thermostable enzyme (ITS) and can be explored for its applications in bakery and food industry. The effect of various metal ions and inhibitors revealed that CGTase is not a metallo enzyme as no metal ions significantly enhanced its activity and the enzyme retained 90 % activity in presence of EDTA which are similar to the reports of CGTase of *Amphibacillus* sp. and *Bacillus pseudoalkaliphilus* (Ibrahim *et al*., 2012; Atanasova *et al*., 2009). The metal ions like Fe^{+3} and Zn^{+2} catalyze the oxidation of the amino acid residues present in active centre including tryptophan, glutamic acid, aspartic acid and histidine, which are essential for cyclization reaction, causing the reaction rate of CGTase to decrease (Martins and Hatti-Kaul, 2002), cause transition state stabilization, as well as substrate binding and guiding linear starch chain into the active site (Uitdehaag *et al.*, 1999; Knegtel *et al.,* 1995; Penninga *et al.*, 1996 and Nakamura *et al.*, 1992). Thus, the cyclization reaction rate of CGTase will be decreased tremendously if these crucial amino acid residues are not well preserved. This suggests presence of glutamic and aspartic acid in active centre. The MALDI- TOF MS analysis of CGTase of *E. aurantiacum* showed the presence of acidic amino acids in peptide 1 (Asp 39, Glu 40, Asp 91 and Glu 253). Since CGTase produced by *E. aurantiacum* has optimum activity at alkaline pH, the presence of acidic amino acids in the enzyme protein is apparent.

The K_m and V_{max} values of CGTase produced by *E.aurantiacum* are 0.022 mg/ml and 57 µg of β- cyclodextrin/ml/min respectively show greater affinity of the CGTase to soluble starch. The K_{cat} value (turnover number) of CGTase of *E. aurantiacum* was 316.7 s^{-1} which is very high as compared to CGTase of *Bacillus macerans* (K_{cat}= 99.8 s^{-1}) (Jeang *et al.*, 1999).

The molecular weight of CGTase produced by *E. aurantiacum* was estimated as 77.84 k Da which is comparable to most of the CGTases having molecular weight in the range of 70 – 110 kDa (Cao *et al*., 2005 ; Hirano *et al*., 2006; Savergave *et al*., 2008 and Ibrahim *et al*., 2012). The MALDI –TOF MS analysis revealed that

the enzyme had about 40 % homology to sequence of glycosylase of OS-*Colwellia psychrerythraea* suggesting that the CGTase produced by *E. aurantiacum* may be a novel enzyme which can be confirmed in future using N- terminal amino acid sequencing. The isoelectric point was predicted as 9.1. CGTase produced by *E. aurantiacum* isolated from Lonar lake is thus a novel intermediate thermostable and alkaline enzyme produced extracellularly with high affinity for substrate.

5.5 Conclusions

Purification and characterization of alkaline CGTase from *E. aurantiacum* resulted in 25 fold purification. The enzyme was purified to homogeneity with a molecular weight of 77.84 kDa. The enzyme was completely inhibited by Fe, partially inhibited by DTT and not inhibited by metal ions. Calcium ions enhanced activity of CGTase. The enzyme was detected in starch and phenolphthalein indicator gels successfully. The enzyme was moderately thermostable suggesting its application in food industry.

Chapter 6

Application of cyclodextrin glycosyl transferase produced by *E. aurantiacum*

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6.1 Introduction

The major application of CGTase is the production of cyclodextrins. CGTase is used to carry out enzymatic transglycosylation and to improve solubility of compounds like rutin, curcumin, stevioside and naringin (Szetjli, 2004).

CGTases can also be used as an antistaling agent in food industry. After bread is baked, a series of physicochemical changes develop in the loaf that leads to deterioration of quality during storage. A change in crumb firmness, called retrogradation, is one of the major factors affecting consumer acceptance. Therefore, several additives such as chemicals, small sugars and enzymes have been included in bread mix in efforts to preserve the softness of bread for longer storage periods. The starch and amylose molecules in bread gradually lose water and bind together increasing the crystalline nature of bread. α-Amylases have an antistaling effect on bread because they retard retrogradation of the loaf (Min *et al.,*1998) . Effective antistaling enzymes, such as amylases, hydrolyze amylose and amylopectin chains into smaller molecules, leading to less crystallization and limited crystal size. Low molecular weight dextrins produced by antistaling enzymes decrease the retrogradation rate of bread by inhibiting the interaction between starch and the continuous protein matrix .In addition, shorter amylopectin chains form more complexes of amylopectin and lipid, consequently retarding retrogradation of bread. CGTases produce circular cyclodextrins that form complexes with lipids which delays crystallization and retards retrogradation. There are very few reports on application of CGTase as an antistaling agent in bread (Lee *et al*., 2002).

The staling in bread is studied by analysis of texture of bread measured in form of chewiness, hardness, crispiness, gumminess, stickiness, cohesiveness, firmness and fracturability. These parameters are measured using a texture analyser. It is an instrument that measures the response of a sample to an applied force by means of tension, compression, penetration. The texture analyser has a probe that travels from start point to the sample at pre-test speed. When the probe measures the trigger force, the speed changes to the test speed and force, distance and time is recorded. The probe travels into the sample at the test speed until the test is complete.When test is finished, probe returns to the start at the post-test speed.

The Texture Profile Analysis (TPA) graph is generated and calculation are performed by the software using the generated graph.The graph is used to calculate hardness, fracturabilty, cohesiveness, springiness, chewiness, gumminess and resilience.

The cyclodextrins are chemically modified to produce derivatives of cyclodextrins .Large ring cyclodextrins (LR-CD) containing upto 31 glycosyl units have been purified and characterized.

Cyclodextrins are used for the stabilization of active compounds, reduction in volatility of drug molecules, and masking of odors and bitter tastes. Normally drug substance has to have a certain level of water solubility to be readily delivered to the cellular membrane, but it needs to be hydrophobic enough to cross the membrane. The majority of pharmaceutical active agents do not have sufficient solubility in water and these insoluble drugs can be complexed with CD's to increase their solubility (Tonkova, 1998).

CDs have many industrial applications, but their use is commercially restricted due to high price of CD's. Immobilization of the enzyme cyclodextrin glycosyl transferase (CGTase) has been pursued as a means of reducing the production cost of cyclodextrins (CDs) from starch. The immobilization of bacteria in polymers can facilitate the recovery of the free enzyme activity, maintain an appropriate pH, affinity for the substrate, and enzyme stability because in this case immobilization may cause benign changes in enzyme microenvironment, or in the of mobility of the protein. The extension of these changes depends upon the enzyme, support and reaction conditions used for immobilization. Among these factors, the choice of support is the most important factor. An ideal support for enzyme immobilization must be selected considering some essential properties, such as, chemical stability, hydrophilic behavior, rigidity, mechanical stability, large surface area, and resistance to microbial attack. There are not many publications describing immobilization of CGTase producers and CGTase. *Bacillus* spp. producing CGTase have been entrapped in different matrices, but with limited success (Ishmail *et al*., 1996; Park *et al.,* 2000). Immobilization of the CGTase producer and exploring the enzyme production using agro- waste based media or medium components can minimize the cost of CD production.

In this chapter, the application of CGTase in production of cyclodextrins, immobilization of *E. aurantiacum* for the production of cyclodextrins from agro waste based medium and the antistaling property of CGTase in bread are described.

6.2 Materials and Methods

6.2.1 Production of β- cyclodextrin

6.2.1.1 Micro organism and culture condition

Exiguobacterium aurantiacum MCM-B 1021 was employed for the present studies. The organism was cultivated in 250 ml flasks containing 50 ml nutrient broth of pH 10 in an orbital shaker at 150 rpm at 37°C for 24 hours. After incubation, the cells were centrifuged at 10,000 rpm for 20 min, the pellet was suspended in saline and cell density was measured spectrophotometrically (Schimadzu, Japan) at 540 nm. The optical density was adjusted to 1.0 which corresponded to 10^8 CFU/ml as enumerated by standard plate count using spread plate method.

6.2.1.2 Production of CGTase

CGTase was produced in 250 ml Erlenmeyer flask containing 50 ml production medium optimized by Taguchi DOE containing (g/L) soluble potato starch 10, peptone 10, yeast extract 5 and sodium chloride 5. The pH was maintained as 10 and the culture was incubated in an orbital shaker at 150 rpm at 37°C for 24 hours. The inoculum (10 % v/v) having cell density of 10^8 CFU/ml was inoculated in the production medium and the flasks were incubated in an orbital shaker at 37°C at 150 rev/min. After 24 h, the broth was centrifuged at 10,000 g for 30 min (Kubota, Japan) and the supernatant was used as crude enzyme for production of CD. The CGTase produced in supernatant was estimated by phenolphthalein assay (Goel and Nene, 1995).

6.2.1.3 Estimation of β- cyclodextrin

The reaction mixture contained 100 µl of supernatant in which 1 ml of 1 % (w/v) soluble potato starch in 0.005 M TrisCl buffer of pH 8.5 was added and incubated at 60° C for 20 min. After incubation, this reaction mixture was cooled in ice, 4 ml of 1mM phenolphthalein reagent was added to the tubes and the absorbance was measured immediately at 550 nm. The amount of β-CD produced was estimated from standard graph of 50 – 200 µg /ml of standard β-CD (Sigma-Aldrich) against decrease in absorbance.

6.2.1.4 Optimization of production of cyclodextrin

The effect of starch concentration and enzyme concentration on the production of cyclodextrin was investigated by varying the starch and enzyme concentration. The starch concentration was varied from 1% to 10% (w/v). The enzyme concentration was varied from 1 to 12 U/g substrate. The reaction was performed by adding 0.1 ml enzyme to 1 ml starch in 50 mM Tris Cl buffer of pH 8. The reaction mixture was incubated at 60°C for 20 min and the cyclodextrin in the supernatant was estimated as described above.

6.2.1.5 Production of β- cyclodextrin under optimized conditions

The β- cyclodextrin was produced using optimum concentration of starch and enzyme under appropriate condition and the percentage conversion of starch to CD was estimated.

6.2.1.6 Detection of cyclodextrin by HPLC and FEG-SEM

The β- Cyclodextrin produced was detected by HPLC (Perkin Elmer, U.S.A)) using Amino column (5 µm, 250 X 4.6) with acetonitrile: water (65:35) as mobile phase and an online Perkin Elmer series-200 Refractive Index detector. About 10 µl of supernatant containing β- cyclodextrin was mixed with equal volumes of HPLC grade acetonitrile (Merck) and centrifuged at 20,000 g for 15 min at room temperature and then filtered through a 0.45 µ filter (Millipore, USA). The sample was injected in the

column and the output was read using a TotalChrom Navigator Software. The pressure was maintained between 2800 to 3070 psi. For microscopic observation, the cyclodextrin produced was precipitated by addition of complexing agent (5 (w/v) trichloroethylene and toluene) and incubation at 4°C for 2 h. After incubation, the reaction mixture was centrifuged at 5, 000 g for 30 min and the precipitate was dried in a hot air oven at 80°C. The cyclodextrin powder was observed using a JSM-7600F Field Emission Gun Scanning Electron Microscope (FEG-SEM) with a resolution of 1nm and accelerating voltage of 15 kV under high vacuum of ~ 10^{-5} Pa.

6.2.1.7 Production of cyclodextrin from different sources of starch

The effect of type of starch on production of CD's was studied. The reaction mixture contained 100 µl crude enzyme and 1 % (w/v) starch substrate viz. raw potato starch (Hi media), corn starch (Hi media), soluble potato starch (Hi media), wheat starch (Food grade), rice starch (Food grade), and sago starch (Food grade). The conditions used for CD production were as mentioned in 6.2.1.4. The percentage of starch converted into CD's was calculated by amount of total CD's formed (g) divided by amount of starch used (g) multiplied by 100.

6.2.2 Production of cyclodextrin by CGTase produced by immobilized *E. aurantiacum*

6.2.2.1 Inoculum

E. aurantiacum was cultivated in 250 ml flasks containing 50 ml nutrient broth of pH 10 in an orbital shaker at 150 rpm at 37°C for 24 hours. After incubation, the cells were centrifuged at 10,000 g for 20 min, the pellet was suspended in saline and cell density was measured spectrophotometrically (Schimadzu, Japan) at 540 nm. The optical density was adjusted to 1.0 and was used for immobilization.

6.2.2.2 Immobilization of *E. aurantiacum* by entrapment method

The cells were immobilized by entrapment method in alginate matrix. Saline suspension of *E. aurantiacum* (25 ml) was added to 4 % sterile molten sodium

alginate in 1:1 proportion. This mixture was dropped from a height of 15 – 20 cm in a chilled solution of 6 % (w/v) calcium chloride with the help of a sterile 22 gauge syringe. The beads formed were soaked in distilled water and kept overnight in refrigerator in 50 mM $CaCl_2$ for hardening.

6.2.2.3 Batch production of CD by immobilized cells in a bench scale packed bed reactor

The packed bed reactor was set up by packing hardened beads in an alcohol rinsed glass burette with a bed volume of 20 ml and filled with sterile 50 mM phosphate buffer pH 7 overnight. After appropriate incubation, the buffer was drained and the beads in the column were repeatedly washed with sterile distilled water. After washing, sterile 1 % starch solution was added in the column and after 20 minutes (reaction time for CGTase) one ml aliquots were collected. The enzyme activity of aliquots was tested by phenolphthalein assay. The process was repeated for 3 runs. The production of CD/ CGTase by immobilized cells was compared to the production by free cells.

6.2.2.4 Production of cyclodextrin from economical media

Agro based waste like potato peels from a wafer company was used as a substrate for economical production of CGTase. Unused Potato peels were obtained from Hot chips, Kondhwa, Pune, dried, powdered and incorporated in optimized production medium at 1 % concentration. The medium was inoculated with 16 h culture of *E. aurantiacum* at 10 % (v/v) concentration having 10^8cells/ml and incubated at 37°C for 24 h in an orbital shaker at 150 rpm. The culture broth was centrifuged and cell free supernatant was analyzed for CGTase and CD.

6.2.3 Application of CGTase as an antistaling agent in bread

As the enzyme CGTase was to be used as an antistaling agent in bread, the in vivo oral toxicity was tested at National Toxicology Centre, Pune, India.

6.2.3.1 *In vivo* toxicity assay of CGTase in Swiss albino mice

The acute oral toxicity of purified CGTase (as used in bread making) was determined following the OECD guidelines, 423, Adopted 17 December 2001.The study was conducted step wise on two sets of three female Swiss

albino mice (total six) aged 6 to 8 weeks with a weight of 18 – 22 g. In step 1, three female mice were administered CGTase diluted in water at a dose of 2000 mg/ kg body weight. The mice were deprived of feed 3-4 h before and 2 h after dosing. Water was allowed *ad libitum*. In step 2, three female mice were administered the enzyme diluted in water at a dose of 2000 mg/kg body weight. All animals were observed for toxic symptoms and mortality at ½, 1,2,3,4 and 24 hours and later twice a day for 14 days to determine their health, general behavior and moribund condition. Any abnormality observed after dosing was recorded and subsequent progress monitored. The weight of the animals was observed and a gross necroscopy was performed of all the animals that died during the course of study and sacrificed at the termination of the test.

6.2.3.2 Analysis of individual component of flour used for bread making

The different constituents of refined wheat flour (maida) used for bread making were tested in an InfraRed Infratec® 1241 Grain analyzer (FOSS). The flour sample was added in circular quartz cuvettes, placed on conveyer belt and then analyzed. The protein content, moisture content, ash content, water content and gluten content of the flour was estimated. The samples were tested in duplicate.

6.2.3.3 Preparation of dough

The dough for basic white loaf bread was prepared using 100 parts of refined white wheat flour, 0.4 parts of yeast, 3.2 parts of sugar, 3.9 parts of shortening, 1.9 parts of defatted milk powder , 1.07 parts of salt and 70 parts w/w water. Three sets for preparing bread were as follows : Control Bread without any antistaling agent as per above mentioned formulation, bread containing above formulation with 415 U CGTase and bread containing above formulation with 0.02 g (w/w) commercial α- amylase (Unizyme) as positive control.

6.2.3.4 Bread baking method

Basic white loaf bread was baked in an automatic home bread maker (Panasonic SD- 253). The baking process of basic white loaf bread consists of mixing the flour, kneading the dough, proofing, fermentation and baking at 180°C. After baking, the bread was allowed to cool in the instrument, demoulded, wrapped in Ziplock© bags and was stored at 10°C in the refrigerator until further use.

6.2.3.5 Analysis of texturial properties of bread

The textural changes in the three types of breads were evaluated using TA XT plus 2i Texture Profile analyzer with a P25 probe. The loaves were manually sliced into thickness of 16- 17 mm. The crust, first and last two slices were discarded. The texturial properties were analyzed using 36 mm cylindrical aluminum probe and a cross head speed of 1.7 mm/sec to compress crumb samples to 40% original height. Measurement was carried from two slices in triplicate from centre of the loaf. The texturial properties were analyzed using AACC BRD two bite test – BRD2_P35R. The hardness was determined by maximum peak force determined by the first cycle (first bite). Springiness (mm) is calculated as distance of the detected height of product during second compression divided by original compression distance (length 2/ length 1). Cohesiveness (diamensionless number) was calculated as ratio of the area of positive force during second compression to that of the first compression (area 2/area 1). Gumminess is calculated as product of hardness X springiness, chewiness is calculated as product of gumminess X springiness. Resilience is calculated as area during withdrawal of first compression divided by area during first compression. Adhesiveness is the negative force area of the first byte.

6.2.3.6 Analysis of bread firmness

Bread firmness was determined using the AACC (74-09) Standard method using a 36 mm cylindrical aluminium probe with a crosshead speed of 1.7 mm/sec and 5 kg load cell. The sample is placed centrally under the cylindrical probe avoiding any irregular or non representative areas of

crumb. The force is measured in compression mode with a pre test speed of 1.0 mm/sec, test speed of 1.7 mm/sec, post test speed of 10 mm/sec, strain value of 40 % with a auto-5g trigger, auto tare mode and data acquisition at 250 pps (points per second).

6.2.3.7 Image and pore size analysis of bread

The images of bread slices were captured at 300 dpi (dots per inch) using a digital SLR camera (Nikon) and the image of bread was imported in Rhino 3D software and scaled to the actual size in mm. Six pores were randomly selected on the image in an area of 50 mm X 50 mm and their pore sizes were measured using the software. The loaf volume was calculated by conventional method.

6.2.3.8 Detection of CD in bread

The sample for HPLC analysis of CD was prepared by adding 10 g bread crumb to 100 ml distilled water and stirring it vigorously for 1 hr. The sample was centrifuged at 10,000 g for 20 min and the supernatant was mixed with equal volume of acetonitrile (HPLC grade).The sample was then filtered through a 0.22 µ filter (Millipore) and used for analysis. CD was detected by HPLC as described earlier (6.2.1.6).

6.3 Results

6.3.1 Production of β- cyclodextrin

6.3.1.1 Optimization of production of cyclodextrin

The optimization of concentration of enzyme and amount of starch for production of β -cyclodextrin was studied. The amount of cyclodextrin produced was quantitated by phenolphthalein assay. The absorbance of the sample decreased with increase in amount of cyclodextrin production as the phenolphthalein molecules are trapped in the hollow CD structure decreasing the pink color of the sample. Thus lesser the absorbance, higher is the cyclodextrin production. The effect of different concentrations of starch and CGTase on production of CD is illustrated as a scatter plot (Fig. 6.1.)

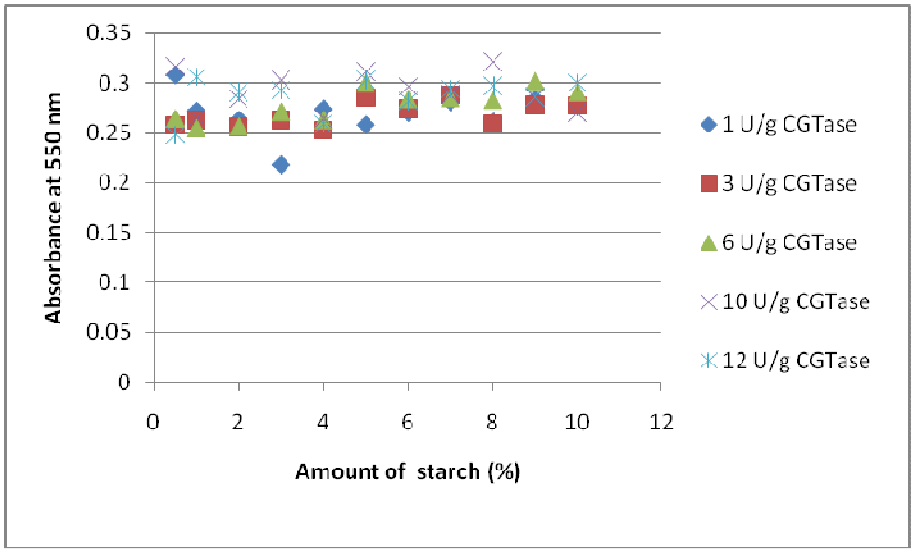


Fig. 6.1 : Scatter plot of effect of different concentrations of starch and enzyme on production of cyclodextrin

The optimum concentration of starch for cyclodextrin production was 3 % with enzyme concentration of 1 U/g. The concentration of starch used for CD production is between 1 to 2.5 % (Alves *et al*., 2007). Increasing the concentration of starch does not increase CD production.

6.3.1.2 Production of β- cyclodextrin under optimized conditions

Cyclodextrin was produced in optimum conditions using 3 % soluble starch and 1 U/g CGTase at 60°C for 20 min. The yield of cyclodextrin was 0.33 – 1.5 mg/ ml. Most alkaliphiles produce CD in the ranger of 0.1 – 5 mg/ml (Alves-Prado *et al*., 2008; Biwer *et al*., 2002). Alves-Prado *et al*. (2008) have reported production of 1 mg / ml CD from maltodextrin.

6.3.1.3 Detection of cyclodextrin by HPLC and FEG-SEM

The cyclodextrin were detected using colorimetry, HPLC and Scanning electron Microscopy. The amount of β cyclodextrin produced was estimated by PHP assay as 155 µg/ml. The cyclodextrin were detected using HPLC, the retention time for α- CD, β- CD and γ- CD were 2.8, 3.1 and 5.7 min respectively. Glucose and maltose were also eluted at 0.78 and 1.5 min respectively.

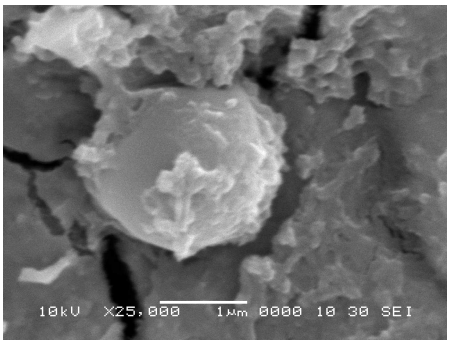


Fig. 6.2 : Scanning electron micrograph of CD-TCE complex produced by *E. auranticum*

The SEM of β-CD- TCE complex showed a typical spherical structure with size ranging from 2- 4 µm (Fig. 6.2). The scanning electron micrograph was compared to images published by other researchers. The morphology of CD complex showed similarity to the CD complex observed by Pandit *et al.* (2011).

6.3.1.4 Production of cyclodextrins using different sources of starch

It was observed that soluble starch is a better substrate for production of CGTase with higher enzyme activity and production of β- CD (Fig. 6.3). Cereal starches like rice and wheat showed less production of CGTase .This may be due to presence of amylase lipid complex present in cereals which is resistant to degradation by CGTase. Tuber starch like sago starch also showed very less yield. Soluble starch is therefore a better substrate for production of CGTase.

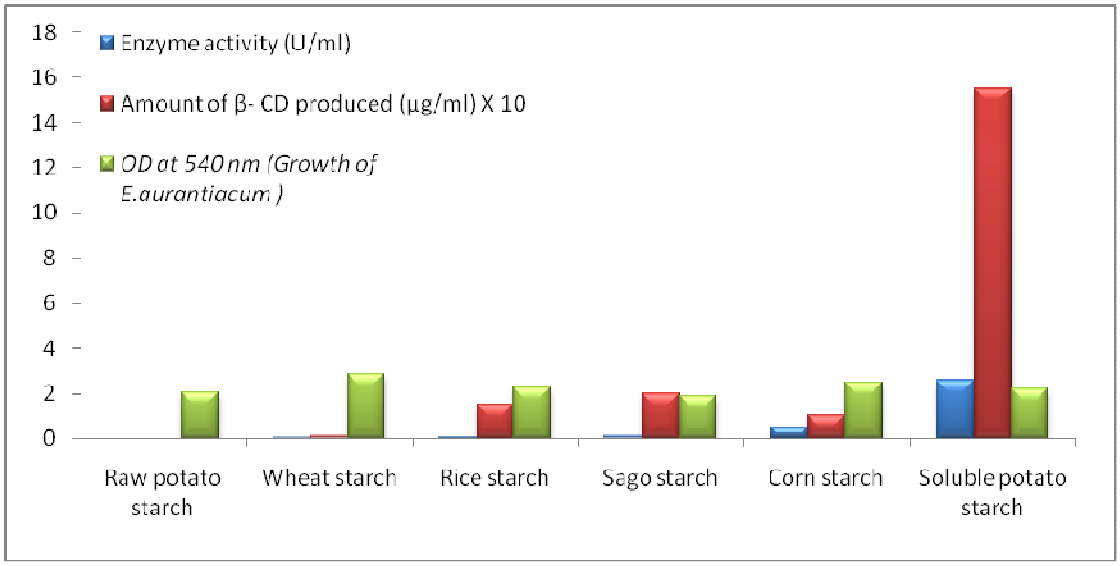


Fig. 6.3 : Effect of different starch substrates on production of CD and CGTase

The conversion of starch to cyclodextrin was 1% with soluble starch. Gawande and Patkar (1998) have observed a low CD conversion using CGTase from *Klebsiella pneumoniae* AS-22 on a corn starch solution. Using CGTase from *Brevibacterium* sp no. 9605, Mori *et al*. (1994) have observed 1% lower conversion when using corn starch than when using sweet potato starch. Similar results were observed by Goel and Nene (1995) who obtained a better CD conversion using CGTase from *Bacillus firmus* in cassava starch than in corn starch.

6.3.2 Production of cyclodextrin by CGTase produced by immobilized *E. aurantiacum*

6.3.2.1 Immobilization of *E. aurantiacum* by entrapment method

E. aurantiacum was immobilized in alginate matrix for production of CD/CGTase. The gel matrix without cells is observed in the scanning electron micrographs as shown in Fig. 6.4. The scanning electron micrograph represented in Fig. 6.4 b show the cells of *E. aurantiacum* entrapped in Ca-alginate matrix. The immobilized whole cells can be effectively used for production of cyclodextrin.

Fig. 6.4 a : Scanning electron micrograph of calcium alginate matrix

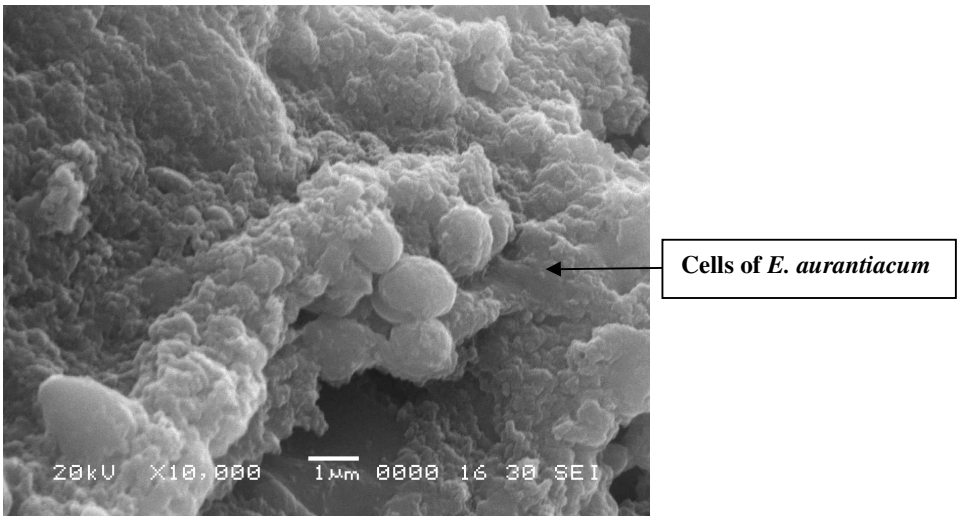


Fig. 6.4 b : Scanning electron micrograph of *E. aurantiacum* entrapped in alginate matrix

E. aurantiacum was seen trapped in the calcium alginate matrix (Fig. 6.4 b). Fig. 6.4 a, shows that there are sufficient lacunae in the matrix for proper transport of nutrients, diffusion of gases and release of enzyme and CGTase out of the matrix. There are various methods and matrices used for immobilization of cells for CGTase production. Abdel-Naby (1999) immobilized CGTase from *Paenibacillus macerans* NRRLB-3186 in aminated polyvinylchloride (PVC) of three different hydrocarbon chain lengths, by covalent binding with glutaraldehyde as a bifunctional reagent.

Loofa sponges are also used for immobilization of cells for CGTase production (Delani *et al*., 2012).

6.3.2.2 Batch production of CD by immobilized cells in packed bed reactor

The production of CD was carried out in packed bed reactor using immobilized cells of *E. aurantiacum* (Fig. 6.5).

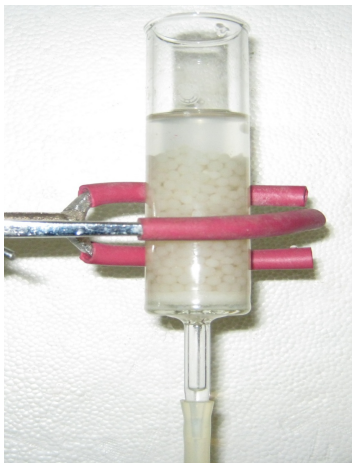


Fig. 6.5 : Packed bed reactor with immobilized cells of *E. aurantiacum* for production of CD

The CD produced by immobilized cells was compared to CD produced by free cells and it was observed that immobilized cells could be used for more number of runs (3 runs) as compared to free cells (Fig. 6.6).

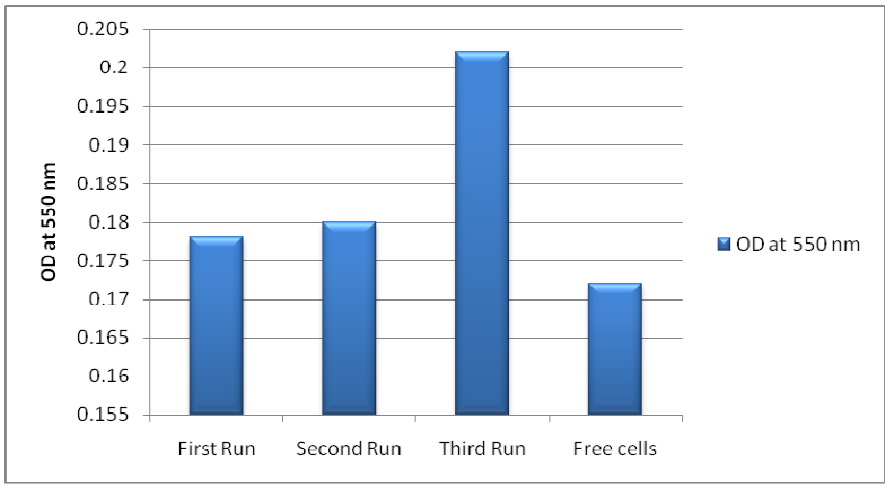


Fig. 6.6 : Production of CD by immobilized and free cells of *E.aurantiacum*

The activity of immobilized *E. aurantiacum* cells for the production of CD was explored by using the cell-immobilized beads, respectively, for three cycles. Immobilized cells show lesser production of CGTase as compared to free cells.

The advantage of an immobilized system is production of CD for more number of runs in batch mode of operation. The prolonged use of the cells and ease of separation of the product is the advantage of application of immobilized cells over free cells.

6.3.2.3 Production of cyclodextrin from economical media

The waste product of a potato chips industry, i.e. potato peel powder was used as a substrate for economical production of CD by immobilized *E. aurantiacum* in a packed bed reactor. The immobilized cells were effectively used for the production of β -cyclodextrins from potato peel waste with an activity of 5 .1 U/ml and yield of 150 µg/ml β- CD as compared to 6 U/ml (162 µg/ml β-CD) for free cells. The β –cyclodextrins produced were detected by HPLC, scanning electron microscopy and colorimetric estimation. An efficient application of this process at industrial level requires stability of the cells which is achieved in this system.

6.3.3 Application of CGTase as an antistaling agent in bread

6.3.3.1 *In vivo* toxicity assay of CGTase in Swiss albino mice

The assessment of the toxicity of CGTase was critical as it had to be used in preparation of bread for human consumption. The *in vivo* toxicity was performed in stepwise manner in two groups of female mice containing three animals in each group. A dose of 2000 mg/ kg of CGTase caused no mortality in the mice (Table 6.1)

Table 6.1 : Effect of CGTase on mortality of mice

Group No	Dose mg/kg	No. of animals treated	No. of animals died	Mortality (%)	Clinical signs	Necroscopy finding after sacrifice
1	2000	3	0	0	Nil	NAD
2	2000	3	0	0	Nil	NAD

NAD: No abnormality detected

All animals appeared normal and showed no clinical signs of intoxication after dosing till the end of the study. Similar results were observed in second group. There was a mean increase in the body weight of all the animals in both the groups of 25.5 – 24.6 % as shown in Table 6.2.

Table 6.2 : Effect of CGTase on body weight of mice

Group	Sex	Mean Body weight (%)		
		Day 0	Day7	Day 14
1	Female	21.6	25.4	29.6
2	Female	21.2	24.6	29.2

There was a mean increase in the body weight of all the animals in both the groups of 25.5 – 24.6 % as shown in Table 6.2. The LD_{50} in females in GHS category (Globally Harmonized System of Classification and Labelling of Chemicals) is > 2000 – 5000 mg/ kg body weight with a LD_{50} cut off at 5000 mg/kg body weight. No abnormality was detected in the animals. It was concluded that the CGTase enzyme was "SAFE" for use as determined by the toxicity studies conducted in compliance with OECD guidelines 423 following Good laboratory Practice regulations. As the enzyme is safe for use it was used as an additive for baking bread.

6.3.3.2 Analysis of individual component of flour used for bread making

The individual components of the refined white flour (maida) were determined by Infratec Grain analyzer (FOSS). Samples of each pack of Reliance refined flour (India) 500 g were tested in duplicate. A total of six packets were analyzed and the results are summarized in Fig. 6.7.

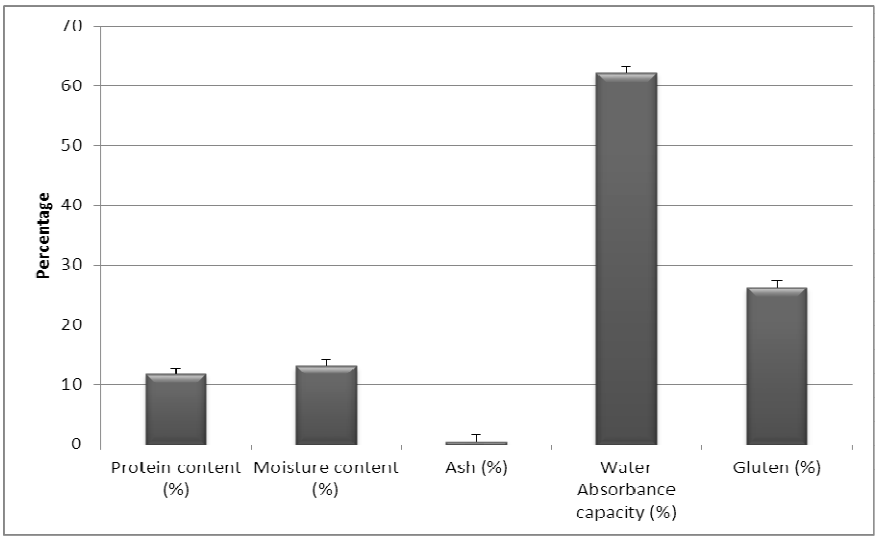


Fig. 6.7 : Individual component analysis of flour used for baking bread

Good quality raw material ensures quality products. The raw material i.e., flour used for making bread contained 11.3 % protein, 13.1 % moisture content, 0.6 % ash, 62 % water absorbance capacity and 26 % gluten. The standard composition of flour consists of protein (10–12%), starch (70–75%), ash and non-starch polysaccharides (2–3%) and water (14%), small amounts of vitamins, minerals and enzymes (Charley and Weaver, 1998). Higher the protein content, harder will be the dough. Hence in refined flour a protein content of approximately 11 – 12 % is maintained which is obtained in the refined flour used for bread making.

High gluten content is preferred for baking bread and the flour analysis reveal gluten content in range of 24 – 29 % which is good for baking bread. The individual component analysis of the flour reveals that it is suitable to be used for making bread.

6.3.3.3 Bread baking and determination of its volume and height

The dough was prepared and bread was baked under standard conditions. After baking, the bread was cooled, demoulded and its height, weight and breadth was recorded (Fig. 6.8).

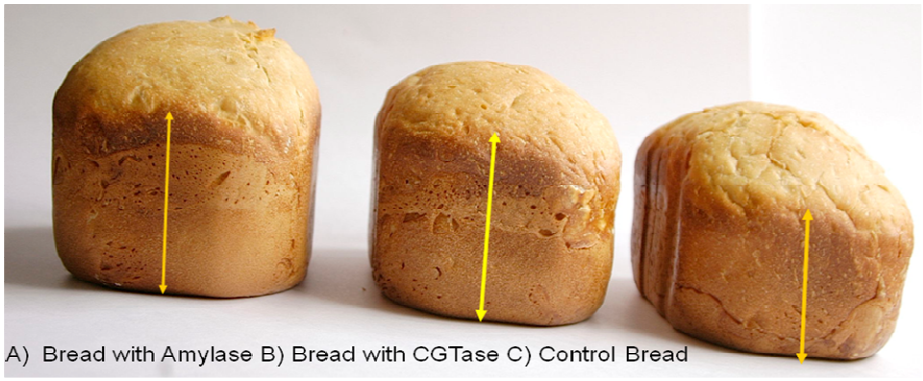


Fig 6.8 : Difference in height of bread with amylase, bread with CGTase and control bread.

It was observed that bread containing amylase had maximum height (10 cm) followed by bread containing CGTase (9cm). The control bread baked without the addition of any enzyme had the least height (7.5cm). The breadth and width of all the three types of breads were same as they were baked in the same mould. The comparison of height and corresponding volume of the three types of bread is depicted in Fig. 6.9.

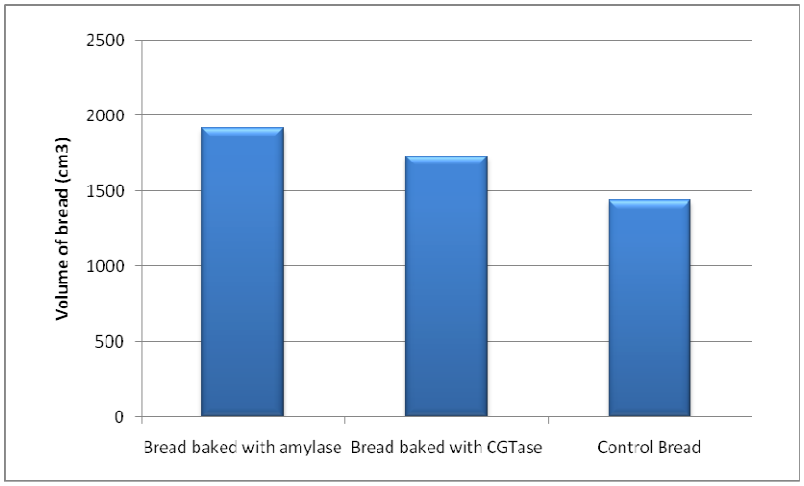


Fig. 6.9 : Difference in volume (cm^3) in three types of bread

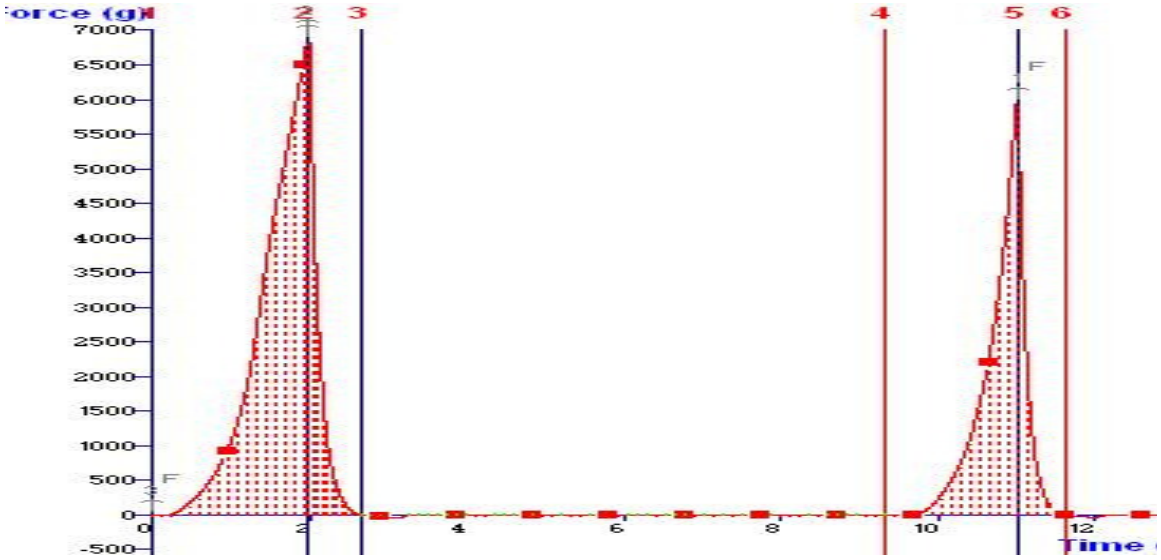
It was observed that bread containing amylase had greater height and thus greater volume than control bread. However bread containing CGTase also has greater volume than control bread but lesser volume than amylase containing bread as in Fig 6.9. The volume of the loaf depends on yeast, carbon dioxide produced by yeast, gluten content of bread and bubbles formed. It has long been known that the protein content of flour has a direct effect on the size of bread produced with it. Amylase hydrolyses carbohydrate to amylose and amylopectin leading to generation of small dextrins easily available to yeast. The starch and protein network of dough holds the gas within and the gas bubbles survive longer in bread baked with amylase. Though the uncontrolled hydrolysis of amylase leads to greater volume, it also generates irregular size pores, bubbles and airpockets. This is clearly visible by the ruptured surface of amylase containing bread (Fig. 6.10).

Fig. 6.10 : Surface topology of the three types of bread, a) Bread with amylase, b) bread with CGTase and c) Control bread.

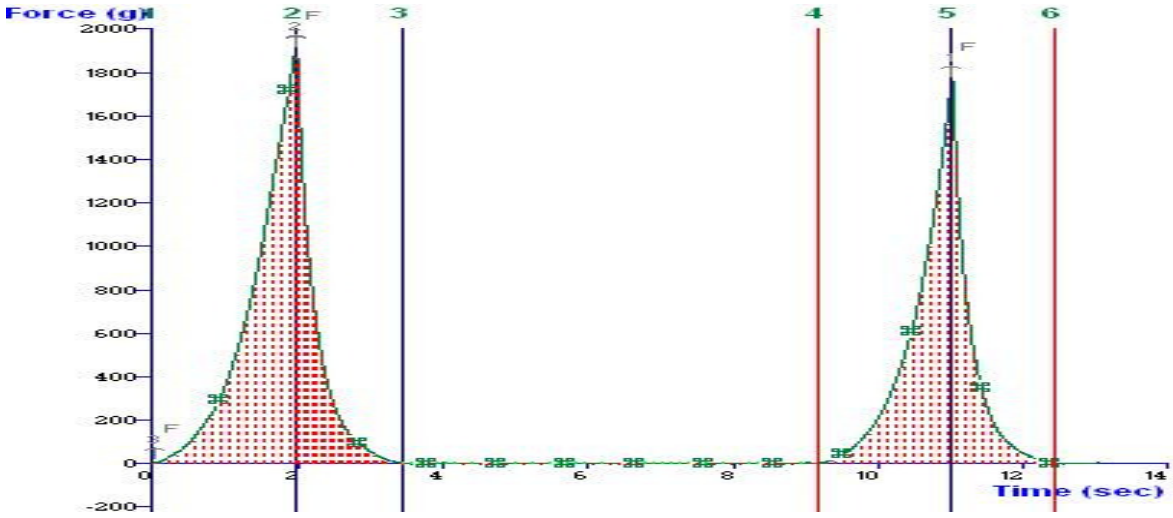
It is observed from Fig. 6.10, that bread containing amylase had a ruptured surface which is undesirable while the surface of bread baked with CGTase as well as control bread had an even surface. As CGTase is an intermediate thermostable enzyme (ITS), the hydrolysis of starch is controlled as the enzyme is inactivated at baking temperatures. CGTase hydrolyses starch and forms cyclodextrins which lead to the increase in loaf volume (Lee *et al*., 2002).

6.3.3.4 Analysis of texturial properties of bread

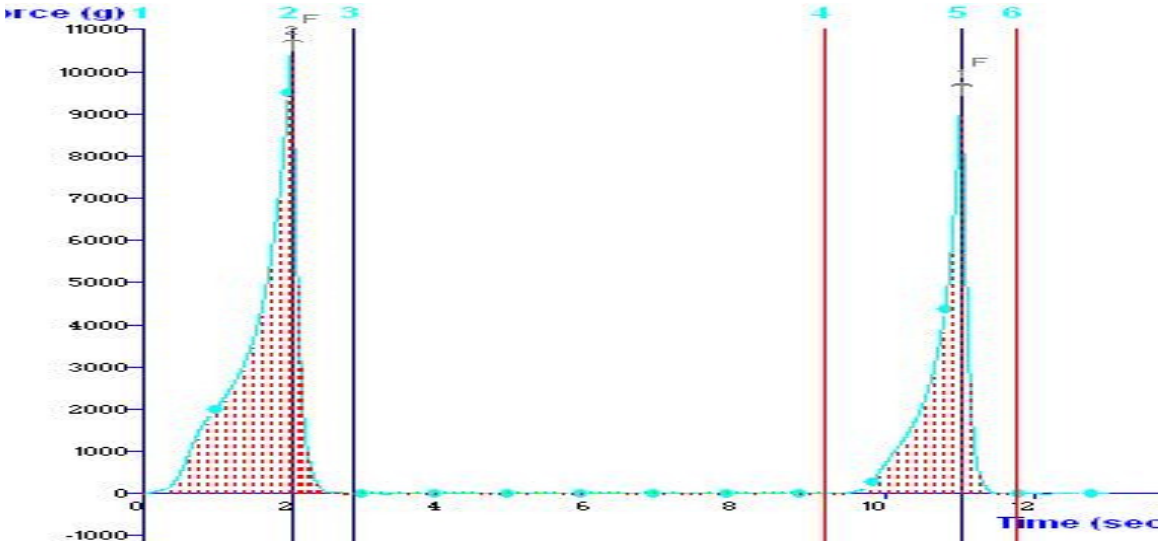
Texturial properties of all the three types of bread viz., bread containing amylase, bread containing CGTase and control bread (without any additives) were analyzed by the two bite test. The graphs of the Texture Property Analysis (TPA) generated by the two bite test were used for calculation of hardness, adhesiveness, cohesiveness, resilience, springiness, gumminess and chewiness. The TPA two bite test graphs used for calculations are shown in Fig. 6.11, a - f.



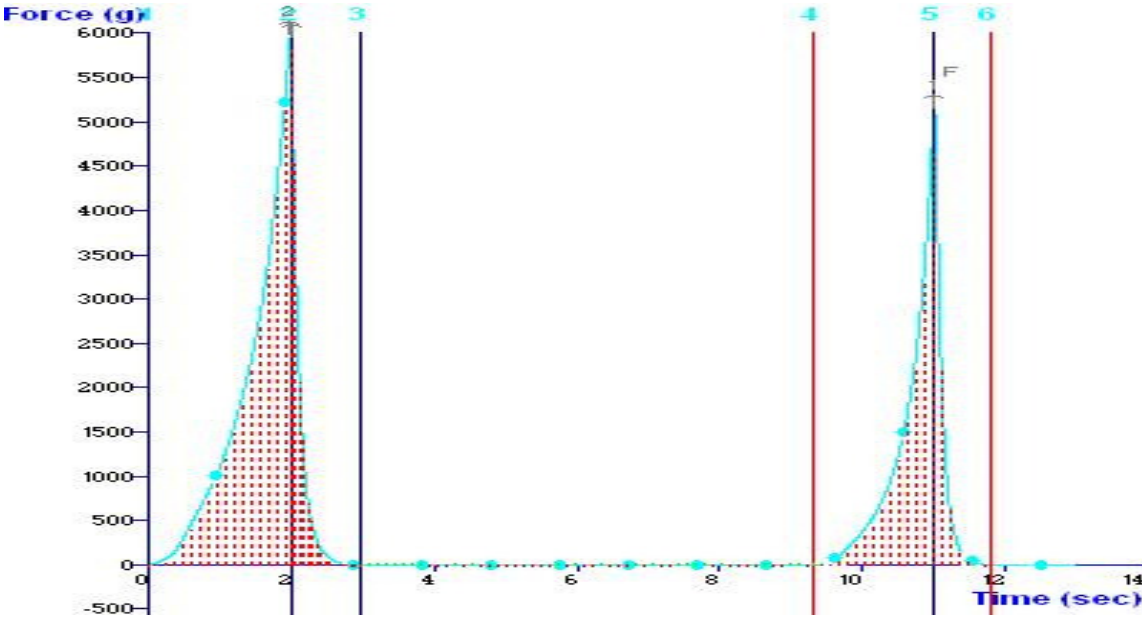
a) TPA of control bread on day 0



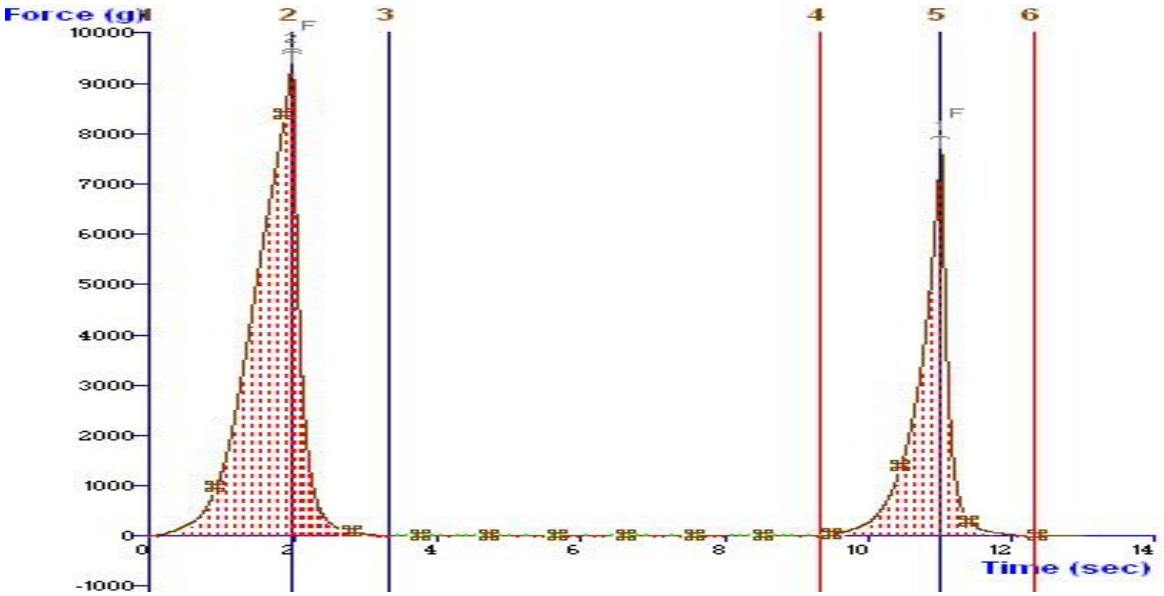
b) TPA of bread containing CGTase on Day 0



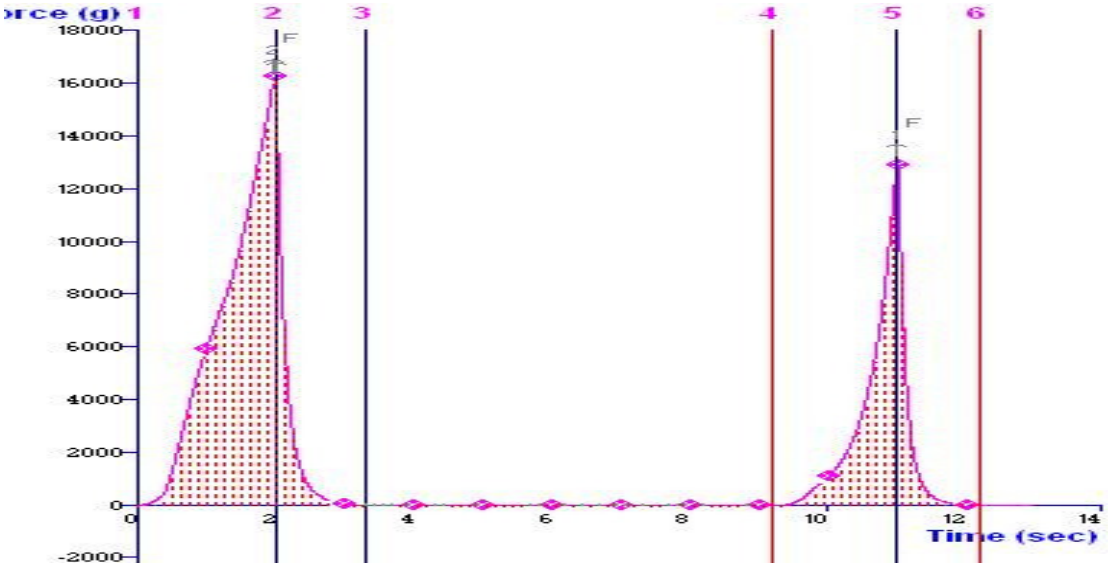
c) TPA of bread containing amylase on Day 0



d) TPA of Control bread on Day 6



e) TPA of bread containing CGTase on Day 6



f) TPA of bread containing amylase on Day 6

Fig. 6.11 : Texture profile analysis (TPA) of Control bread , bread containing CGTase and amylase on day 0 and day 6.

The TPA graphs revealed significant information related to the effect of antistaling enzymes on bread. The adhesiveness of control bread on day 0 is the least as evidenced by the absence of negative area in Fig. 6.11 a. It is

observed from Fig. 6.11 b , that the area 1 of the TPA graph of bread containing CGTase is maximum which demonstrates that its texture is more hard as compared to the other two bread on the day of baking (Day 0). It is observed from Fig 6.11 c , that the hardness of amylase containing bread is less as compared to control and the bread shows absence of fracturability. Retrogradation in bread begins after the water content starts decreasing from the bread. Changes such as crystallisation of amylose and other subunits increases the hardness. It can be observed from TPA graphs of all the breads on Day 6 (Fig. 6.11 d, e and f) that the area 2 of bread containing CGTase decreased significantly demonstrating lesser hardeness. This shows that bread containing CGTase was more softer as compared to other breads (Fig. 6.11, e). The control bread showed a significant fracture in area 1 (Fig. 6.11, e). The TPA graph of bread containing amylase showed that there was no significant reduction in area 1 on day 6 and the bread was hard on day 6 inspite of addition of amylase.

Hardness was monitored for six days after which the bread was spoilt and was unfit for human consumption. The effect of antistaling enzyme on hardness of bread are illustrated in Fig.6.12.

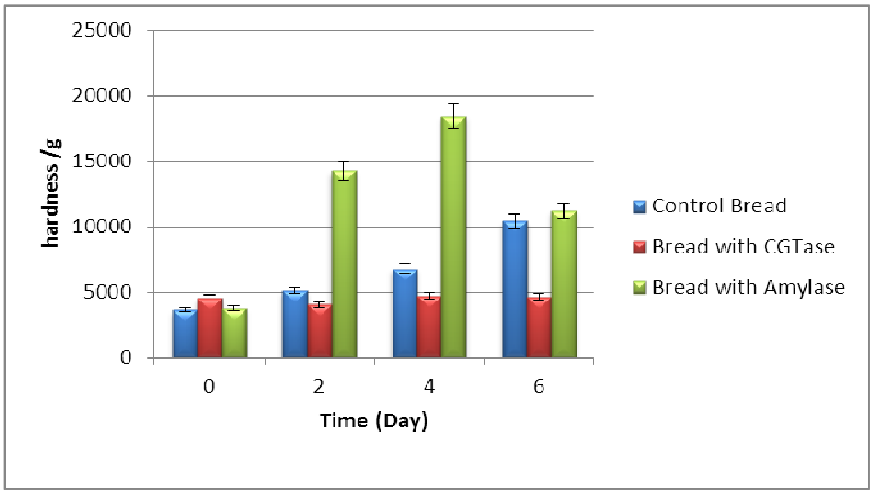


Fig. 6.12 : Effect of CGTase on hardness of bread

It was observed that the bread containing CGTase was more hard as compared to other breads on the day of baking. But the hardness of bread with CGTase was almost constant up to six days. The bread containg

amylase was softer on day 0 , but its hardness increased with time and was highest in comparison with the other breads on the sixth day. As the control bread did not contain any antistaling enzyme, its hardness increased with time. It can be concluded from Fig. 6.12 that CGTase containing bread was softer in comparison with the other breads. CGTase produces cyclodextrins from starch and causes formation of lipid- CD complex which retains moisture. Bread containing amylase was soft on the day of baking, but the amylose produced by amylase starts crystallising and loosing moisture, thus increasing its hardness. The softness of bread is the most important criteria in consumer acceptance of bread. Thus it is important that the bread remains softer for a longer time. Addition of CGTase from *E. aurantiacum* maintains the softness of bread and thus is ideal for bakery industry.

The effect of antistaling enzyme on adhesiveness of bread is illustrated in Fig. 6.13.

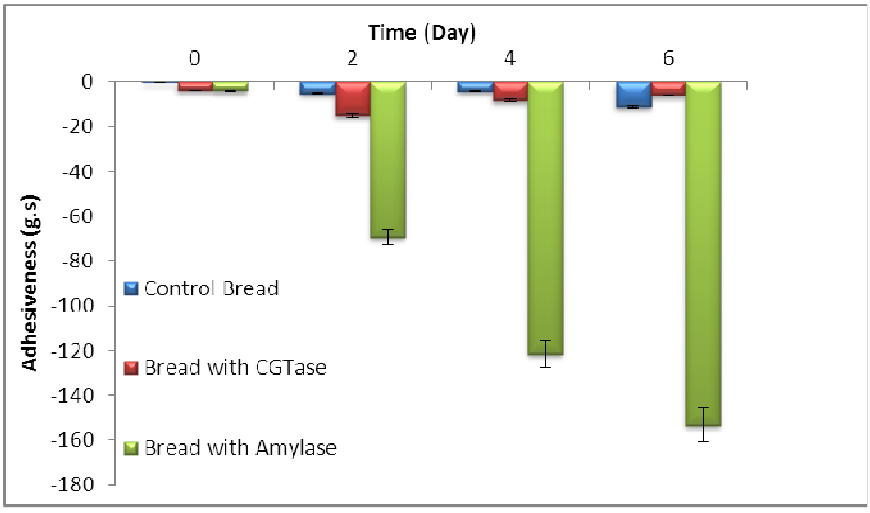


Fig. 6.13 : Effect of CGTase on adhesiveness of bread

Adhesiveness is highly undesirable in bread. It was observed that on addition of amylase, the adhesiveness of bread increased due to over hydrolysis and uncontrolled starch degradation by thermostable amylase (Fig. 6.13). The adhesiveness of CGTase was the least on the day of baking and also after six days. The adhesiveness of control bread increased with

time but was lesser than bread with amylase. These results prove that CGTase is a better enzyme for preventing the development of adhesiveness as compared to amylase.

The effect of antistaling enzyme on cohesiveness is depicted in Fig. 6.14.

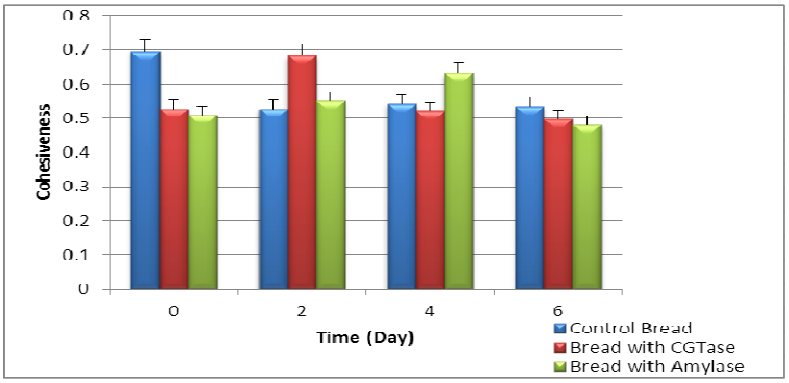


Fig. 6.14 : Effect of CGTase on cohesiveness of bread

Cohesiveness is a diamensionless number which indicates the uniform binding of all the molecules in the bread and absence of lump formation. There was no significant difference in cohesiveness of bread due to addition of the antistaling enzyme. However, the cohesiveness of control bread was greater in comparison to the other two breads (Fig. 6.14).

The effect of antistaling enzyme on gumminess of bread is illustrated in Fig. 6.15.

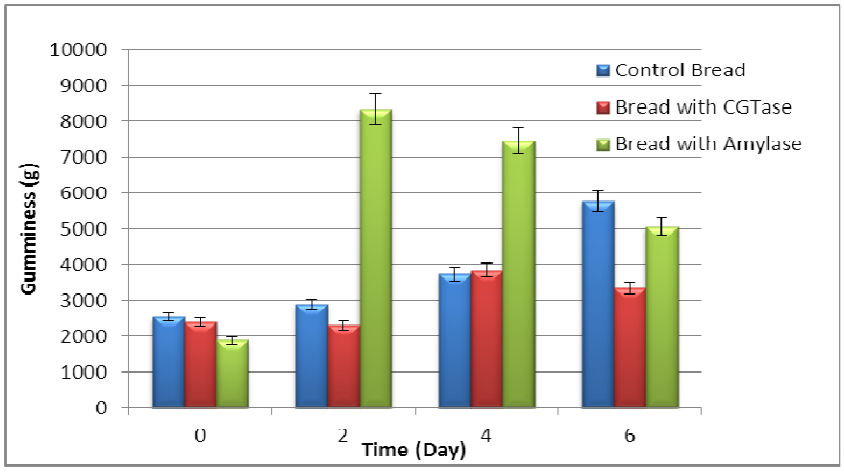


Fig. 6.15 : Effect of CGTase on gumminess of bread

It was observed that the gumminess of bread containing amylase increased upto the second day and decreased therafter till the sixth day. This may be due to the conformational changes in the starch and lipid -gluten complex by amylase. Bread with CGTase demonstrated the least gumminess as seen in Fig. 6.15 which enhances the consumer acceptance of bread.

The effect of antistaling enzyme on chewiness on bread is presented in Fig. 6.16.

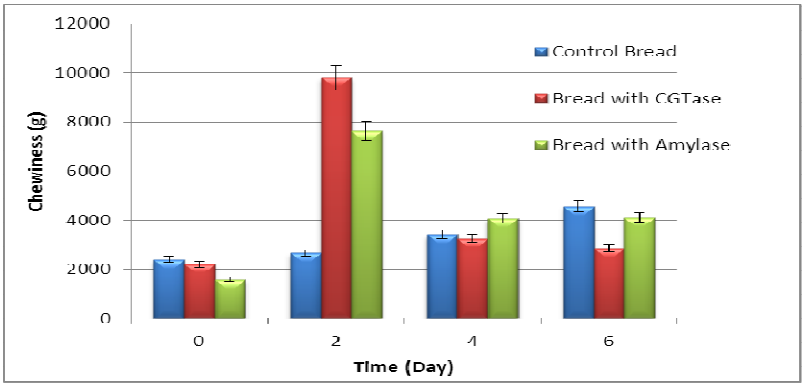


Fig. 6.16 : Effect of CGTase on chewiness of bread

The texture of bread containing CGTase was very chewy on day 2 as compared to other breads (Fig. 6.16). However the chewiness of the bread with CGTase decreased after second day and was least on the sixth day.

The effect of antistaling enzyme on resilience of bread is depicted in Fig. 6.17.

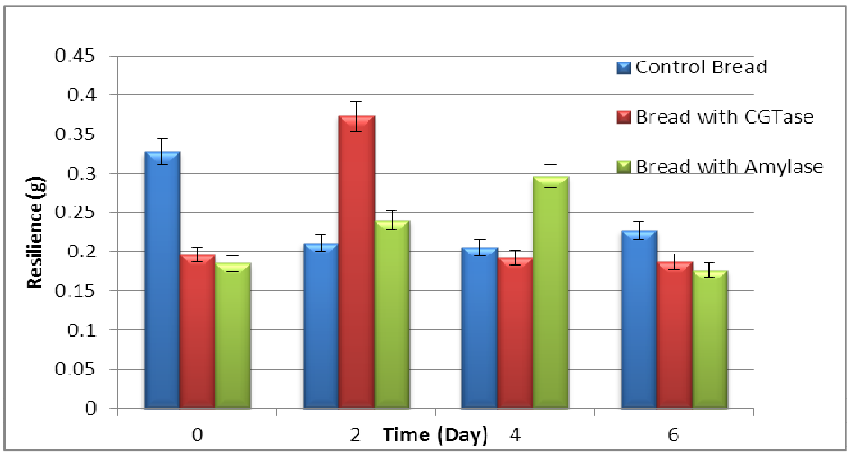


Fig. 6.17 : Effect of CGTase on resilience of bread

Resilience is the ability of the product to regain its original position after application of pressure. The resilience of bread containing antistaling enzymes was lesser as the bread was soft (Fig.6.17). The resilience of control bread was higher due to its hard texture. The overall texturial analysis showed that bread containing CGTase was much softer and more acceptable as compared to the bread containing commercially used antistaling enzyme, amylase.

6.3.3.5 Analysis of bread firmness

The firmness of Control bread, bread containing amylase and bread containing CGTase was studied on day 0, 2,4,6,8 or till the bread was spoilt. The results of analysis of firmness are expressed as force and are represented in Fig. 6.18.

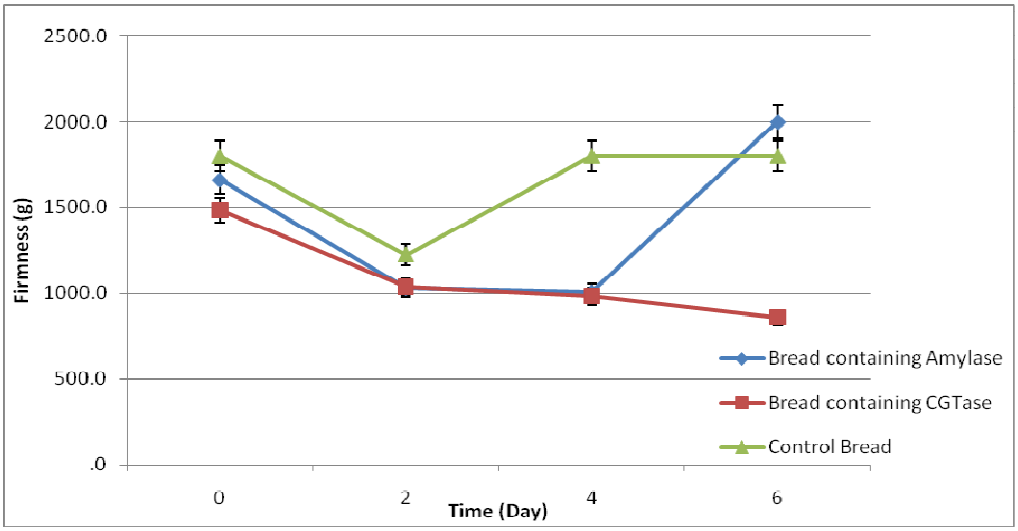
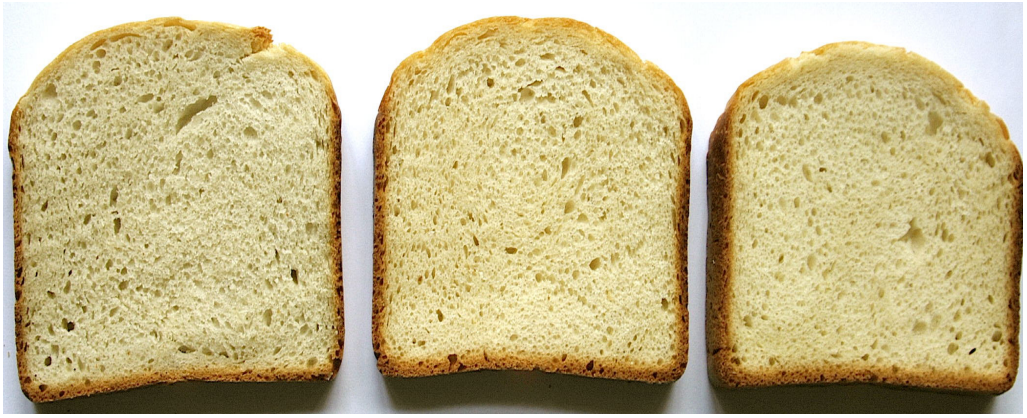


Fig. 6.18 : Effect of CGTase on firmness of bread

It is clearly observed from the above figures that control bread is more firm as compared to breads containing enzymes. Amylase containing bread was softer till the fourth day but the firmness increased on the sixth day. CGTase containing bread was softer for a longer time (8 days) as compared to control.

6.3.3.6 Image and pore size analysis of bread

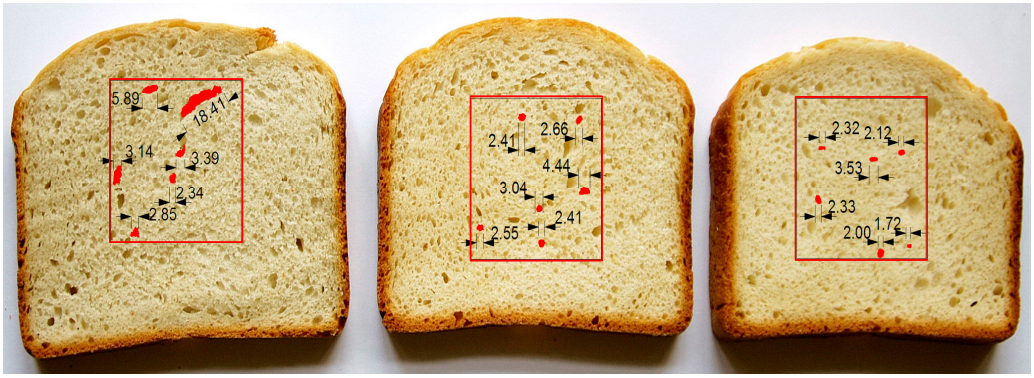
The slices of bread were photographed and the images were analyzed for studying the microstructure of bread. The microstructure of bread is shown in Fig. 6.19.



a) **b)** **c)**

Fig. 6.19 : Microstructure of crumb of bread containing a) amylase b) CGTase and c) control bread.

Large air pockets were observed in bread containing amylase. This explains the increase in volume as well as decrease in gumminess and cohesiveness due to the spongy nature of the bread. However, the bread containing amylase had uneven crust surface as evident by ruptured surface, thus decreasing its consumer acceptance. However, the microstructure of control bread was much better as compared to amylase containing bread. Even pores are formed in the control bread (Fig. 6.19,c) due to carbon dioxide released by yeast. The microstructure of CGTase containing bread did not contain airpockets and was comparable to the control bread**.** The pore size analysis was performed on Rhino 3 D software. Significant variation was observed in the pore sizes in the three types of breads. Large pores, uneven distribution of irregular size pores and large pockets were observed clearly in bread containing amylase (Fig. 6.20).



a) **b)** **c)**

Fig. 6.20 : Pore size analysis of a) Bread with amylase, b) Bread with CGTase and c) Control Bread

On comparison of the pore sizes of all the bread slices, the bread with CGTase was more even as compared to all other breads. The control bread also had even but smaller pores. The pore size of bread with amylase was maximum as illustrated in Fig.6.21.

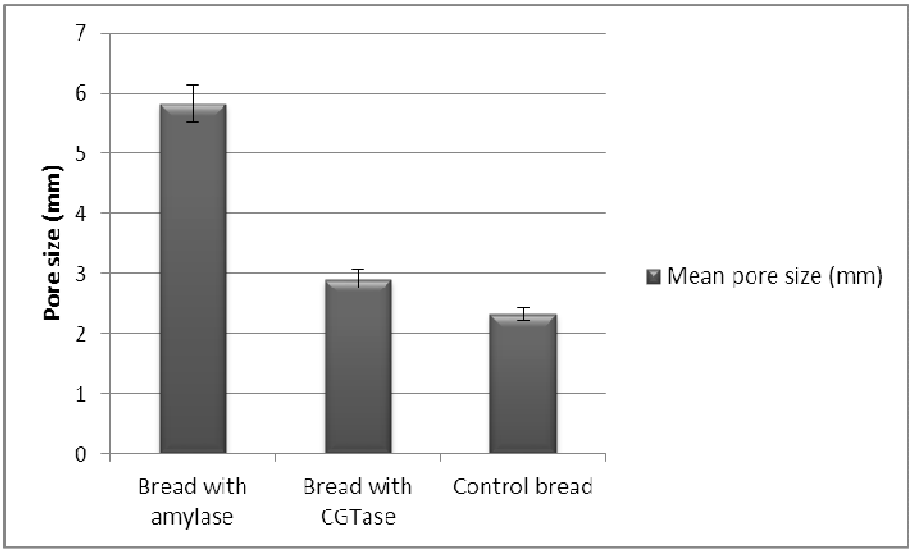


Fig. 6.21 : Effect of CGTase on pore size of bread

During fermentation of the dough, yeast produces carbon dioxide in all the breads. When the temperature increases, amylase and CGTase degrade starch and release oligosaccharides for metabolism of yeast. Amylase rapidly generates amylose and amylopectin which is broken to maltose and glucose. These monosaccharides are utilized by yeast resulting in higher

production of CO_2 leading to large pore size and airpockets in bread with amylase. At high temperatures, CGTase hydrolyses starch to produce CD. CD is not easily metabolized by yeast, and it forms complexes with lipids to maintain even pore size. Smaller monosaccharides are not formed, thus formation of large air pockets is averted.

6.3.3.7 Detection of CD in bread

CD's were detected in the bread baked with CGTase by HPLC at 3.1 min. This shows that the CD's were not degraded at baking temperatures and they maintained the texture and pore size of bread effectively. However, there was no CGTase activity in the baked bread, as CGTase produced by *E. aurantiacum* was degraded at baking temperature.

It is thus observed that the texture of bread prepared using CGTase is softer, is not adhesive, cohesive, gummy, chewy and firm in comparison with bread prepared with amylase and control bread. The CGTase produced by *E. aurantiacum* can thus be used as an antistaling agent in bread making.

6.4 Discussion

The most important application of CGTase is the production of cyclodextrins (CDs) which in turn have vast industrial applications. However the applications are restricted due to the cost of CD.

There is an increasing need for production of alkaliphilic CGTases with novel properties as they are better suited for industrial productions (Horikoshi, 1999).
The thermotolerant and alkaliphilic CGTase produced by *E. aurantiacum* isolated from soda lake of Lonar, India was used for production of cyclodextrins. The production of CD's by *E. aurantiacum* CGTase was optimized. The optimum concentration of starch and enzyme was found to be 3 % and 1 U respectively. Rauf *et al.* (2008) have reported the enzyme and starch concentration are the most significant parameters influencing the production of β- CD. Alves- Prado *et al.* (2008) have reported optimum concentration of starch as 1 % for production of CD's.

The effect of starches on CD and CGTase production was studied. It was observed that *E. aurantiacum* produced maximum enzyme in the presence of soluble potato starch followed by rice, corn, sago and wheat starch. The organism was not able to degrade raw potato starch. There are many reports on effect of starch on CGTase production. Gawande and Patkar (1998) observed low production of CD using corn starch. Similar results were obtained by Goel and Nene (1995) and Mori *et al.* (1994). Wong *et al*. (2008) have produced CD's using sago starch as source of carbon. Sago starch is extracted from tapioca which is a root. Most CGTases are capable of hydrolyzing tuber (potato and sweet potato) and root (tapioca, cassava) starches. Cereal starches (wheat, rice and corn) are not easily hydrolyzed as the lipids and starch form a stable complex and are enclosed in a spherosome dispersed in the endosperm (Alves-Prado *et al*., 2008).

Nogrady *et al.* (1995) found that CGTase was the only starch degrading enzyme in *Bacillus macerans* which hydrolysed the α-1, 4-glucosidic linkages of starch and therefore they suggested that CGTase was thought to play a primary role in the degradation of both amylase and amylopectin. However in earlier report by Jin-Bong *et al*. (1990), soluble starch was more suitable for *B*. *stearothermophillus*. Some reports have mentioned that xylose and glucose were the best sources for production of CGTase by *B. cereus* (Jamuna *et al.,* 1993). In some cases, production of CGTase was enhanced with addition of starch (Thatai *et al*., 1999). The content and structural characteristics of amylose and amylopectin present in starches may vary depending of their botanical sources. The ratio amylose/amylopectin is an important factor to consider for CD production. The helicoidal structure of amylose with loops of six to seven glucose units can contribute with action of CGTase on α- and β-CD formation. However, another factor that should be considered is the lipid concentration of the starch. Root starches (cassava) and tuber starches (sweet potato) show low lipid quantities, less than 0.1%, while in cereal starches (corn), the lipid quantities are high, around 0.5 to 1.0% (Alves- Prado *et al*., 2008). *E. aurantiacum* demonstrated optimum CGTase production in the presence of soluble starch.

The production of CD's by *E. aurantiacum* using soluble starch can be economized by immobilizing the cells. The advantages of using immobilized cells are prolonged and repeated use of the cells, reduced risk of contamination and ease of

separation of cells from fermentation medium (Delani *et al*., 2012). There are a very few reports on immobilization of CGTase producers. *E. aurantiacum* cells immobilized in calcium alginate were successfully used for batch production of CGTase for three runs. Abdel-Naby *et al.* (2011) have reported biosynthesis of CGTase using immobilized *Bacillus cereus* cells. Vassileva *et al*. (2005) have produced CGTase from immobilized cells of *B. circulans* while Delani *et al.* (2012) have improved the production of CGTase by alkaliphilic *B. firmus* and *B. sphaericus* by immobilization on synthetic and loofa sponges. The production of CD's by immobilized cells was further economized by using potato peels which is a waste product of potato chips manufacturing units. The process for production of CGTase using immobilized *E. aurantiacum* cells and potato peel powder as substrate was an inexpensive method for biosynthesis of CD's.

Many undesirable changes take place in bread during storage which is called staling. Staling is defined as increase in firmness and loss in freshness due to gradual changes in the structure of starch (Min *et al*., 1998). The changes in starch take place due to inter or intramolecular association of starch molecules via hydrogen bonds that changes the amorphous state of starch to highly crystalline state (Hebeda *et al*., 1991). This change in bread firmess during staling is called as retrogradation. Enzymes with Intermediate Temperature Stability (ITS) like CGTase could be used as antistaling agents in bread and other products because they have optimum activity at gelatinizing temperature of starch and are inactivated at baking temperatures (Lee *et al.,* 2002). Enzymes like amylase can decrease retrogradation by hydrolyzing starch, amylose and amylopectin to smaller molecules leading to lesser crystallization (Boyle and Hebeda., 1990; Martin and Hoseney., 1991). Lee *et al.* (2002) have reported the use of CGTase produced by *B. stearothermophilus* as an antistaling enzyme. They reported that maltose and water soluble dextrin were most effective in preserving crumb softness. However, the use of α-amylase has become limited despite its antistaling effect because too much of the enzyme can cause stickiness in bread. De Stefanis and Turner (1981) explained that it is the production of branched maltooligosaccharides by α-amylase that leads to gumminess in bread.

The studies on purification and characterization of CGTase produced by *E. aurantiacum* revealed that it is a novel ITS and alkaline enzyme (extremozyme).

The application of CGTase produced by *E. aurantiacum* as an antistaling agent in bread making is demonstrated through the present studies. Bread baked with CGTase had higher volume than the control bread. Mustaers *et al.* (1995) reported that the loaf volume of bread increased due to production of CD's in bread. The texture profile analysis revealed that bread baked with CGTase from *E. aurantiacum* had softer texture in comparison to the control bread and bread baked with amylase. These results are in accordance with the results obtained by Lee *et al.* (2002) and Shim *et al.* (2004). Gujral *et al.* (2003) have improved the quality of rice bread using CGTase. Besides retrogradation, the porosity and microstructure also influence consumer acceptance of bread. Bread baked with CGTase from *E. aurantiacum* had more even pore size, a better microstructure when compared to the other breads. Hence, CGTase could be effectively used as an antistaling agent in bread. To the best of our knowledge, this is the first report on application of CGTase produced by *E .aurantiacum* as an antistaling enzyme in bread.

Conclusions

The application of CGTase from *E. aurantiacum* in production CD's has been demonstrated in the present studies. Biosynthesis of CD by CGTase is important as CD's have numerous applications in pharmaceutical and cosmetic industries. The production process was made economically feasible by using immobilized cells and inexpensive potato peels (waste of a potato chip manufacturing unit) as substrate. The immobilized cells were used for three runs in batch production mode. This process can be used as an economical alternative for production of cyclodextrins. The application of CGTase as an antistaling enzyme in bread was studied. The bread prepared using CGTase was softer, better in texture, even in pore size and had more shelf life than amylase containing bread. ITS-CGTase from *E. aurantiacum* can thus have potential industrial applications for production of CD's and immense applications as antistaling enzyme in bakery industry. To the best of our knowledge, this is the first report on production of CGTase by immobilized cells of *E. aurantiacum* and application of CGTase as an antistaling enzyme in bread.

SUMMARY AND CONCLUSIONS

The results obtained in the present study are briefly summarized as follows:

CGTase is an important enzyme due to its application in manufacture of cyclodextrins. The literature survey reveals that extremophiles especially alkaliphiles are candidate organisms for the industrial production of CGTase. There were studies on aerobic cultivable microbial diversity of alkaliphiles from Lonar lake, India; but there was no report on the occurrence of CGTase in organisms isolated from Lonar Lake. Thus it was expected that studies on CGTase producing alkaliphilic organisms from Lonar Lake may pave way for discovery of a novel CGTase with immense application for production of CDs. The present investigation was undertaken to assess the alkaliphiles isolated from Lonar lake for production of CGTase and to explore their biotechnological potential and application.

The first objective was the screening of aerobic alkaliphilic bacteria previously isolated and identified from Lonar Lake for CGTase production using phenolphthalein plate and colorimetric assay. 15 isolates from Lonar lake showed starch hydrolytic activity and 6 were positive for CGTase production on Phenolphthalein methyl orange medium. The result of production of CGTase was confirmed by phenolphthalein assay. The six isolates showing positive CGTase activity were *Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1. *Bacillus* sp is a common producer of CGTase and *Paenibacillus sp.* has also been reported for CGTase production . To the best of our knowledge, this is the first report of alkaliphilic *Exiguobacterium aurantiacum, Paenibacillus sp L55* and Lake Bogoria isolate 25 B1 for CGTase production. The enzyme activity, specific activity and production of β-cyclodextrin by *Exiguobacterium aurantiacum* was higher in comparison to Lake Bogoria isolate 25 B1, hence *E. aurantiacum* and *Paenibacillus sp*. L55 it were selected for further studies on optimisation for production of CGTase.

The second objective was optimization of production for CGTase production using *E. aurantiacum* and *Paenibacillus sp*. L55 in 250 ml shake flask fermentation. The Preliminary optimisation of fermentation parameters using one parameter at a time indicated the optimum inoculum medium, inoculum size, inoculum age and agitation selected for CGTase production using *E. aurantiacum* to be Nutrient agar of pH 10, 10%, 24 h and 150 rpm respectively. Similar results were obtained for *Paenibacillus* sp. L55, however optimum pH for production of CGTase was found to be 9.

Optimisation of four important factors which influence fermentation were selected and multilevel testing of 2 factors at a time was studied. Inoculum density was found to be a statistically significant parameter. The optimum inoculum density was found to be 10^8 CFU/ml and optimum medium to flask ratio was found to be 1:5 for both the organisms. Effect of time of fermentation and temperature of fermentation was studied similarly by testing these two factors at multilevel. The optimum time of fermentation for CGTase production is 24 hrs as confirmed by the growth curve studies of *E. aurantiacum*. The optimum temperature for production of CGTase by *E. aurantiacum* was 37°C and *Paenibacillus* sp. L55 was 30°C.

Taguchi DOE revealed that starch is the most significant factor for the CGTase production followed by peptone and yeast extract. pH showed least impact among the factors studied with the assigned variance of values. The error observed was very low which indicated the accuracy of the experimentation. Based on the equation for prediction, the production of CGTase by *E. aurantiacum* can be increased from 4.19 U/ml (which is average activity obtained from the nine trials) in an optimised batch submerged shake flask level fermentation to 6.15 U/ml. CGTase concentration of 6.15 U/ml is 96 % of the predicted >6.4 U/ml with the modified culture conditions while the activity of CGTase using *Paenibacillus* sp. L55 increased from 3 U/ml to 5.15 U/ml. Taguchi experimental design was thus used in combination with conventional and statistical methods successfully to optimise fermentation parameters for production of CGTase and enhance activity of the enzyme. Based on the enzyme activity and growth rate, *E. aurantiacum* was selected for further studies.

The fourth objective was scale-up production of CGTase using *E. aurantiacum* in 3 L and 14 L fermentor. Various parameters like aspect ratio, aeration and agitation were studied. The yield of CGTase was enhanced from 6.15 U/ml in 250 ml shake flask to 7.7 U/ml in 3 L and upto 8.2 U/ml in 14 L fermentor. The production of CGTase was attained in 4 hours in 14 L fermentor which demonstrates that the production process is of a remarkably short time indicating its industrial potential.
Purification and characterization of CGTase was the fifth objective of the present investigation. CGTase produced by *E. aurantiacum* was successfully purified using Starch Adsorption chromatography with 25 fold purification. A single band of 77.84 kDa on SDS PAGE indicates the homogeneity and possible molecular weight. CGTase demonstrated pH optimum 9, pH stability8 – 9, temperature optimum 50°C and temperature stability 50 – 70°C. The enzyme demonstrated intermediate thermal stability (ITS) suggesting its application as an antistaling enzyme in food industry.

The Cyclodextrins were produced by action of CGTase on raw potato starch (Hi media), corn starch (Hi media), soluble potato starch (Hi media), wheat starch (Food grade), rice starch and sago starch. The percentage of starch converted into CDs was calculated by ratio of total grams of CDs formed divided per gram of starch and multiplied by 100. The enzyme activity of CGTase was assessed by phenolphthalein assay. The cyclodextrins were detected by HPLC.

The CGTase was produced by immobilized cells of *E. aurantiacum* entrapped in calcium alginate beads in a packed bed reactor and was compared to the CGTase produced by free cells. It was observed that the immobilized cells could be used for more number of runs (3 runs) as compared to free cells.

For economical production of CGTase, agro-waste like potato-peel waste was used as the sole carbon substrate. The immobilized cells were effectively used for the production of β -Cyclodextrins from potato peel waste with an activity of 5 .1 U/ml as compared to 6 U/ml for free cells. The β –Cyclodextrins produced were detected by HPLC, microscopy and colorimetric estimation. Thus immobilized whole cells can be effectively used for economical production of cyclodextrins from agro – waste based materials.

CGTase produced by *E. aurantiacum* demonstrated moderate thermostability due to which it can be used as an antistaling enzyme in bread. The firmness and textural properties of bread containing CGTase were studied. It was observed that loaf volume, texture of bread and pore size of bread baked with CGTase was better than control bread. CGTase can thus be used to enhance texture and freshness of bread and has a potential application in bakery industry.

Conclusions

- Alkaliphilic microorganisms were found to be suitable candidates for production of CGTase. Alkaliphilic bacteria isolated from Lonar lake, India were exploited for production of CGTase.
- The process parameters were optimized using statistical methods to obtain optimum yield of the enzyme.
- Fermentation parameters like aeration, agitation, inoculum density and the carbon source starch were found to be statistically significant parameters which influenced production of CGTase by *E. aurantiacum.*
- An optimized and rapid fermentative process for production of CGTase by *E. aurantiacum* was thus developed successfully using starch based medium.
- The enzyme was moderately thermostable suggesting its application in food industry.
- Production of CGTase using *E. aurantiacum* was economized by using cheap substrates and immobilization of the culture producing CGTase.
- The CGTase produced by *E. aurantiacum* was used as an antistaling enzyme in bread successfully. ITS-CGTase from *E. aurantiacum* can thus have potential industrial applications for production of CD's and immense applications as antistaling enzyme in bakery industry.

Contributions made to the body of knowledge

1. *Exiguobacterium aurantiacum* and *Lake Bogoria* isolate 25 B1 are reported to produce CGTase for the first time to the best of our knowledge.
2. First report on production of CGTase from Lonar lake isolate, *Paenibacillus* sp. L55.
3. A process for rapid production of CGTase (within 5 hr) in 14 L SS fermentor by *Exiguobacterium aurantiacum* is reported.
4. A mesophilic organism, *E. aurantiacum* is reported for production of novel Intermediate thermo stable (ITS) and alkalitolerant CGTase.
5. Economical production of cyclodextrin using agro- based waste as substrate was investigated.
6. A process for production of CD using immobilized *E. aurantiacum* in packed bed reactor was studied.
7. Application of CGTase produced by *E. aurantiacum* as an antistaling agent in bread making is reported for the first time.

Future prospects

1. The amino acid sequence of CGTase produced by *E. aurantiacum* was partially obtained using MALDI-TOF MS. N- terminal amino acid sequencing may reveal more information on the characteristics of protein.

2. The sequencing of *cgt* gene may provide more insights on the functions and features on the enzyme. Degenerate primers using the conserved sequence of known CGTases have been designed. These studies could be extended further.

3. The literature suggests that there is no significant difference in the taste of bread prepared using CGTase. However, sensory studies may be undertaken to confirm the same.

APPENDIX

1. Horikoshi I medium (g/L)

Glucose	10
Peptone	5
Yeast extract	5
KH_2PO_4	1
$MgSO_4.7H_2O$	0.2
Na_2CO_3	10
Agar	20

Na_2CO_3 (10 % w/v) was sterilized separately and added to the autoclaved medium. pH of the media was 10.

2. Horikoshi II medium (g/L)

Soluble starch	10
Peptone	5
Yeast extract	5
KH_2PO_4	1
$MgSO_4.7H_2O$	0.2
Na_2CO_3	10
Agar	20

Na_2CO_3 (10 % w/v) was sterilized separately and added to the autoclaved medium. pH of the media was 10.

3. Nutrient agar (NA) (g/L)

Peptone	5
Yeast extract	1.5
Beef extract	1.5
Sodium chloride	5
Agar	20

Na_2CO_3 (10 % w/v) was sterilized separately and added to the autoclaved medium. pH of the media was 10.

4. Starch agar (g/L)

10 g Soluble starch in 1 L Nutrient agar.

5. Davis Mingioli's synthetic medium +0.5% peptone as nitrogen source

K_2HPO_4	7
KH_2PO_4	3
$MgSO_4.7H_2O$	0.1
Peptone	1.0

Trace element solution

($FeSO_4.7H_2O$ 0.5, $ZnSO_4.7H_2O$ 0.5, $MnSO_4.3H_2O$ 0.5, H_2SO_4 0.1N 10ml, pH adjusted to 10.0 with 10 % Na_2CO_3.

6. Phenolphthalein methyl orange medium (g/L)

Soluble starch	10
Peptone	5
Yeast extract	5
K_2HPO_4	1
$MgSO_4.7H_2O$	0.2
Na_2CO_3	10
Phenolphthalein	0.3
Methyl Orange	0.1

pH adjusted to 10.0 with 10 % Na_2CO_3.

7. Estimation of β- CGTase (Goel and Nene, 1995)

Procedure

i. Pipette out different volumes of β- CD (20, 40 ,60,100,120,140,160,200 µg/ml) into a series of test tubes and make up the volume to 1 ml with 50 mM Tris Cl buffer of pH 8.5.

ii. To each tube add 4 ml of phenolphthalein reagent and mix well.

iii. Cover the tubes with aluminium foil on top and keep them at room temperature for 20 min.

iv. Measure the O.D. at 550.

v. Plot a standard graph of concentration of β- CD vs. % decrease of OD

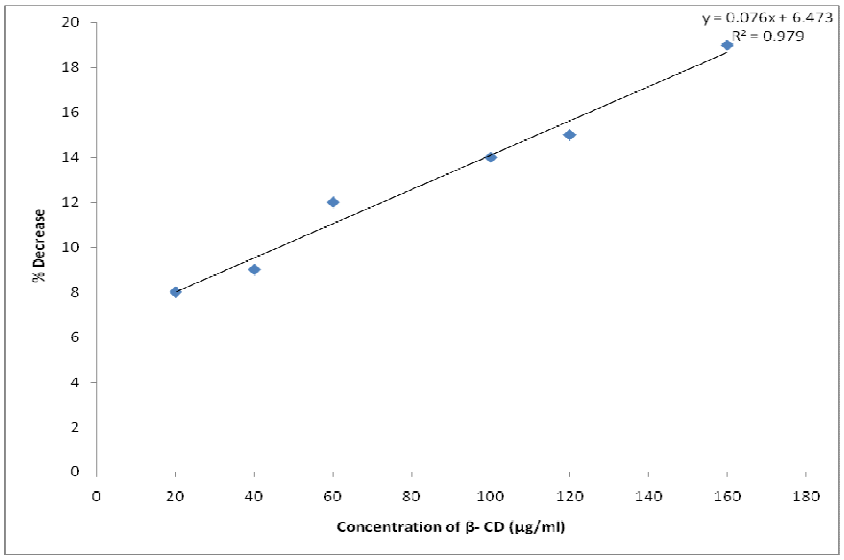


Fig. A1 : Standard graph for calculation of CGTase activity

$$\text{CGTase activity} = \frac{\%\text{ OD decrease X amount of β- CD equivalent to \% decrease X } 10^6}{\text{Molecular weight of (β- CD, 1135) X time (20 min)}}$$

% OD decrease = {OD of blank (control) – OD test / OD of blank }X 100

8. Estimation of proteins (Bradford, 1976)

i. Pipette out into a series of tubes (40, 80,120, 160, 200 µg/ml) standard protein solution (Bovine serum albumin, 200 µg/ml) and make the total volume upto 1ml with distilled water

ii. To each tube, add 5 ml of Bradfords reagent (Biorad ready to use Bradfords reagent) and mix for 5 min.

iii. Measure the blue colored formed at 595 nm

iv. Use a reagent blank without protein is used (distilled water + reagent)

v. Plot a standard graph of concentrations of standard BSA vs. O.D at 595 nm

vi. Calculate the protein content from the standard graph

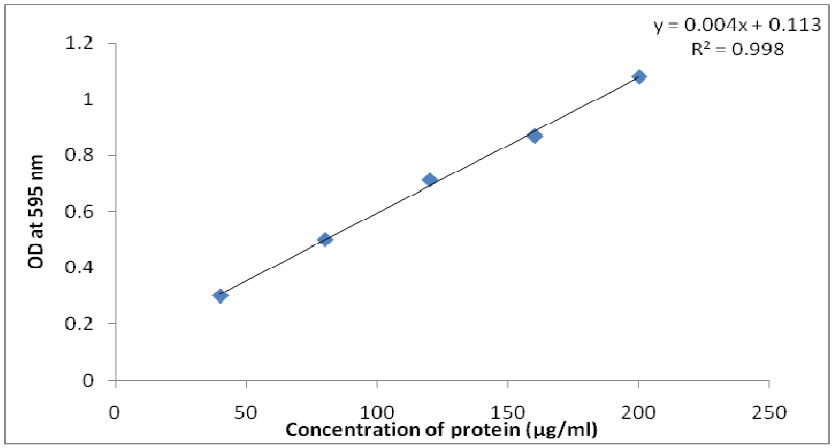


Fig. A2 : Standard plot of concentration of protein vs OD at 595 nm

9. Estimation of starch (Smith *et al*., 1948)

i. Pipette out into a series of tubes (2, 4, 6, 8, 10 mg/ml) standard starch solution (soluble potato starch, 10 mg/ml) and make the total volume upto 1ml with distilled water

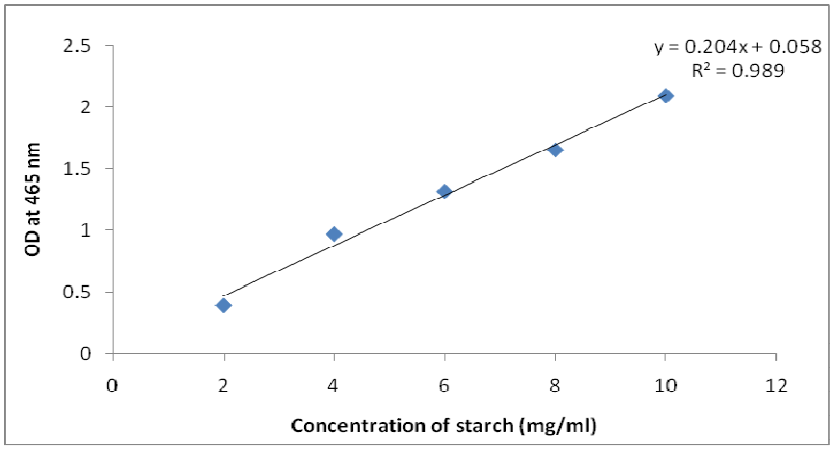
ii. To each tube, add 4 ml of Iodine reagent [0.01 M Iodine (0.3 8 g) in 0.25 M KI (12.45 g in 300 ml distilled water)] and mix for 5 min.

iii. Add 5ml of distilled water to the blue colored formed and measure at OD at 465 nm.

iv. Use a reagent blank without starch (distilled water + reagent).

v. Plot a standard graph of concentrations of standard starch vs. O.D at 465 nm.

vi. Calculate the starch content from the standard graph.



Abdel Fattah YR,Saeed HM, Gohar YM and El-Baz MA. (2005). Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. *Process Biochemistry.* 40: 1707–1714.

Abdel-Naby M A. (1999). Immobilization of *Paenibacillus macerans* NRRL B-3186 cyclodextrin glucosyltransferase and properties of the immobilized enzyme.*Process Biochemistry*. 34: 399-405.

Abdel-Naby MA, El-Refai HA, Abdel-Fattah AF. (2011). Biosynthesis of cyclodextrin glucosyltransferase by the free and immobilized cells of *Bacillus cereus* NRC7 in batch and continuous cultures. *J Appl Microbiol*. 111(5):1129-37.

Abelian VA, Balaian AM, Manukian LS, Afian KB, Meliksetian V,S,, Andreasian NA, Markocian AA.(2002).Characteristics of cyclodextrin glucosyltransferases of various groups of micro organisms. *Prikl Biokhim Mikrobiol*.38, 616-24.

Aeckersberg F, Bak F. and Widdel F. (1991). Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. *Arch. Microbiol.* **156**, 5-14.

Ahn JH, Hwang JB and Kim SH.(1990).Cyclodextrin Glucanotransferase from *Bacillus stearothermophilus*: Purification by Affinity Chromatography and Its Properties. *Kor. J. Appl.Microbial. Biotechnol*. 18: 585-590.

Alves-Prado HF, Carneiro AA, Pavezzi FC, Gomes E, Boscolo M, Franco CM, da Silva R.(2008). Production of cyclodextrins by CGTase from *Bacillus clausii* using different starches as substrates. *Appl Biochem Biotechnol*. 146(1-3):3-13.

Amemura A, Chakraborty R, Fujita M, Noumi T, and Futai M. (1988). Cloning and nucleotide sequence of the isoamylase gene from *Pseudomonas amyloderamosa* SB-15. *J. Biol. Chem.* 263: 9271-9275.

American Association of Cereal Chemists (AACC).1995. www.scisoc.org/aacc

Antranikian G, Ruepp A, Gordon PMK, Ballschmiter M, Zibat A, Stark M, Sensen CW, Frishman D, Liebl W and Klenk H-P. (2009). Rapid Access to Genes of Biotechnologically Useful Enzymes by Partial Genome Sequencing: The Thermoalkaliphile *Anaerobranca gottschalkii*. *J Mol Microbiol Biotechnol.* 16: 81-90.

Atanasova N, Kitayska T, Yankova D, Safarikova M, Tonkova A.(2009).Cyclodextrin glucanotransferase production by cell biocatalysts of alkaliphilic bacilli. *Biochem. Eng. J*.46:278–285.

Aziz SA, Noi SA, Hassan O,Karim MIA, Alitheen NB, Kamaruddin K and Illias RMD. (2007). A rapid screening method for CGTase producing bacteria using different starches as carbon source. *Malays. Appl. Biol. 36: 1–5*

Bakhtiari MR, Faezi MG, Fallahpour M, Noohi A, Moazami N , Amidi Z.(2006). Medium optimization by orthogonal array designs for urease production by *Aspergillus niger* PTCC5011. *Process Biochem*.41: 547–551.

Bautista V, Esclapez J, Pérez-Pomares F, Martínez-Espinosa RM, Camacho M, Bonete MJ. (2012). Cyclodextrin glycosyltransferase: a key enzyme in the assimilation of starch by the halophilic archaeon *Haloferax mediterranei*. *Extremophiles*. 16(1):147-59.

Biwer A, Antranikian G, Heinzle E. (2002). Enzymatic production of CDs. *Appl Microbiol Biotechnol*. 59:609–617

Blackwood AD and Bucke C. (2000). Additional of polar organic solvents can improved the product selectivity of cyclodextrin glycosyltransferase solvent effects on CGTase. *Enzyme and Microbial Technology*. 27: 704-708.

Bonilha PRM, Menocci V, Goulart A J, Polizeli MLTM, Monti R.(2006). Cyclodextrin glycosyl transferase from *Bacillus licheniformis*: optimization of production and its properties. *Braz. J. Microbiol* 37, 317-323.

Bosshard P, Zbinden R, Altwegg M. (2002). International Journal of Systemic and Evolutionary Biology. 52: 2241 -2249.

Bovetto LJ, Backer DP, Villette JR, Sicard PJ and Bouquelet SJ. (1992). Cyclomaltodextrin Glucanotransferase from *Bacillus circulans E 192*. *Biotechnology and Applied Biochemistry*. 15:48-58.

Boyle PJ and Hebeda RE.(1990). Antistaling enzyme for baked goods. *Food Technology*.44:129.

Bradford M.M.(1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*.72:248–254.

Casas JA, de Lara SG, Garcia-Ochoa F. (1997). Optimization of a synthetic medium for *Candida bombicola* growth using factorial design of experiments. *Enzyme and Microbial Technology*. 21:221–9.

Cao X, Jin Z, Wang X, Chen F. (2005). A novel cyclodextrin glycosyl transferase from an alkaliphilic *Bacillus* species: Purification and characterization. *Food Res. Int.* 38:309–314.

Cavalli R, Peira E, Caputo O, Gasco MR.(1999). Solid lipid nanoparticles as carriers of hydrocortisone and progesterone complexes with beta-cyclodextrins. *Int J Pharm* 182, 59 – 69.

Charley H and Weaver C. (1998). Foods: A Scientific Approach, 3rd Edition. PrenticeHall, Inc.

Chen QH, He GQ, Ali MAM. (2002). Optimization of medium composition for the production of elastase by *Bacillus* sp. EL31410 with response surface methodology. *Enzyme and Microbial Technology*. 30: 667–72.

Chien A, Edgar DB, Trela JM. (1976). Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *J Bacteriol*. 127: 1550±1557

Choi J, Lee J, Cjoi K. (1996). *Klebsiella oxytoca* No 19-1 capable for producing α-cyclodextrin. US patent 5492829.

Cockshott AR, Sullivan GR. (2001). Improving the fermentation medium for Echinocandin B production. Part 1. Sequential statistical experimental design. *Process Biochem.* 36:647–60.

Cooper, F.M., Fernstrom , G.A. and Miller, S.A.(1944). Performance of gas-liquid contactors. *Ind. Eng. Chem.*36, 504-509.

Dasu VV, Panda T and Chidambaram M. (2003). Determination of significant parameters for improved griseofulvin production in a batch bioreactor by Taguchi's method. *Process Biochem.* 38: 877-880.

Davies GJ, Wilson KS, Henrissat B. (1997). Nomenclature for sugarbinding subsites in glycosyl hydrolases. *Biochem J*. 321:557–559

Davis BJ. (1964). Disc electrophoresis. 2. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci*. 121 (A2):404-427.

Delani TCO, Pazzetto R, Mangolim CS, Fenelon VC, Moriwaki C and Matioli G. (2012). Improved production of cyclodextrins by alkalophilic *bacilli* immobilized on synthetic or loofa sponges.*Int. J. Mol. Sci.*13:13294-13307.

De Stefanis VA and Turner EW. (1981). US Patent 4 299 848.

Dey G, Mitra A, Banerjee R, Maiti BR. (2001). Enhanced production of amylase by optimization of nutritional constituents using response surface methodology. *Biochem Eng J*.7:227–31.

Duchene D. (1987). Cyclodextrins and their Industrial Uses Editions de Sante, Chapter 6: 211-257.

Fava F, Gioia D, Marchetti L. (1998).Cyclodextrin effects on the ex-situ bioremediation of a chronically polychlorobiphenyl-contaminated soil. *Biotechnol Bioeng.* 58:345–55.

Ferrarotti SA, Rosso AM, Marechal MA, Krymkiewicz N and Marechal LR.(1996). Isolation of two strains (s-r type) of *Bacillus circulans* and purification of a cyclomaltodextrin glucanotransferase. *Cell. Mol. Biol.*42:653-657.

Freitas TL, Monti R and Contiero J. (2004). Production of CGTase by a Bacillus Alkalophilic CGII strain isolated from waste water of a manioc flour industry. *Braz. J. Microbiol*. 35: 255-260.

French D. (1957). The Schardinger dextrins. *Adv Carbohydr Chem.* 12:189–260.

Fujiwara S, Kakihara H, Woo KB, Lejeune A, Kanemoto M, Sakaguchi K, Imanaka T .(1992). Cyclization characteristics of cyclodextrin glucanotransferase are conferred by the NH2-terminal region of the enzyme. *Appl Environ Microbiol.* 58:4016–4025.

Fujita Y, Tsubouchi H, Inagi Y, Tomita K, Ozaki A and Nakanishi K.(1990).Purification and properties of cyclodextrin glycosyltransferase from *Bacillus* sp. AL-6. *J. Ferment. Bioeng.*70:150-154.

Gawande BN, Singh RK, Chauhan AK, Goel A, Patkar AY.(1998). Optimization of cyclomaltodextrin glucanotransferase production from *Bacillus firmus*. *Enzyme and Microbial Technology.* 22: 288-291

Gawande BN and Patkar A. (2001). *Alpha-Cyclodextrin Production using Cyclodextrin Glycosyltrans-ferase from Klebsiella pneumoniae AS-22*. *Starch*.53: 75-83.

Gawande, BN, Patkar, AY. (2001). Purification and properties of a novel raw starch degrading Cyclodextrin glucanotransferase from *Klebsiella pneumonia* AS-22. *Enzyme Microb Technol.* 28:735-743.

Goel A. and Nene S. (1995). *A Novel Cyclomaltodextrin Glucanotransferase from Bacillus firmus that Degrades Raw Starch*. *Biotechnology Letters.*17 (4): 411-416.

Gujral HS, Guardiola I, Carbonell JV, Rosell CM. (2003). Effect of cyclodextrin glycosyl transferase [corrected] on dough rheology and bread quality from rice flour. *J Agric Food Chem.* 18, 51(13):3814-8.

Haga K, Harata K, Nakamura A, Yamane K.(1994). Crystallisation and preliminary X-Ray studies of Cyclodextrin glucanotransferase from alkaliphilic *Bacillus* sp 1101. *J Mol Bio*. 237:163- 4.

Hao DC, Zhu PH and Yang SL. (2006). Optimization of recombinant cytochrome P450 2C9 protein production in *Escherichia coli* DH5a by statistically-based experimental design. *World J. Microbiol. Biotechnol*. 22, 1169-1176.

Harada J, Takaku S, Watanabe K. (2012). An on-demand metalloprotease from psychro-tolerant *Exiguobacterium undae* Su-1, the activity and stability of which are controlled by the Ca^{2+} concentration. *Biosci Biotechnol Biochem.*76 (5):986-92.

Harada T, Misaki A, Akai H, Yokobayashi K, Sugimoto K .(1972). Characterization of *Pseudomonas* isoamylase by its actions on amylopectin and glycogen: comparison with *Aerobacter* pullulanase. Biochim Biophys *Acta*. 268:497–505.

Harata K, Haga K, Nakamura A, Aoyagi M, Yamane K. (1996). X-ray structure of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011. Comparison of two independent molecules at 1.8 A resolution. *Acta Crystallogr D Biol Crystallogr*.52:1136-45.

Hebeda R E, Bowles LK. and Teague W M .(1991). Use of intermediate temperature stability enzyme for retarding staling in baked goods. *Cereal Foods World*. 36:619

Heravi KM, Eftekhar F, Yakhchali B. and Tabandeh F.(2008). Isolation and identification of a lipase producing *Bacillus* sp. from soil. *Pak J Bio Sci.*11 (5), 740 –745.

Higuti IH, Grande SW, Sacco R, Nascimento AJ. (2003). Isolation of alkalophilic CGTase producing bacteria and characterization of cyclodextrin glycosyltransferase. *Braz. Arch. Biol. Technol.*46:183–186.

Higuti IH, Silva PAD, Papp J, Okiyama VMDE, Andrade EAD, Marcondes ADA and Nascimento AJD.(2004). Colorimetric determination of α and β-cyclodextrins and studies on optimization of CGTase production from *B. firmus* using factorial designs. *Braz. Arch. Biol.* Technol. **47**: 837–841.

Civil Engineering and Environmental Systems. 21 (2): 79 -90.

Hirano K, Ishihara T, Ogasawara S, Maeda H, Abe K, Nakajima T .(2006). Molecular cloning and characterization of a novel γ-CGTase from alkalophilic *Bacillus* sp. *Appl. Microbiol. Biotechnol.* 70: 193–201.

Horokoshi, K. (1999). Alkaliphiles: Some applications of their products in Biotechnology. *Microbiol Mol Biol Rev*.63:735-50.

Huang D, Cui F, Li Y, Zhang Z, Zhao J, Han X, Xiao X, Qian J, Wu Q, Guan G. (2007). Nutritional requirements for mycelial biomass and exopolymer production by *Hericium erinaceus* CZ-2. *Food Technol. Biotechnol*.45 (4): 389–395.

Hujanen M, Linko S, Linko YY, Leisola M.(2001). Optimisation of media and cultivation conditions for l(+)(S)-lactic acid production by *Lactobacillus casei* NRRL B-441. *Appl Microbiol Biotechnol* .56:126–30.

Ibrahim A, Al-Salamah A, El-Tayeb MA, El-Badawi YB and Garabed Antranikian. (2012). A Novel Cyclodextrin Glycosyltransferase from Alkaliphilic *Amphibacillus* sp. NPST-10: purification and properties. *Int J Mol Sci*. 13(8): 10505–10522.

Ibrahim HM, Yusoff WMW, Hamid AA and Omar O. (2010). Enhancement of cyclodextrin glucanotransferase production by *Bacillus* G1 using different fermentation modes. *Biotechnology*. 9: 506–512.

Ishmail AS, Sobieh UI and Abdel-Fattah AF. (1996). Biosynthesis of cyclodextrin glycosyl transferase and cyclodextrin *Bacillus macerans* 314 and properties of the crude enzyme.*Chem Eng J*.61: 247-253.

Irie T, and Uekama K. (1999). Cyclodextrins in peptide and protein delivery. *Adv Drug Deliv Rev* 36: 101–123.

Jamuna R, Saswathi N, Sheela R, Ramakrishna SV. (1993). Synthesis of cyclodextrin glucosyl transferase by *Bacillus cereus* for the production of cyclodextrins. *Appl Biochem Biotechnol*. 43:163–176

Janecek S. (1997). α-Amylase family: molecular biology and evolution. *Prog Biophys Molec Biol.* 25:67–97

Jeang CL, Wung CH, Chang BY, Yeh SS, Lour DW.(1999). Characterization of the Bacillus macerans cyclodextrin glucanotransferase overexpressed in *Escherichia coli*. *Proc Natl Sci Counc Repub China B*. 23(2):62-8.

Jemli S, Ben Messaoud E, Ben Mabrouk S, Bejar S. (2008). The cyclodextrin glycosyltransferase of *Paenibacillus pabuli* US132 strain: molecular characterization and overproduction of the recombinant enzyme. *J Biomed Biotechnol*. 692573: 1-9.

Jin-Bong H, Kim SH, Lee TK and Yang HC. (1990). Production of cyclomaltodextrin from *Bacillus stearothermophilis*. *Korean Journal Applied Biotechnology*, 18(6): 578-584.

Jones BB. (1941). Factors for converting percentages of nitrogen in foods and feeds into percentage of protein. *US. Dept. Agric. Circ*.18322.

Joshi AA**,** Kanekar PP, Kelkar AS, Sarnaik SS, Shouche Y and Wani Y. (2007). Moderately halophilic, alkalitolerant *Halomonas campisalis* MCM B-365 from Lonar lake, India. *Journal of Basic Microbiology.* 47: 213-221.

Joshi AA, Kanekar PP, Kelkar AS, Shouche YS, Vani AA, Borgave SB, Sarnaik SS. (2008). Cultivable bacterial diversity of alkaline Lonar Lake, India. *Microb Ecol.* 55:63-72.

Kabaivanova L, Dobreva E and Miteva V. (1999). Production of cyclodextrin glucosyltransferase by *Bacillus stearothermophilus* R2 strain isolated from a Bulgarian hot spring. *Journal of Applied Microbiology*.86: 1017-1023.

Kanekar PP, Nilegaonkar SS, Sarnaik SS, Kelkar AS. (2002). Optimisation of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. *Bioresour Technol*. 85, 87-93.

Kaneko T, Hamamoto T, Horikoshi K. (1988). Molecular cloning and nucleotide sequence of the cyclomaltodextrin glucanotransferase gene from the alkalophilic *Bacillus* sp. strain no. 38-2. *J Gen Microbiol.* 134:97-105.

Katsuragi N, Takizawa N, Murooka Y. (1987). Entire nucleotide sequence of the pullulanase gene of *Klebsiella aerogenes* W70. *J Bacteriol*. 169:2301-2306.

Khairizal M. (2000). Cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp.TS1 bacterium. Universiti Teknologi Malaysia. M.Sc. Thesis.

Khayati G, Pahlevanzadeh H, Ghaemi N and Vasheghani-Farahani E.(2009). Enhancement of 2,3-butanediol production by *Klebsiella pneumoniae* PTCC 1290: Application of Taguchi methodology for process optimization. *African Journal of Biotechnology*.8 (22):6304-6310.

Kim MH, Sohn CB and Oh TK. (1998). Cloning and sequencing of a cyclodextrin glycosyltransferase gene from *Brevibacillus brevis* CD162 and its expression in *Escherichia coli. FEMS Microbiology Letters*. 164: 411-418.

Kimura K, Kataoka S, Ishii Y, Takano T, Yamane K . (1987). Nucleotide sequence of the beta-cyclodextrin glucanotransferase gene of alkalophilic *Bacillus* sp. strain 1011 and similarity of its amino acid sequence to those of alpha-amylases. *J Bacteriol.* 169:4399–4402

Kimura K, Kataoka S, Ishii Y, Takano T, and Yamane K. (1987). Nucleotide sequence of the β-cyclodextrin glucanotransferase gene of alkalophilic *Bacillus* sp. strain 1011 and similarity of its amino acid sequence to those of α-amylases. *J. Bacteriol*. 169: 4399–4402

Kimura K, Kataoka S, Nakamura A, Takano T, Kobayashi S, Yamane K. (1989). Functions of the COOH-terminal region of Cyclodextrin glucanotransferase of alkalophilic *Bacillus* sp. #1011: relation to catalyzing activity and pH stability. *Biochem Biophys Res Commun.*161:1273-9.

Kitamoto N, Kimura T, Kito Y and Ohmiya K. (1992). Cloning and Sequencing of the Gene encoding Cyclodextrin Glucanotransferase from *Bacillus* sp. KC201. *Journal of Fermentation and Bioengineering*. 74(6): 345-351.

Kitahata S. (1995). Catalytic reaction mechanism of amylases and related enzymes. In: Amylase Research Society of Japan (eds) Enzyme chemistry and molecular biology of amylase and related enzymes. CRC, Boca Raton: 6–17.

Klein C, Hollender J, Bender H. and Schulz GE. (1992). Catalytic center for cyclodextrin glycosyltransferase derived from X-ray structure analysis combined with site-directed mutagenesis. *Journal of Biochemistry*.31: 8740-8746.

Knegtel RMA, Strokopytov B, Penninga D, Faber OG, Rozeboom HJ, Kalk KH, Dijkhuizen L and Dijkstra BW. (1995). Crystallographic Studies of the interaction of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 with natural substrates and products. *The Journal of Biological Chemistry*. 270: 29256-29264.

Knegtel RMA, Wind RD, Rozeboom HJ, Kalk KH, Buitelaar RM and Dijkhuizen BW. (1996). Crystal structure at 2.3 Å resolution and revised nucleotide sequence of the thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1. *Journal of Molecular Biology*. 256: 611-622.

Kobayashi S.(1996). Cyclodextrin producing enzyme (CGTase). In:Park K, Robyt J, Choi Y (eds) Enzymes for carbohydrate engineering. Elsevier, Amsterdam, pp 23–41

KrishnaPrasad K, Venkata, Mohan S, Sreenivas, Rao R, Ranjan Pati B and Sarma PN. (2005). Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. *Biochem Eng J*.24:17-22.

Kulkarni SO, Kanekar PP, Jog JP, Patil PA, Nilegaonkar SS, Sarnaik SS, Kshirsagar PR.(2011). Characterisation of copolymer, poly (hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) produced by *Halomonas campisalis* (MCM B-1027), its biodegradability and potential application. *Bioresour Technol*. 102(11):6625-8.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature.227:680–685.

Larsen KL, Duedahl-Olesen L, Christensen HJS, Mathiesen F, Pedersen LH and Zimmermann W. (1998). Purification and characterization of cyclodextrin glycosyltransferase from *Paenibacillus* sp. F8. *Carbohydr. Res.*, 310, 211-219.

Lawson CL, van Montfort R, Strpkoptov B, Rozeboom HJ, Kalk KH, de Vries G, Penninga D, Dijkhuizen L and Dijkstra BW. (1994). Nucleotide sequence and X-ray structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form. *Journal of Molecular Biology*. 236: 590-600.

Lee SL, Chen WC. (1997). Optimization of medium composition for the production of glycosyltransferase *by Aspergillus niger* with response surface methodology. *Enzyme Microbial Technol*. 21:436–40.

Lee SH, Kim YW, Lee S, Auh JH, Yoo SS, Kim TJ, Kim JW, Kim ST, Rho HJ, Choi JH, Kim YB, Park KH.(2002). Modulation of cyclizing activity and thermostability of cyclodextrin glucanotransferase and its application as an antistaling enzyme. *J Agric Food Chem*.50:1411–1415.

Leemhuis H, Rozeboom HJ, Dijkstra BW and Dijkhuizen L. (2003). The fully conserved Asp residue in conserved sequence region I of the α-amylase family is crucial for the catalytic site architecture and activity. *FEBS Letters.* 541: 47-51.

Li C, Bai J, Cai Z, Ouyang F. (2002). Optimization of a cultural medium for bacteriocin production by *Lactococcus lactis* using response surface methodology. *J Biotechnol*. 93:27–34.

Li ZF, Wang M, Wang F, Gu ZB, Du GC, Wu J, Chen J (2007) γ-Cyclodextrin: a review on enzymatic production and applications. *Appl Microbiol Biotechnol.* 77:245–255.

Lima HO, De Moraes FF, Zanin GM. (1998). Beta-cyclodextrin production by simultaneous fermentation and cyclization. *Appl Biochem Biotechnol* .70–72:789–804.

Liu CH, Chu IM, Hwang SM. (2001). Factorial designs combined with the steepest ascent method to optimize serum-free media for CHO cells. *Enzyme Microbial Technol*. 28:314–21.

Loftsson T and Duchêne D. (2007). Cyclodextrins and their pharmaceutical applications. *Int J Pharm*.329: 1-11.

Maekelae M, Mattsson P, Schinina ME, Korpela. (1988). Purification and properties of cyclomaltodextrin glucanotransferase from an alkalophilic *Bacillus*. *Biotechnol Appl Biochem.* 10: 414-427.

Mahat MK, Illias RM, Rahman RA, Rashid NA, Mahmood NAN, Hassan O, Suraini AA, Kamaruddin K.(2004). Production of cyclodextrin glucanotransferase (CGTase) from alkalophilic Bacillus sp. TS1-1: media optimization using experimental design .Enzyme and Microbial Technology.35: 467- 473.

Martins RF and Hatti-Kaul R. (2002). A new cyclodextrin glycosyltransferase from an alkaliphilic *Bacillus agaradhaerens* isolate: purification and characterisation. *Enzyme and Microbial Technology*. 30: 116-124.

Martins RF and Hatti-Kaul R.(2003). *Bacillus agaradhaerens* LS-3C cyclodextrin glucosyltransferase: activity and stability features. *Enzyme and Microbial Technology*. 33: 819 827.

Martin M and Hoseney RC. (1991). A mechanism of bread firming. Role of starch hydrolyzing enzymes. *Cereal Chem*. 68:503-507.

Matioli G, Zanin GM, Guimaraes MF and Moraes FF. (1998). Production and purification of CGTase of alkalophilic *Bacillus* isolated from Brazilian soil. *Appl. Biochem. Biotechnol.* 70 : (2), 267-275.

Matsuura Y, Kusunoki M, Harada W and Kakudo M. (1984). Structure and possible catalytic residues of Taka-amylase. *A. J. Biochem*. 95 :697-702.

Matsuzawa M, KawanoM, Nakamura N, and Horikoshi K. (1975). An improved method for the production of Schardinger b-dextrin on an industrial scale by cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. *Starch* 27,410–413.

Menocci V, Antonio JG, Paulo RA, Olga LT, Daniela PM, Jonas C. and Rubens M.(2008). Cyclodextrin glycosyl transferase production by new *Bacillus.* sp strain isolated from Brazilian soil. *Brazilian Journal of Microbiology.* 39:682-688.

Min BC, Yoon SH, Kim JW, Lee YW, Kim YB and Park KH. (1998). Cloning of novel maltooligosaccharide-producing amylases as antistaling agents for bread. *Journal of Agricultural and Food Chemistry*. *46* (2): 779-782

Montgomery DC. (1976). *Design and Analysis of Experiments.* John Wiley and Sons, New York.

Mori S, Hirose S, Oya T and Kitahata S. (1994). Purification and properties of cyclodextrin glucanotrasferase from *Brevibacterium* sp. no. 9605. *J. Biosci. Biotechnol. Biochem*.58: 1968-1972.

Motallebi M, Afshari Azad H and Zamani MR. (2008) .Polygalacturonase production by *Sclerotinia sclerotiorum*, causal agent of canola stem rot: parameter optimization using Taguchi approach. *World Applied Sciences Journal.* 3 (1): 96-101.

Munro I C, Newberne PM, Young VR, Bär A. (2004). Safety assessment of γ-cyclodextrin. *Regul Toxicol Pharm* 39:S3–S13.

Mutsaers JHGM and Van Eijk JH.(1995). Bread improving composition. Eur. Patent, EPO687414.

Myung HK, Cheon BS and Tae KO. (1998). Cloning and Sequencing of a cyclodextrin glycosltransferase gene from *Brevibacillus brevis* CD 162 and its expression in *Escherichia coli. FEMS Microbiology letters*.164: 411-418.

Nakajima R, Imanaka T, Aiba S. (1986). Comparison of amino acid sequences of eleven different α-amylases. *Appl Microbiol Biotechnol.* 23:355–360

Nakamura, N. and Horikoshi, K. (1976). Purification and properties of cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. *Agriculture Biology Chemistry.* 40: 935-941.

Nakamura A, Haga K, Ogawa S, Kuwano K, Kimura K, Yamane K. (1992). Functional relationships between Cyclodextrin glucanotransferase from an alkaliphilic *Bacillus* and alpha- amylases. Site – directed mutagenesis of the conserved two Asp and one Glu residues. *FEBS Letts.* 296: 37- 40.

Nakamura A, Haga K, Yamane K. (1993). Three histidine residues in the active centre of cyclodextrin glucanotransferase from alkaliphilic *Bacillus* sp. 1011: effects of replacement on pH dependence and transition – state stabilization. *Biochemistry.* 32: 6624-31.

Nogrady N, Pocsi I and Szentirmai A. (1995). Cyclodextrin glycosyltransferase may be the only starch degrading enzyme in *Bacillus macerans*. *Biotechnol. Applied Biochem*.21: 233-243.

Noi-Ai, Sauvaphap, Suraini A, Norjahan A, Osman H and Mohamed A K. (2008). Optimisation of cyclodextrin glycosyltransferase production by response surface methodology approach. *Biotechnology*.7 (1):10-18

Nomoto M, Chen CC and Sheu DC. (1986). Purification and Characterization of Cyclodextrin Glucanotransferase from an Alkalophilic Bacterium of Taiwan. *Journal of Agricultural Biology Chemistry*. 50(11): 2701 – 2707.

Norman BE, Jorgensen ST. (1992). *Thermoanaerobacter* sp. CGTase its properties and application. *Denpun Kagaku*. 39: 101-108

Pandit V, Gorantla R, Devi K, Pai R S, Sarasija S. (2011). Preparation and characterization of pioglitazone cyclodextrin inclusion complexes. Journal of Young Pharmacists.3 (4): 267-274.

Pakzad SR, Ajdary S, Moazami N and Haghighi S. (2005). A Novel Method to Detect β-Cyclodextrin Glucosyl Transferase (β-CGTase) Activity on Polyacrylamide Gels. *Iranian Biomedical Journal. 9 (2): 87-90.*

Park CS, Park KH, Kim SH. (1989). A rapid screening method for alkaline β cyclodextrin-methyl orange containing solid medium. *Agric. Biol. Chem.*53:1167–1169.

Park KH, Kim TJ, Cheong TK and Kim JW. (2000). Structure, specificity and function of cyclomaltodextrinase, a multispecific enzyme of the α-amylase family. *Biochimica et Biophysica Acta*. 1478: 165-185.

Penninga D, van der Veen B, Knegtel RMA, van Hijum SAFT, Rozeboom HJ, Kalk KH, Dijkstra BW and Dijkhuizen L. (1996). The raw starch binding domain of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251. *The Journal of Biological Chemistry*. 271 (51): 32777-32784.

Phadke MS. (1989). Quality Engineering using robust design.1989. Englewoods cliffs, NJ. Prentice Hall.

Pongsawasdi P, Yagisawa M. (1988). Purification and some properties of cyclomaltodextrin glucanotransferase from *Bacillus circulans*. *Agric. Biol. Chem*. 52 :109–1103.

Prakasham RS, Subba Rao C, SreenivasRao R, Sarma PN. (2007). Enhancement of acid amylase production by an isolated *Aspergillus awamori*. *J. Appl. Microbiol.*102: 204-211.

Pujari V, Chandra TS. (2000). Statistical optimization of medium components for enhanced riboflavin production by a UV-mutant of *Eremothecium ashbyii*. Process Biochem.36:31–7.

Qi Q, Zimmermann W. (2005). Cyclodextrin glucanotransferase: from gene to applications. *Appl Microbiol Biotechnol*.66:475–485.

Rashid N, Cornista J, Ezaki S, Fukui T, Atomi H and Imanaka T. (2002). Characterization of an archael cyclodextrin glucanotransferase with a novel C-terminal domain. *Journal of Bacteriology.* 184(3): 777-784.

Rao RS, Prakasham RS, Prasad KK, Rajesham S, Sarma PN and Rao LV.(2004). Xylitol production by *Candida* sp.: parameter optimization using Taguchi approach. *Process Biochem* 39:951-956.

Rauf ZA, Rosli Md. I, Nor MM, Hassan O. (2008).Experimental design to optimization of beta cyclodextrin production from ungelatinized sago starch. *European Food Research and Technology*. 226:1421-1427.

Ravinder K, Tadimalla P, Katikala P and Nalli V. (2012). Immobilization of cyclodextrin glycosyltransferase from newly isolated, mutated *Bacillus* sp. TPR71HNA6 by entrapment technique. *Advances in Applied Science Research*. 3 (4):2288-2298

Rendleman JA. (2000). Hydrolytic action of α-amylase on high-amylose starch of low molecular mass. *Biotechnology and Applied Biochemistry*. 31: 171-178.

Rosso AM, Ferrarotti SA, Krymkiewicz N, Nudel BC. (2002). Optimisation of batch culture conditions for cyclodextrin glycosyl transferase production from *Bacillus circulans* DF 9F. *Microbial Cell factories.*1:3.

Rushton, J, Costich E and Everett H. (1950). Power characteristics of mixing impellers. *Chem. Eng. Prog.* 46(9):467–476.

Savergave LS, Dhule SS, Jogdand VV, Nene SN, Gadre RV. (2008). Production and single step purification of cyclodextrin glycosyltransferase from alkalophilic *Bacillus firmus* by ion exchange chromatography. *Biochem. Eng.J*. 39:510–515.

Schwimmer S. (1953). Evidence for the purity of schardinger dextrinogenase. *Arch. Biochem. Biophys.* 43:108-117.

Schmid, G. (1989). Cyclodextrin glucosyltransferase production: yield enhancement by overexpression of cloned genes. *Tibtech*. 7: 244-248.

Schmid G. (1996). Preparation and industrial production of cyclodextrins. In: Atwood J, Davies ED, MacNicol DD, *et al*.(eds) Comprehesive supermolecular chemistry. Kluwer, Dordrecht: 41–56

Schmidt AK, Cottaz S, Driguez H, Schulz GE. (1998). Structure of cyclodextrin glycosyltransferase complexed with a derivative of its main product beta-cyclodextrin. *Biochemistry*.37:5909–5915.

Shim JH, Kim YW, Kim TJ, Chae HY, Park JH, Cha H, Kim JW, Kim YR, Schaefer T, Spendler T, Moon TW, Park KH. (2004). Improvement of cyclodextrin glucanotransferase as an antistaling enzyme by error-prone PCR. *Protein Eng Des Sel*. 17:205–211.

Sin K, Nakamura A, Kobayashi K, Masaki H and Uozumi T. (1991). Cloning and Sequencing of a cyclodextrin glucanotransferase gene from *Bacillus obhensis* and its expression in *Escherichia coli. Applied Microbiology and Biotechnology*. 35:600-605.

Sivaramakrishnan S, Gangadharan D, Madhavan K, Soccol CR and Pandey A.(2006). α-Amylases from Microbial Sources, *Food Technol. Biotechnol. 44* (2):173–184.

Schmidt AK, Cottaz S, Driguez H and Schulz GE. (1998). Structure of cyclodextrin glucosyltransferase complexed with a derivative of its main product β-cyclodextrin. *Journal of Biochemistry*.37: 5909-5915.

Smith B and Roe J. (1948). A photometric method for determinarion of α-amylase in blood and urine with use ofstarch-iodine colour. *Journal of Biological Chemistry.*53-59.

Sohn CB, Kim SA, Park YA, Kim MH, Moon SK and Jang SA. (1997). Characterisation of cyclodextrin glycosyl transferase from *Bacillus firmus*. *J Korean Soc. Food Sci. Nutr*. 26:351-357.

Sreenivas RR, Prakasham RS, Krishna PK , Rajesham S, Sarma PN ,Venkateswar RL. (2004). Xylitol production by *Candida* sp. : Parameter optimization using Taguchi approach. *Process Biochem.* 39:951–956.

Stanbury PF, Whitaker A and Hall SJ. (1995). Principles of Fermentation Technology. Butterworth- Heinemann. An imprint of Elsevier, Oxford, MA. ISBN: 978-0-7506-4501-0.

Stefanova ME, Tonkova AI, Miteva VI, Dobreva EP. (1999). Characterization and cultural conditions of a novel cyclodextrin glucanotransferase producing *Bacillus stearothermophilus* strain. *J Basic Microbiol* .39:257–63.

Stokopytov B, Penninga D, Rozeboom HJ, Kaljk KH, Dijkhuizen L and Dijkstra BW. (1995). X-Ray structure of cyclodextrin glycosyltransferase complexed with acarbose implications for the catalytic mechanism of glycosidases. *Journal of Biochemistry* 34: 2234-2240.

Svensson B, Jensen MT, Mori H, Bak-Jensen KS, Bønsager B, Nielsen PK, Kramhøft B, PrÆtorius-Ibba M, Nøhr J, Juge N, Greffe L, Williamson G. and Driguez H. (2002). Fascinating facets of function and structure of amylolytic enzymes of glycoside hydrolase family 13. *Biologia Bratislava.* 57(11):5-19.

Szejtli J. (1997). Utilization of Cyclodextrins in Industrial Products and Processes. *Journal of Material Chemistry*. 7(4): 575-587.

Szejtli J. (1998). Introduction and General Overview of Cyclodextrin Chemistry. *Chemical Reviews.* 98: 1743-1753.

Szejtli J. (2004). Past, present, and future of Cyclodextrin research. *Pure Appl Chem.* 6:1825–1845.

Tachibana Y, Kuramura A, Shirasaka N, Suzuki Y, Yamammoto T, Fujiwara S, Takagi M and Imanaka T.(1999). Purification and characterization of an extremely thermostable cyclomaltodextrin glucanotransferase from a newly isolated hyperthermophilic archaeon, *Thermococcus* sp. *Applied and Environmental Microbiology*. 65: 1991-1997.

Takada M, Nakagawa Y and Yamamoto M. (2003). Biochemical and genetic analyses if a novel γ-Cyclodextrin glucanotransferase from an alkalophilic *Bacillus clarkii* 7364. *Journal of Biochemistry.* 133: 317-324.

Takano T, Fukuda M, Monma, M., Kobayashi, S., Kainuma, K. and Yamane, K. (1986). Molecular Cloning, DNA Nucleotide Sequencing and Expression in *Bacillus subtilis* Cells of *Bacillus macerans* Cyclodextrin Glucanotransferase Gene. *Journal of Bacteriology*. 166(3): 1118 –1122.

Takata H, Kuriki T, Okada S, Takesada Y, Iizuka M, Minamiura N and Imanaka T. (1992). Action of neopullulanase. *The Journal of Biological Chemistry*. 267 (26):18447-18452.

Tao, B. Y. (1991). Cyclodextrin Glucanotransferases: Technology and Biocatalyst Design. In : Himmel, M. E. and Leatham, G. F. *Enzyme in Biomass Conversion*. Washington, D.C.:American Chemical Society:372-383.

Terada Y, Yanase M, Takata H, Takaha T, Okada S.(1997). Cyclodextrins are not the major cyclic α-1, 4-glucans produced by the initial action of cyclodextrin glucanotransferase on amylose. *J Biol Chem*. 272:15729–15733.

Techapun C, Poosaran N, Watnabe M and Sasaki K. (2003). Thermostable and alkaline-tolerant microbial cellulose-free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocesses: A review. *Process Biochem*. 38 : 1327- 1340.

Tilden EB and Hudson CS. (1939). *J Am Chem Soc*.61: 2900- 2902

Thatai A, Kumar M, Mukherjee M. (1999). Single step purification process for cyclodextrin glucanotransferase from a *Bacillus* Sp. isolated from soil. *Preparative Biochemistry and Biotechnology*. 29: 35 – 47.

Tomita K, Kaneda M, Kawamura K and Nakanishi K. (1993). Purification and Properties of a cyclodextrin glucanotransferase from *Bacillus autolyticus* 11149 and selective formation of β-Cyclodextrin. *Journal of Fermentation and Bioengineering*. 25: 89-92.

Tonkova, A. (1998). Bacterial cyclodextrin Glucanotransferase. *Enzyme Microbial Technology*. 22: 678 – 686.

Ulukanli Z and Diĝrak M. (2002). Alkaliphilic Micro-organisms and Habitats. *Turk Journal Biology*. 26: 181-191.

Uitdehaag, JCM, Kalk KH, van der Veen BA, Dijkuizen L and Dijkstra BW. (1999). The cyclization mechanism of cyclodextrin glycosyltransferase (CGTase) as revealed by a γ-cyclodextrin-CGTase complex at 1.8-Å resolution. *The Journal of Biological Chemistry*. 274 (49): 34868 – 34876.

Uitdehaag JCM, van der Veen BA, Dijkhuizen L and Dijkstra BW. (2002). Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the α-Amylase Family. *Enzyme and Microbial Technology*. 30: 295-304.

van der Maarel MJEC, van der Veen BA, Uitdehaag JCM, Leemhuis H, Dijkhuizen L. (2002). Properties and applications of starch-converting enzymes of the a-amylase family. *J. Biotechnol*. 94: 137-155.

van der Veen BA, van Aleebek GJWM, Uitdehaag JCM, Dijkhuizen L. (2000). The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms. *European Journal of Biochemistry.* 267: 658-665.

van der Veen BA, Leemhuis H, Kralj S, Uitdehaag JCM, Dijkstra BW, Dijkhuizen L. (2001). Hydrophobic amino acid residues in the acceptor binding site are main determinants for reaction mechanism and specificity of Cyclodextrin glycosyltransferase. *The Journal of Biological Chemistry*. 276(48): 44557-44562.

Van Reit K. (1983). Mass transfer in fermentation. *Trends Biotechnol*. 1(4): 113-116.

Vassileva A, Burhan N, Beschkov V, Spasova D, Radoevska S, Ivanova V, Tonkova A. (2003). Cyclodextrin glucanotransferase production by free and agar gel immobilized cells of *Bacillus circulans* ATCC 21783. *Process Biochem*.38:1585 – 1591.

Villiers A. (1891). Sur la transformation de la fécule en dextrine par le ferment butyrique. *Compt. Rend. Fr. Acad. Sci*.435-8.

Volkova DA, Lopatin SA and Varlamov VP. (2000). One –step affinity purification of cyclodextrin glucanotransferase from *Bacillus* SP. 1070. *BIOCATALYSIS: Fundamentals and applications*.67-69.

Wani AA, Surakasi VP, Siddharth J, Raghavan RG, Patole MS, Ranade D, Shouche YS. (2006). Molecular analysis of microbial diversity associated with the Lonar soda lake in India, an impact crater in a basalt area. *Res Microbiol*.157:928–937.

Wind R, Liebl W, Buitlaar R, Penninga D, Spreinat A, Dijkhuizen L and Bahl H. (1995). Cyclodextrin formation by thermo stable α-Amylase of *Thermoanaerobacterium thermosulfurigenes* EM1 and reclassification of the enzyme as cyclodextrin glycosyl transferase. *Appl Env Microbiol.* 61:1257 – 1265.

Wind RD, Uitdehaag CM, Buitelaar RM, Dijkstra BW and Dijkhuizen L. (1998). Engineering of cyclodextrin product specificity and pH optima of the thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1. *The Journal of Biological Chemistry*. 273 (10): 5771 – 5779.

Wong TE, Rahman RA, Ismail AF, Salleh MM, Hassan O, Kamaruddin K. (2008). Initial Screening of fermentation variables for the production of cyclomaltodextrin glucanotransferase (CGTase) from local isolated *Bacillus stearothermophillus* HR1. Developments in Chemical Engineering and Mineral Processing.13(5-6): 541 – 547.

Yagi Y, Kouno K, Inui TA. A process producing cyclodextrin. Patent 1987; No. 4,317,881 U.S.A.

Yenpetch W, Packdibamrung K, Zimmermann W, Pongsawasdi P. (2011). Evidence of the involvement of asparagine deamidation in the formation of cyclodextrin glycosyltransferase isoforms in *Paenibacillus* sp. RB01. *Mol Biotechnol*. 47 (3):234-42.

Yong J, Choi JN, ParkSS, Park CS, Park KH and Choi YD. (1996). Secretion of heterologous cyclodextrin glycosyltransferase of *Bacillus* sp. E1 from *Escherichia coli*. *Biotechnology Letters*. 18: 1223-1228.

Yim DG, Sato HH, ParkYH and Park YK. (1997). Production of cyclodextrin from starch by Cyclodextrin Glycosyltransferase from *Bacillus firmus* and Characterization of Purified Enzyme. *Journal of Industrial Microbiology and Biotechnology*.18: 402-405.

Zain W, Salwanis W, Illias Rosli ,Md Salleh, Hassan M, Abdul Rahman, Roshanida and Aidil AH. (2007). Production of cyclodextrin glucanotransferase from alkalophilic *Bacillus sp. TS1-1:* Optimization of carbon and nitrogen concentration in the feed medium using central composite design. *Biochemical Engineering Journal*. 33: 26-33.

Zhou Y, Lee YS, Park IH, Sun ZX, Yang TX, Yang P, Choi YL, Sun M. (2012). Cyclodextrin glycosyltransferase encoded by a gene of *Paenibacillus azotofixans* YUPP-5 exhibited a new function to hydrolyze polysaccharides with β-1,4 linkage. *Enzyme Microb Technol.* 50 (2):151-7.

Zheng X, Wu D, Li Z, Chen J, Wu J. (2011). Enhanced storage stability of recombinant enzyme preparation of alpha-CGTase from *Paenibacillus macerans* by chemical additives. *Sheng Wu Gong Cheng Xue Bao.* 27 (2):185-95

Publications

Papers Published in Journal

R.S.Thombre, P.P.Kanekar and J.M.Rajwade. Production of Cyclodextrin glycosyl transferase from alkaliphilic *Paenibacillus* sp L55 MCM B- 1034 isolated from alkaline Lonar lake, India. *International Journal of Pharma and Biosciences*. (2013) 4(1): B1-9. (In press)

Thombre R S and Kanekar P P. (2011). Studies on cyclodextrin glycosyl transferase (CGTase) producing alkaliphilic bacteria from Lonar Lake, *Journal of Microbial Biotechnology*.10:S1 /Special Issue 2011,doi: 10.4172/1948-5948.1000001.

Book Chapter Accepted for Publication

P.P.Kanekar, A.A.Joshi, S.O.Kulkarni, S.B.Borgave, S.S.Sarnaik, S.S.Nilegaonkar, A.S.Kelkar, **R.S. Thombre**. (2013).Biotechnological potential of alkaliphilic microorganisms in Biotechnology and Bioinformatics- Current Trends and Future perpectives to be published by Society for Applied Biotechnology and Springer, Germany. (In press)

Oral Papers Presented in Conferences

R.S.Thombre, P.P.Kanekar, " Cyclodextrin glycosyl transferase production by alkaliphiles" at World Congress on Biotechnology organized by OMICS Publication group, Los Angelos, USA at Hitech Centre, Banjara Hills, Hyderabad from 21 – 23 March 2011

R.S.Thombre, P.P.Kanekar. Immobilisation of *Exiguobacterium aurantiacum* for production of cyclodextrins at International Conference on Biotechnology for Better Tomorrow organised by Dr. Babasaheb Ambedkar Marathwada University, Aurangabad from 6 – 9 February 2011.

R.S.Thombre, P.P.Kanekar, S.S.Sarnaik, Screening of alkaliphilic bacterial diversity of Lonar lake for industrially important cyclodextrin production at International Conference on Biodiversity and its Conservation organized by Modern College, Pune from 28 to 30 Jan 2011. Received the **"BEST ORAL PRESENTATION AWARD".**

R.S.Thombre, P.P.Kanekar,S.S.Sarnaik, Studies on CGTase producing bacteria from Lonar lake at National Seminar for women ,"WE –MEET" ,organized by College of Engineering , Pune on 7 Sept 2010 . Received **"BEST PAPER AWARD"**

Posters Presented in Conferences

R.S.Thombre, P.P.Kanekar, M.Oak. Application of CGTase produced by *Exiguobacterium aurantiacum* as antistaling agent in Bread making, at Indian Convention of Food Scientist and Technologist (XXI ICFOST) organized by AFSTi at Yashada, Pune on 20 – 21 Jan 2012. **BEST POSTER AWARD**.

Rebecca Thombre, P.P.Kanekar, S.S.Sarnaik. Studies on CGTase producing bacteria from Lonar lake" at 50 th National Conference of Association of Microbiologists of India (AMI) at NCL, Pune from 15 -18 Dec 2009.