Studies on enzyme Cyclodextrin Glycosyltransferase from *Klebsiella pneumoniae pneumoniae* AS-22

A
THESIS SUBMITTED TO THE
UNIVERSITY OF PUNE
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

BY

BHARAT N. GAWANDE

CHEMICAL ENGINEERING DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE 411 008 (INDIA)

JANUARY 2000

CONTENTS

Dr. Anant Patkar
Scientist

Chemical Engineering Division
National Chemical Laboratory
Dr. Homi Bhabha Road
Pune – 411 008, INDIA

Tel: +91-20-5893317 / 58993318
Extn. 2348
Fax: +91-20-5893041
E-mail: patkar@che.ncl.res.in

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled '**Studies on enzyme Cyclodextrin Glycosyltransferase from *Klebsiella pneumoniae pneumoniae* AS-22**' submitted by Mr. Bharat Nathu Gawande was carried out by the candidate under my supervision/guidance at National Chemical Laboratory. Such material as has been obtained from other sources, has been duly acknowledged in the thesis.

Date: Dec 25, 99

(Dr. Anant Patkar)
Research Guide

Acknowledgement

It is my great pleasure to express a deep sense of gratitude to my research guide Dr. Anant Y. Patkar for his excellent guidance, constant encouragement and parental care during the course of this investigation. His determination and ambition to achieve goals in life really fascinated and inspired me.

I am equally obliged to Mr. Sanjay Nene, Head, Biochemical Engineering group for his encouragement and useful suggestions during the progress and completion of this project.

I am grateful to Dr. R. V. Gadre and Mr. V. V. Jogdand for their valuable suggestions, constructive discussions, keen interest and constant encouragement during the tenure of this project. I thank all other Scientists and staff members of Biochemical Engineering Group for their suggestions and constant support in completing this work.

I thank Dr. D. D. Deobagkar and Dr. Mrs. D. D. Deobagkar, University of Pune, for allowing me to work for couple of months in their laboratory. I also thank all my M. Sc. teachers from University of Pune who helped me to rise up to this level.

I thank National Facility for peptide sequencing, Prof. A. K. Lala, Indian Institute of Technology, Bombay, for carrying out N-terminal sequencing.

I wish to sincerely thank Anuj Goel, my senior, who helped me during my initial days and developed my interest in cyclodextrins. Anuj induced the perseverance and strong determination in me. Rohit Gujar, K. D. Deshpande and C. J. Jagtap deserve a special thank for their help in dealing with instruments. I thank Madhuri Shinde for helping me in learning HPLC technique.

I admire the co-operation of Rajesh, Deba, Avinash, Prakash, Sangeeta, Neelima and all other colleagues and friends at Chemical Engineering Division. I would like to express my appreciation to Vasant Jadhav, Deepak Musale, Gopalkrishna and S. S. Hegade for their help and co-operation.

No words can suffice to acknowledge the encouragement and moral support given by my parents. I am grateful to my brother and sisters for their constant support, blessings and love. I appreciate my wife, Trupti, for her support and helping hand in completion of this project in time.

I wish to thank Dr. B. D. Kulkarni, Head, Chemical Engineering Division, the Director, National Chemical Laboratory for permitting me to work in this laboratory and to submit this work in the form Ph. D thesis.

Finally, the financial assistance, from the Council of Scientific and Industrial Research, India, is gratefully acknowledged.

Bharat Gawande

Abstract

The enzyme cyclodextrin glycosyltransferase (CGTase; E.C. 2.4.1.19) synthesizes cyclic malto-oligosaccharides, called cyclodextrins (CDs), from starch or other α-1,4-glucans. CDs are of three major types: α, β and γ containing six, seven and eight glucose molecules, respectively, linked by α(1→4) glycosidic bonds. A CD molecule has a torus-shaped structure with a hydrophobic interior cavity and hydrophillic exterior surface. Therefore, they can form inclusion complexes with a variety of hydrophobic guest compounds or functional moieties and thereby change their physical and chemical properties. CDs have great economic importance and are widely used in pharmaceutical, agricultural, chemical, cosmetic and food industries.

A novel α-CGTase producing organism was isolated from soil samples and taxonomically identified as *Klebsiella pneumoniae pneumoniae* AS-22. The enzyme is a true CGTase and does not possess detectable amylase activity. This CGTase produces predominantly α-CD from starch with negligible amounts of malto-oligosaccharides even at high starch concentrations. It is also capable of degrading raw starch to produce predominantly α-CD. The organism grows very fast with a doubling time of less than 20 min.

This CGTase was purified 736-fold in a sequence of steps consisting of ultrafiltration, affinity and gel filtration chromatography. The specific cyclization activity of the pure enzyme preparation was 523 U/mg of protein. No hydrolysis activity was detected when soluble starch was used as the substrate. The pure protein had a molecular weight of 75 kDa, as estimated by SDS-PAGE and gel filtration. The isoelectric point of the pure protein was 7.3. The enzyme possessed highest activity in the pH range of 5.5-9.0 and was most stable in the pH range 6-9. It was most active in the temperature range of 35-50 °C. However, it rapidly lost activity above 30 °C. Presence of soluble starch and calcium chloride improved the temperature stability of the enzyme up to 40 °C. CGTase was almost 100% stable at 30 °C for a month when stored in presence of 30% (v/v) glycerol. The K_m and k_{cat} values for the pure enzyme were 1.35 mg/mL and 249.13 μM/mg/min respectively, with soluble starch as the substrate. The N-terminal

sequence of the protein showed some homology with another *Klebsiella* CGTase, but no homology with CGTases from other organisms. Preliminary chemical modification studies with carboxylates and tryptophan residues indicated involvement of these residues in CGTase catalytic function. The enzyme predominantly produced α-CD without using any complexing agents. Soluble starch (100 g/L) and raw wheat starch (125 g/L) were converted to 21 and 20% (w/w) CDs, respectively, with 10 U of enzyme/g starch. The ratio of α : β : γ-CD using gelatinized soluble starch and raw wheat starch were, 81 : 12 : 7 and 89 : 9 : 2, respectively.

CGTase production was optimized in shake flasks using a statistical experimental design approach. Effect of various components in the basal medium like carbon, nitrogen, phosphorus and mineral sources, as well as initial pH and temperature, on enzyme production were tested. The optimum concentrations of the selected media components were determined using factorial designs. Two level factorial designs in five variables, namely dextrin, peptone, yeast extract, ammonium dihydrogen orthophosphate and magnesium sulphate concentrations, were constructed. The optimum medium composition was found to be 49.3 g/L dextrin, 20.6 g/L peptone, 18.3 g/L yeast extract, 6.7 g/L ammonium dihydrogen orthophosphate and 0.5 g/L magnesium sulphate. The cell growth and CGTase production profiles were studied with the optimized medium in shake flask and in 1 L fermenter. The enzyme production was found to be growth associated both in shake flasks and fermenters, but was slower in shake flasks. Maximum CGTase activity obtained in the fermenter was 32.5 U/mL in 16 h in optimized medium as compared to 3.75 U/mL in basal medium in 8 h of incubation. The optimized medium resulted in about 9-fold increase in the enzyme activity as compared to that obtained in the basal medium in shake flasks as well as in fermenters.

Alpha-cyclodextrin production from raw starch or dextrin was investigated in the presence of various complexing agents. The addition of n-butanol resulted in maximum conversion of raw wheat starch to α-CD, whereas presence of n-hexanol lead to maximum conversion of dextrin to α-CD. In presence of 2% (v/v) n-butanol, with 125 g/L raw wheat starch and 20 U enzyme/g starch, 42.5% (w/w) conversion to CDs was obtained. The ratio of α : β : γ-CD at this stage was 97 : 3 : 0 with negligible

amounts of malto-oligosaccharides. The production of α-CD was optimized using two-level factorial designs in three variables: dextrin, n-hexanol and enzyme concentrations. The optimum concentrations for α-CD production were found to be: dextrin 500 g/L, n-hexanol 3% (v/v), enzyme 15 U/g dextrin. At this optimum condition, maximum conversion of 12.1% (w/w) of dextrin to total-CDs was achieved and the ratio of α : β : γ-CD was 91 : 3 : 6 with negligible amounts of malto-oligosaccharides. This CGTase was found to be strongly inhibited by α-CD; 50% inhibition was observed at 4 g/L initial concentration of α-CD. The effectiveness of ultrafiltration membrane bioreactor for continuous removal of product was also tested.

CGTase production was extensively studied in batch, fed-batch and continuous mode of operations in automated bioreactors. The fermentation parameters were optimized to achieve maximum growth and enzyme production. Cell growth was faster at 37.5 °C while CGTase production was maximum at 30 °C. The initial pH of 7.0 produced higher amounts of enzyme than controlled pH of 7.0 in basal medium. However, at higher substrate concentration, controlled pH of 7.0 resulted in maximum CGTase production. Low agitation speed caused dissolved oxygen limitation resulting in less growth and enzyme production. Results indicated that an optimum substrate concentration is required for maximum CGTase production; higher concentrations resulted in reduced enzyme production. Induction of CGTase using various carbon sources was also investigated. Results indicated that CDs are likely to induce CGTase production in this organism. No enzyme production was obtained when glucose (G_1) was used as a carbon source. Glucose strongly repressed CGTase production even in the presence of starch or α-CD. CGTase was produced at a very low level when maltotriose (G_3) was used as a carbon source. However, in the presence of malto-oligosccharide mixture (G_4-G_{10}) 65% of CGTase activity was detected as compared to that observed in starch-based medium. Comparable amount of enzyme was produced when α-CD was used as a carbon source; very low levels were produced using β- or γ-CD. High cell density culture was achieved with controlled feeding of glucose-based medium (76 g/L dry cell weight). Controlled feeding of dextrin in fed-batch operation produced 1.7-fold more CGTase and 1.73-fold more dry cell mass as compared to that in batch operation. Continuous culture studies

experiments were performed with feed containing tapioca starch or dextrin. The volumetric enzyme productivity in continuous culture was 1.66-fold higher with tapioca starch and 3.52-fold higher with dextrin as compare to that in batch fermentations.

CHAPTER 1

General Introduction

I. Cyclodextrins

1. Historical background

Cyclodextrins (CDs, cyclomaltodextrins, cycloamyloses) are non-reducing, cyclic malto-oligosaccharides made up of glucopyranose units linked together by α-(1,4)-glycosidic bonds (Figure 1.1). CDs are generally designated by a Greek letter to denote the number of glucopyranose units in them. Thus, α-, β- and γ-CD contain six, seven and eight glucopyranose units, respectively.

Villiers was the first to discover CDs more than a century ago (Villiers 1891). He observed that small amount of crystalline non-reducing dextrins (3 gm in 1000 gm of starch) were formed along with reducing dextrins from the digest of *Bacillus amylobacter* (*Clostridium butyricum*) on starch. These new dextrins had a composition represented by a multiple of the formula $(C_6H_{10}O_5)_2 . 3H_2O$. Villiers named his crystalline dextrins as "Cellulosine" for the simple reason that they resembled cellulose in their non-reducing and acid hydrolysis nature. Later on, in 1903, Schardinger, while working with bacteria responsible for food spoilage, obtained Strain II which possessed considerable starch fermenting property. When grown on starch, this strain gave alcohol-insoluble starch and two types of crystalline dextrins, A and B. Schardinger observed that his crystalline dextrin B was most likely identical with "cellulosines" reported by Villiers. Unfortunately Schardinger could not maintain this Strain II and lost it. Subsequently, in 1904, Schardinger isolated a new strain, which had a pronounced disintegrating or rotting action on carbohydrate-containing media. It produced acetone and ethyl alcohol from sugar- or starch-containing plant materials. He named this organism as *Bacillus macerans* (*macerare* means to rot).

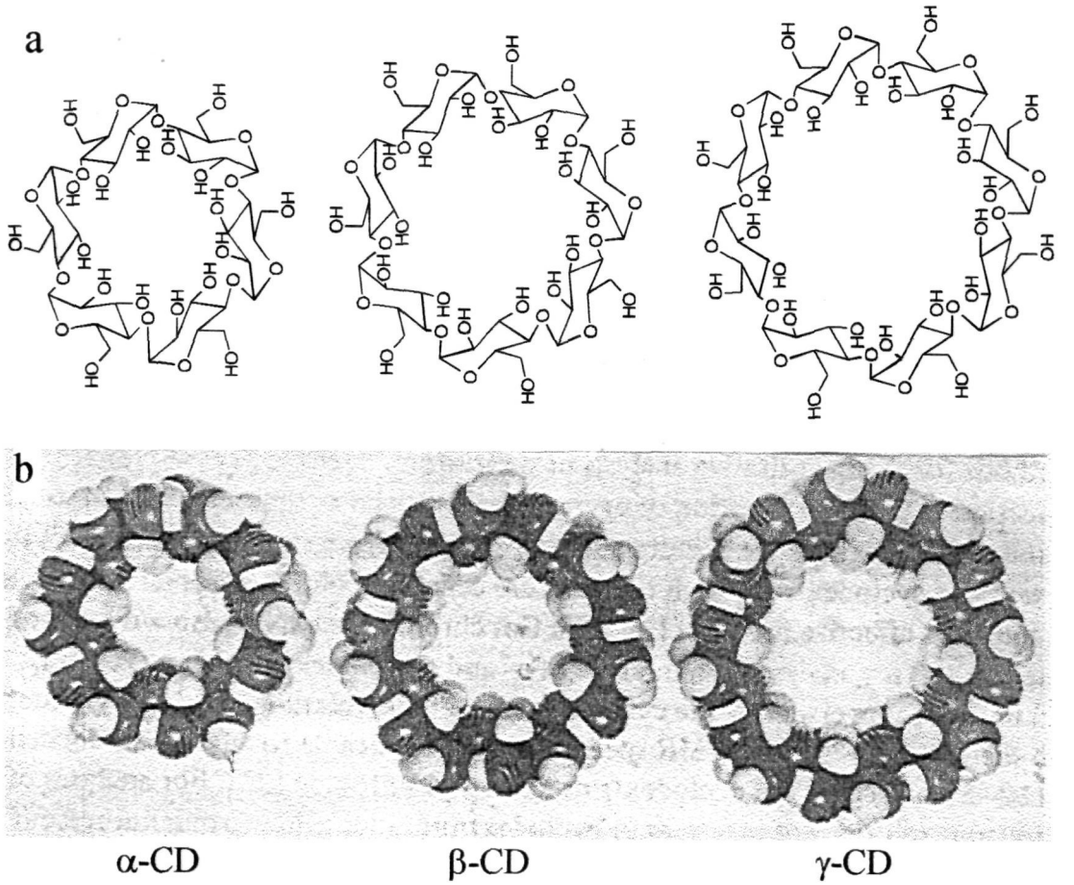
In 1908, Schardinger reported production of crystalline non-reducing polysaccharides from starch by the action of *Bacillus macerans*. The two types of dextrins were named as 'crystalline amylose' and 'crystalline amylodextrin'. Schardinger further studied and renamed these crystalline dextrins as 'dextrin α' and 'dextrin β' (Schardinger 1911). He observed upto 25-30% conversion of potato starch paste to crystalline dextrins (the major one being dextrin α) under the influence of action of *B. macerans*.

Pringsheim and co-workers published a lot of work on Schardinger dextrins between 1912-1934, but their work was of limited value (French 1957; Szejtli 1996a). Freudenberg and Jacobi (1935) developed a fractionation scheme for dextrins and also discovered γ-dextrin. The cyclic nature of Schardinger dextrins, containing glucose units linked by α, 1,4-glycosidic bonds, was shown by Freudenberg and coworkers (Freudenberg et al., 1936, 1938, 1939; Freudenberg and Rapp 1936; Freudenberg and Meyer-Delius 1938). The accurate molecular weights of α- and β-dextrin was determined by French and Rundle (1942) using X-ray crystallography. They named α-dextrin as cyclohexaamylose and β-dextrin as cycloheptaamylose. Freudenberg and Cramer (1948) and French et al. (1950) discovered the molecular structure of the γ-dextrin and the ability of the cyclodextrins to form inclusion complexes.

The new members of the Schardinger dextrin series, containing 9-12 residues (δ-, ε-, ζ-, η-cyclodextrins) were reported by Pulley and French (1961). The molecular size and structure of these higher CDs was also studied by French et al. (1965). The cyclodextrins, with less than six glucose residues, cannot be formed because of steric reasons as noted by Sundararajan and Rao (1970). The higher members in the cyclodextrin series were isolated and purified (13-21 residues) by Miyazawa et al. (1995) and Endo et al. (1995, 1997a and b, 1998). Recently Terada et al. (1997) have reported synthesis of large ring cyclodextrins (LR-CDs) up to 60 residues, from synthetic amylose substrate by purified enzyme from *Bacillus* sp. A2-5a.

2. *Structure and properties*

Cyclodextrins have a peculiar doughnut-shaped or torus-shaped ring structure with all the glucose units in $^4C_1$ chair conformation (Figure 1.1). The secondary hydroxyl groups (on the C2 and C3 atoms of the glucose units) are located on the wider edge of the ring while primary hydroxyl groups (on the C6 atom of the glucose units) are located on the other edge, resulting in the hydrophillic exterior. The internal cavity of the torus is lined with electron-dense glycosidic bridges and apolar hydrogens on C3 and C5 atoms making it hydrophobic in nature.



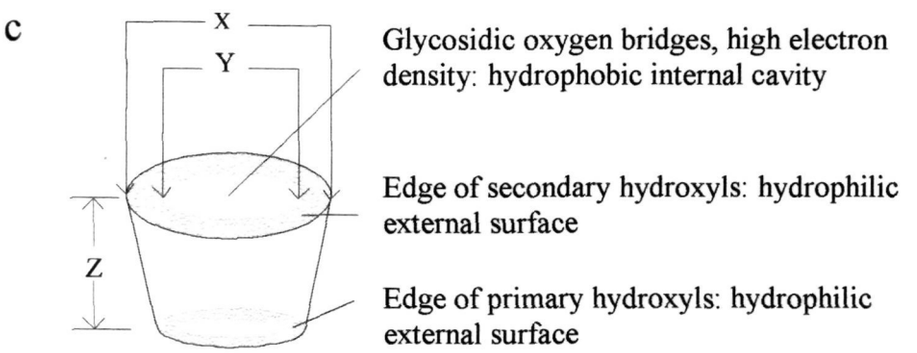


Figure 1.1 (a) Structures of α-, β- and γ-CDs, (b) their molecular models viewed from the secondary hydroxyl side of the torus and (c) schematic presentation of three-dimensional shapes and sizes. For sizes of x, y and z, see Table 1.1 (Source: Bender and Komiyama 1978; Szejtli 1996b)

The aqueous solubility of different CDs is in the order $\beta < \alpha < \gamma$ (Table 1.1). The solubilities of CDs strongly depend on the temperature. At 50 °C the solubility of all CDs is about three times more than that at 20 °C. CDs are insoluble in most pure water-miscible solvents but show a maximum on the concentration-solubility curve (Szejtli 1996b). The solubility of β-CD increases as volume of alcohol is increased in a water-alcohol mixture up to 0-30% (propanol or ethanol), then it decreases as alcohol concentration is increased. At 100% alcohol (absolute), no β-CD is soluble. This is called concentration-solubility curve and there is maximum solubility of β-CD at one point on this curve. CDs tend to decompose when heated above 200 °C. CDs are hydrolysed by strong acids like conc. HCl and H_2SO_4, but are 2-5 times stable in acids as compared to acyclic dextrins. CDs are not hydrolysed with bases even at high temperature.

Characteristic	α-CD	β-CD	γ-CD
Molecular weight (Da)	972	1135	1297
Number of glucose units	6	7	8
Solubility in water at 25 °C (g/L)	145	18.5	232
Outer diameter (x) (Å)	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
Cavity diameter (y) (Å)	5.0 ± 0.3	6.25 ± 0.25	7.9 ± 0.4
Height of the torus (z) (Å)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Approx. cavity volume ($Å^3$)	174	262	427
Approx. cavity volume in 1 mol of CD (mL)	104	157	256
Approx. cavity volume in 1 gm of CD (mL)	0.10	0.14	0.20

Source: Szejtli 1996b. The outer diameter (x), cavity diameter (y) and height of the torus (z) are shown in Figure 1.1c

Table 1.1 Characteristics of α-, β- and γ-CD

CDs are completely resistant towards β-amylase attack, as they do not contain end groups susceptible to such enzymatic action. However, α-amylases from *Aspergillus oryzae*, *Bacillus subtilis*, *Peudomonas* sp., *Filobasidium capsuligenum* and *Trichosporon pullalans* as well as glucoamylase from *Flavobacterium* sp. are reported to hydrolyse CDs; the rate of hydrolysis is much slower than that for starch (Saha and Zeikus 1992). The enzyme cyclodextrinase from *Bacillus coagulans*, *Bacillus macerans*, *Bacillus sphaericus* can degrade CDs very rapidly (Saha and Zeikus 1992).

The hydroxyl groups of CDs can be modified or substituted by ether, ester, amino (substituted amino), azido and other groups and may change their physical and chemical properties drastically. e.g. the aqueous solubility of modified CDs can be 500 g/L or higher (solubility of β-CD is 18.5 g/L in water at 25 °C). Crosslinking of β-CD with epichlorohydrin results in a insoluble CD-polymer in water.

3. Formation of inclusion complex

One of the most important characteristics of CDs is the formation of inclusion complexes with a variety of hydrophobic compounds or functional groups (guests). In such complexes the guest compounds or functional groups are encapsulated in the CD (host) cavity (Figure 1.1). The guest compounds range from polar reagents such as acids, amines, small ions and halogen anions to highly apolar aliphatic and aromatic hydrocarbons and even rare gases (Bender and Komiyama 1978). The most important criterion for the formation of inclusion complex is the three dimensional shape and size of the hydrophobic guest molecule.

There are four different effects involved in inclusion complex formation. They are i) Van der Waals interaction between the guest and the host, ii) hydrogen bonding between the guest and the hydroxyl groups of the CD, iii) release of high-energy water molecules from the CD cavity during complex formation and iv) release of strain energy exerted by the water molecules on the macromolecular ring of the CD. The relative extent to which these interactions contribute depends on the nature of included guest molecule. Griffith and Bender (1973) suggested that water molecules in the CD cavity are enthalpy rich, since they can not form their full complement of hydrogen bonds to

adjacent water molecules. Therefore, inclusion complex formation involves replacement of these high enthalpy water molecules by guest compounds, resulting in a favourable enthalpy change. According to Saenger (1980, 1984), CD can release the strain energy of its macrocyclic ring after inclusion complex formation by displacing the water molecules from the CD cavity.

In aqueous solutions, the ratio of guest to host is usually 1 : 1. This is not necessarily true in the crystalline state. X-ray crystallography studies on inclusion complexes have shown three different structures, namely cage, channel and layer structures (Harata 1996). The cage structures are a result of a displaced arrangement of CDs, in which guest molecules are located in discrete small cavities represented by annular apertures. The cage-type structure is observed when the guest molecule is small enough to be fully enclosed within the CD cavity. Small molecules like methanol, 1-propanol, 3-iodopropionic acid, iodine are included in α-CD to form cage type packing structure. The channel structures develop when CDs stack on top of one another to yield endless channels, in which guest molecules are included. Channel type packing structure can be formed by acetate, γ -aminobutyrate, 1-propanesulfonate, iodine-iodine complex and methyl orange with α-CD. The CD molecules are arranged nearly parallel to the ring plane to form a molecular layer and the adjacent two layers are shifted by half a molecule to each other. As a result, both ends of the cavity are open to the intermolecular space of the next layer to trap guest molecule. The layer structure is observed when the guest molecule is too large to be included, *e.g. p*-nitrophenol and *p*-iodoaniline with α-CD.

4. Applications

CDs can form inclusion complex with a variety of guest molecules and thereby change the physical and chemical properties of the included guest molecule. Therefore, CDs find numerous applications in food, pharmaceutical, agricultural, chemical and cosmetics industries.

a. *Cyclodextrins in foods, cosmetics and toiletries* (Hashimoto 1996)

i. Applications to foods

CDs can protect foods against oxidation, light and heat. The flavour components of fruits, spices and essential oils can be stabilized by their encapsulation in CDs. Foods which have a high fat content, like nuts (almond, cashew, pista) are difficult to powderize. CDs are used for powderization of such nuts and are used for making cakes, as seasonings of various dishes and in curry. The purpose is to prevent oxidation of unsaturated fatty acids present in them. Vinegar and other organic acids can be powdered for their safe storage and transport. The natural colours or pigments are very unstable when exposed to light and change in pH; CDs can improve their stability.

- *Removal of unpleasant taste, odour and undesired components*

The peculiar grassy smells in soya beans can be removed by adding CDs during the manufacture of soy products. CDs help in texture improvement in fish paste, sea foods and meat products. The typical smell of stored rice can be eliminated with CDs. The bitter taste in milk casein hydrolysate, orange, citrus and grapefruit juice products can be debittered. The bitterness in coffee, tea, cocoa and cola drinks can be removed by decaffeination with CDs. Most recently CDs have been used for selective removal of cholesterol from different foods such as egg yolk, sunflower oil, animal fats and dairy products.

- *Food quality improvement*

CDs are used to improve emulsification of whipping creams. A CO_2-α-CD complex can be utilized as emulsifying or foaming baking powder for sponge cakes. Sponge cakes manufactured with oil-in-water fat and oil composition containing β-CD had good taste and readily melted in mouth. They are also used to prevent precipitation or turbidity in canned citrus products, soft drinks, coffee and tea extracts. Storage stability of many foods or food components can be improved. Mixture of wheat flour and CD was fermented and baked to manufacture bakery products of good flavour and texture. CDs can increase shelf life of powdered green tea. They are also used for food preservation and in packaging, *e.g.* mushrooms can be preserved in plastic containers containing paper

treated with CDs. Fresh lemons when stored at 25 °C with paper coated with CD-complexes, showed no change in their quality after 60 days.

ii. Cyclodextrins in cosmetics and deodorants

CDs are used to retain fragrance in perfumes, toothpaste, cosmetic creams, lotions, shampoos and dusting powders (talcum powder). Oral deodorizers can be prepared by compounding CDs in the form of powders, tablets, drinks, mouthwashes, chewing gum or toothpaste.

iii. Applications in house hold items and toiletries

CD-complexed fragrance can be utilized in heat volatile solid perfumes in fragrant candles, incenses and detergents. CDs, when added to the rinsing water in a laundry, removes last traces of detergent by acting as defoaming agents and may help to save water. CDs, added in diluted ethanol, can act as a deodorant for toilet air, or spoiled waste. CDs are used as odour-controlling or deodorant component when mixed with conventional deodorants such as terpenes in disposable diapers and sanitary napkins. Fragrant paper can be prepared using CD complexed with perfumes, insecticides, fungicides and bactericides.

b. ***Applications of cyclodextrins in pharmaceuticals*** (Nagai and Ueda 1996; Uekama and Irie 1996)

Natural CDs have been extensively used to improve solubility, stability and bioavailability of drug molecules. CDs are useful in improving oral absorption of poorly water-soluble drugs like steroids, cardiac glycosides, nonsteroidal anti-inflammatory drugs, barbiturates, anti-diabetics, vasodilators, etc. CDs are used for controlled release of drugs to avoid development of tolerance, *e.g.*. nitrates, antibiotics and contraceptive steroids. CDs can be used in drugs either for complexation or as auxiliaries such as carriers, dilutents, solubiliser or tablet ingredients. Following are examples of approved and marketed pharmaceutical preparations using CDs (trade and manufacture names are given in the brackets): PGE_1-α-CD, PGE_2-β-CD (Prostadin, Ono, Japan), piroxicam-β-CD (Brexin, Chiesi, Italy; Cicladol, Masterpharma, Italy), Garlic oil-β-CD (Allidex, Hermes, Germany; Tegra, Bipharm, Germany), Benexate-β-CD (Ulgut, Teikoku, Japan), Iodine-β-CD (Mena-Gargle, Kyushin, Japan), Dexamethasone-β-CD (Glymesason

ointment,Fujinara, Japan), Cefatiam hexetil hydrochloride-β-CD (Pansporin T, Takeda, Japan), Cephalosporin-β-CD (Meiact, Meiji Seika, Japan).

c. ***Applications in agricultural industry*** (Szejtli 1984; Szente and Szejtli 1996)
Molecular encapsulation of pesticides by CDs modifies wettability, solubility, volatility and smell of pesticides and can also improve their stability and bioavailability. CDs are used in insecticide preparation for effective control of termites. A sparingly water soluble and light sensitive fungicide, fenarimol, when complexed β-CD becomes light resistant and water soluble. Maleic acid and fumaric acid-CD complexes are known to function as sustained-release fungicide compositions. CDs have been found to enhance the activity of benomyl, metomeclan and fenarimol fungicides. Insect repellents can be formulated with methylated β-CD. The germination of seeds can be temporarily retarded by treating the seeds with CDs, which helps later on for vigorous plant growth. CDs are complexed with several plant growth regulators and ripening agents, *e.g.*. ethene gas, auxine-like substances, gibberellins and cytokinines.

d. ***Applications in chemical products and processes*** (Szejtli 1996c)
CDs are used to improve relative sensitivity and fogging of silver halide-containing photographic materials, when added to the light sensitive photographic gelatin layers. CD complexes can be utilized as cross-linking agents in foamed polyurethane sheet production. CD complexes are compatible with thermo-plastic resins and help to retain fragrance for a long time. Water-resistant membranes, containing CDs, can separate enantiomers of a racemic mixtures, such as D, L-tryptone. Rubber compositions with improved ozone resistance contain CD complexes of various antioxidants. Biodegradable plastic can be prepared by blending β-CD complex with a plastic substance to protect the plastic from deteriorating agents during useful life of the plastic. However, when discarded, CD can be degraded by microbial action. Epoxy resin adhesives are produced and stored as separated components and are mixed just before use. When CD is complexed with a polymerization agent in such adhesive, only one package composition can be prepared. Adhesive setting time can be reduced to about half of its original by mixing β-CD complex with cyanoacrylate adhesive. Perfumed

textile can be prepared by binding CD to fibres. The odour-absorbing wool and polyester fibres contain CDs incorporated in them.

e. ***Applications in biotechnology*** (Bar 1996)

CDs can help in biotransformation by forming complexes with the substrates or the products. They have been used in Ortho-hydroxylation of 17β-oestradiol by phenol oxidase, microbial conversion of various steroids, hydrolysis of triglycerides and p-nitrophenyl, synthesis of fatty acids, oxidation of cholesterol, etc. Use of CDs instead of serum for some animal cell lines has resulted in reduction in cost, better recovery of protein products like monoclonal antibodies and absence of contaminants like viruses and mycoplasmas. The growth rate of human lymphoblast cells in a serum-free medium supplemented with 100 mg/L of α-CD-fatty acid complexes and 1 g/L α-CD (Yamane et al., 1981) was comparable to that observed in an identical serum supplemented medium. A nutrient medium supplemented with CD-complexed to organic substrates (benzene, phenol, chlorophenol, hexadecane, fatty acids, cholesterol, etc.) has been used for cultivation of microbial cells and mycoplasmas for reduced toxicity and increased bioavailibility substrates. They have also been used for production of antibiotics (lankacidins, prodigiosin, monensin A and nigeriun), toxins (Brodetella toxins, pertussis toxin, etc.) and microbial degradations (β-CD polymer to increase efficiency of phenol degradation in wastewater by *Candila tropicalis*, *Pseudomonas* to remove toluene in presence of β-CD). CDs have been used for product recovery in production of antibiotics and steroids.

f. ***Cyclodextrins as enzyme models*** (Komiyama 1996)

CDs are widely employed as substrate-recognition moieties in artificial enzymes because of their high water solubilities, availability of versatile guest compounds, well studied molecular structure and hydroxyl groups acting as effective catalysts. CDs play an important role in various enzyme mimic models (protease, phosphodiesterase, adenylate cyclase, esterase, transaminase, carbonic anhydrase, glycoxylase models). Peptide bonds in natural dipeptides and tripeptides were efficiently hydrolysed by a combination of cerium ion and γ-CD. An attachment of (NAD) nicotinamide residue, a well-known cofactor for redox reactions, to β-CD provides a superb NADH coenzyme model. They

also mimic some proteins, *e.g.* a modified β-CD was prepared as a mimic of a visual pigment rhodopsin, hemolysis of the carbon-cobalt bond of adenosyl-cobalamin a coenzyme (coenzyme B_{12}) is partially mimicked by the β-CD bearing cob(I)alamin.

g. ***Cyclodextrins in analytical chemistry*** (Snopek et al., 1996)

CDs are used in analytical separation techniques like gas chromatography (GC), thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and various electrophoretic techniques. The water-insoluble β-CD polymer beads are used as TLC sorbents. CDs are also used for TLC separation of racemic, diastereomeric and structural isomers. CDs and their derivatives have been used as stationary phases in GC. They have also been used in HPLC to separate structural and optical isomers, and in capillary electrophoresis to achieve chiral separation.

II. Cyclodextrin glycosyltransferases

Cyclodextrin glycosyl transferase (CGTase, E. C. 2.4.1.19, cyclomaltodextrin glucanotransferase, cyclodextrin synthetase) is the enzyme which converts starch or other 1,4-α-D-glucans to non-reducing cyclic malto-oligosaccharides called cyclodextrins (CDs). CGTases are known to perform four different reactions: cyclization, coupling, disproportionation and hydrolysis (or dextrinization). In the cyclization, reaction (intramolecular transglycosylation), the reducing end sugar is transferred to another sugar residue in the same oligosaccharide chain, resulting in formation of cyclic molecule called cyclodextrin (CD). In coupling reaction, which is the reverse of cyclization reaction, CD is coupled to a linear oligosaccharide (chain) to produce a longer linear chain oligo-saccharide. In the disproportionation reaction, there is a transfer of part of a linear oligosaccharide chain to another linear acceptor chain resulting in a mixture of shorter and longer oligosaccharides. The coupling and disproportionation reactions are due to intermolecular transglycosylation activity of the enzyme. The hydrolysis of starch or long oligosaccharide results in starch degradation or dextrinization, the only reaction which increases the reducing power. The CGTase reactions are outlined below.

$$G_n \underset{\text{Coupling}}{\overset{\text{Cyclization}}{\rightleftharpoons}} cG_x + G_{(n-x)} \qquad (n \geq 8,\ x \geq 6)$$

$$G_n + G_m \underset{}{\overset{\text{Disproportionation}}{\rightleftharpoons}} G_{(n-y)} + G_{(m+y)} \qquad (n \geq 3,\ m \geq 1,\ y \geq 1)$$

where G_n and G_m are 1,4-α-D-glucopyranosyl chains with 'n' and 'm' D-glucopyranosyl residues and cG_x is the CD molecule with 'x' number of glucose residues.

CGTases are named (or classified) according to the type of CD they predominantly produce at the initial stage of the reaction. Thus, α-CD producing enzymes are named as α-CGTase, β-CD producing as β-CGTase and γ-CD producing as γ-CGTase. The β-CGTases occur most commonly in nature; α-CGTases are relatively less common and γ-CGTases are very rare. Only four organisms capable of producing γ-CGTase have been isolated (Table 1.2). Almost all CGTases produce β-CD as the main product when reaction is allowed to reach equilibrium, because formation of β-CD is thermodynamically more favourable than that of α- and γ-CDs (Schmid, 1996a).

1. CGTase producing microorganisms and their occurrence

Tilden and Hudson (1939) reported for the first time that Schardinger dextrins are produced by a cell-free enzyme preparation from *Bacillus macerans* (*Aerobacillus macerans*). This enzyme, which converts starch to Schardinger dextrins or CDs, was initially identified as an amylase (*Bacillus macerans* amylase, BMA) and later named as CGTase. Since then many CGTase producing microorganisms have been isolated by different researchers. All the reported CGTase-producing microorganisms belong to bacterial genera, except for one example of actinomycetes (Abelian et al., 1995b). Many species of genus *Bacillus* and a few species from other genera like *Brevibacterium*, *Brevibacillus*, *Clostridium*, *Corynebacterium*, *Klebsiella*, *Micrococcus*, *Paenibacillus*, *Pseudomonas*, *Thermoalcalibacter*, *Thermoanaerobacter* and *Thermoanaerobacterium* have been reported to produce CGTases.

CGTase producers have been usually isolated from soil or degrading starchy plant materials. For discovering CGTases with novel properties, organisms have also been isolated from hot springs (Kaneko et al., 1990; Do et al., 1993a), deep-sea (Georganta et al., 1993) highly salty (halophillic, Abelian et al., 1995a) or highly alkaline (alkalophillic, Nakamura and Horikoshi 1976a) environments. A large number of alkalophillic CGTase-producing organisms have been reported. Microorganisms, growing at different temperature from 4 °C to 65 °C (psycrophillic, Georganta et al., 1993 to thermophillic, Starnes et al., 1989) and having different oxygen demands (aerobic and anaerobic), are reported to produce CGTase. Table 1.2 presents a comprehensive overview of CGTase producing microorganisms.

Organism	CGTase	Refs.	Organism	CGTase	Refs.
Bacillus macerans (*Aerobacillus macerans*)	α	1	*Bacillus coagulans* A-147	β	42
Bacillus macerans (ATCC 8514)	α	2,3	*Bacillus ohbensis* C-1400[a]	β	8,9,37 87
Bacillus macerans[a] (IFO 3490)	α	3-5, 32	*Bacillus licheniformis* IT25[a]	β	43,86
Bacillus macerans[a] (IAM 1243)	α	3,6,74, 75	*Bacillus firmus* (NCIM 5119)	β	54, 55
Bacillus macerans	α	7	*Bacillus firmus* H609	β	111
Bacillus macerans (IAM 1227)	α	8,9	*Bacillus firmus* No. 324	β	53
Bacillus macerans	α	10	*Bacillus lentus*	β	51,52
Bacillus macerans 314	α	11,12	*Bacillus halophilus*	β	56
Bacillus macerans[a] (NCAIM 00154)	α	13, 14	*Bacillus stearothermophilus* TC-91[a,b]	β	77, 107
Bacillus macerans (NRRL B3185)	α	88	*Bacillus stearothermophilus* 239	β	33, 34
Bacillus macerans (NCIM 2281)	α	15, 16	*Bacillus stearothermophilus* ET1	β	35
Bacillus macerans 17A[a]	α	77	*Bacillus* sp. BE101	β	112
Bacillus circulans DF 9	α	28,29	*Bacillus* sp. No. C1	β	44
Bacillus cereus RJ30 (NCIMB 13123)	α	36	*Bacillus* sp. HA3-3-2	β	45
Bacillus amyloliquifacines	α	38	*Bacillus* sp. A2-5a	β	46
Klebsiella pneumoniae M 5 al[a]	α	63,73	*Bacillus* sp. KC201	β	47
Klebsiella oxytoca 19-1	α	64	*Bacillus* sp. No. 562	β	49,110
Bacillus stearothermophilus TC-60	α	5,31	*Bacillus* sp. 1-1[a]	β	84,85
Bacillus stearothermophilus NO2	α	32	*Bacillus* sp. E1[a]	β	71,72
Thermoalcalibacter bogoriae[a]	α	65,93	*Bacillus* sp. No. 1011[a,b]	β	78,79, 109
Thermoanaerobacter sp.[a] (ATCC 53627)	α	66,67, 91,92	*Bacillus* sp. No. 17-1[a]	β	83
Thermoanaerobacterium thermosulfurigenes EM1[a,b]	α	68,108	*Bacillus* sp. N-227[a]	β	89
Bacillus macerans WKMW 506	β	17	*Bacillus* sp. A11[a]	β	90
Bacillus circulans[a] (ATCC 21783)	β	3,18-23, 81	*Clostridium thermoamylolyticum*	β	57
Bacillus circulans (IFO 3329)	β	8,9	*Pseudomonas* sp. (NRRL B18375)	α/β	58
Bacillus circulans C31	β	24	*Corynebacterium* sp.	β	59
Bacillus circulans E192	β	25	*Paenibacillus* sp. F8	β	61,62
Bacillus circulans N° 76	β	26	*Micrococcus varians* M 849	β	69
Bacillus coagulans	β	41	*Bacillus circulans*	β	27

a: indicates CGTase gene cloned and b: indicates CGTase X-ray crystallographic structure known

Table1.2 Overview of CGTase producing microorganisms (continued)

Organism	CGTase	Refs.	Organism	CGTase	Refs.
Bacillus circulans strain 251[a,b]	β	95,101-106	*Brevibacillus brevis* CD 162[a]	β	94
Bacillus circulans strain 8[a,b]	β	80,96-100	*Bacillus subtilis* No. 313[a]	γ	39,76
Bacillus subtilis No. 1-7	β	40	*Brevibacterium* sp. No. 9605	γ	60
Bacillus megaterium	β	3,6	*Bacillus* sp. AL-6	γ	48
Bacillus autolyticus 11149	β	30	*Bacillus* sp. 290-3	γ	50
Actinomycetes	β/α	70			

a: indicates CGTase gene cloned and b: indicates CGTase X-ray crystallographic structure known

Table1.2 Overview of CGTase producing microorganisms, for references see

1. Tiden and Hudson, 1939
2. DePinto and Campbell, 1968
3. Horikoshi and Akiba, 1982
4. Kitahata et al., 1974
5. Kitahata and Okada 1982b
6. Kobayashi et al., 1978
7. Stavn and Granum 1979
8. Yagi et al., 1986
9. Sato and Yagi 1991
10. Jeang et al., 1992
11. Abdel-Fattah et al., 1994
12. Ismail et al., 1996
13. Nogrady et al., 1995
14. Nogrady et al., 1996b
15. Sreenivasan et al., 1991
16. Desai and Pai 1996
17. Steighardt and Kleine 1993
18. Nakamura and Horikoshi, 1976a
19. Nakamura and Horikoshi 1976b
20. Nakamura and Horikoshi 1976c
21. Nakamura and Horikoshi 1976d
22. Makela et al., 1988b
23. Makela et al., 1990
24. Pongsawasdi and Yagisawa 1988
25. Bovetto et al., 1992a
26. Salva et al., 1997
27. Glickstein and Bar 1996
28. Marechal et al., 1996
29. Ferrarotti et al., 1996
30. Tomita et al., 1993
31. Kitahata and Okada 1982a
32. Fujiwara et al., 1992
33. Ahn et al., 1990
34. Hwang et al., 1990
35. Chung et al., 1998
36. Jamuna et al., 1993
37. Sin et al., 1991
38. Yu et al., 1988
39. Kato and Horikoshi 1986a
40. Georganta et al., 1993
41. Akimura et al., 1991a,b
42. Kaneko et al., 1990
43. Aoki et al., 1987
44. Prema et al., 1990
45. Nomoto et al., 1986
46. Kometani et al., 1994
47. Kitamoto et al., 1992
48. Fujita et al., 1990
49. Yan and Li 1995
50. Englebrecht et al., 1990
51. Sabioni and Park 1992a
52. Sabioni and Park 1992b
53. Yim et al., 1997
54. Goel and Nene 1995a
55. Gawande et al., 1999
56. Abelian et al., 1995a
57. Starnes 1991
58. Allenza et al., 1991
59. Tsuji 1995
60. Mori et al., 1994
61. Larsen et al., 1998a
62. Larsen et al., 1998b
63. Bender 1977a
64. Lee et al., 1992
65. Sjoholm et al., 1998
66. Starnes et al., 1990
67. Norman and Jorgensen 1992
68. Wind et al., 1995
69. Yagi et al., 1980
70. Abelian et al., 1995b
71. Park et al., 1992
72. Yong et al., 1997
73. Binder et al., 1986
74. Takano et al., 1986
75. Takano et al., 1992
76. Kato and Horikoshi 1986b
77. Sakai et al., 1987
78. Kimura et al., 1987
79. Kimura et al., 1990
80. Nitschke et al., 1990
81. Kaneko et al., 1988
82. Paloheimo et al., 1992
83. Kaneko et al., 1989
84. Schmid et al., 1988
85. Schulz et al., 1997
86. Bender 1990
87. Sin et al., 1993
88. Lee and Tao 1994
89. Tang et al., 1990
90. Siripornadulsil et al., 1993
91. Jorgensen et al., 1997
92. Jorgensen et al., 1998
93. Sjoholm et al., 1998
94. Kim et al., 1998
95. Penninga et al., 1995
96. Hofmann et al., 1989
97. Klein and Schulz 1991
98. Klein et al., 1992
99. Parsiegla et al., 1998
100. Schmidt et al., 1998
101. Lawson et al., 1990
102. Lawson et al., 1994
103. Knegtel et al., 1995
104. Strokopytov et al., 1995
105. Strokopytov et al., 1996
106. Mosi et al., 1998
107. Kubota et al., 1991
108. Knegtel et al., 1996
109. Harata et al., 1996
110. Yan and Li 1993
111. Do et al., 1993a
112. Lee and Kim 1991a

2. *CGTase production*

Almost all reported CGTases are inducible enzymes, requiring some form of starch/oligo-saccharide for induction. However, there is one example of constitutively produced CGTase, which was secreted in presence of either glucose or xylose (Jamuna et al., 1993). CGTases are usually secreted out in the extracellular medium. However, there are few reports of detection of intracellular CGTase activity (Bender 1977b; DePinto and Campbell 1968; Nogardy et al., 1996a and b; Vandamme et al., 1984).

The onset of enzyme production is organism-specific, but, in general, the enzyme is released during stationary or lysis phase (Georganta et al., 1993; Jamuna et al., 1993; Makela et al., 1990; Yan and Lin 1993). The cultivation period varies from 8 to 120 h in different CGTase producing organisms (Table 1.3). Fast growing organisms produce CGTase within 12-24 h (Bender 1977a; Lee et al., 1992; Nogrady et al., 1995; Tomita et al., 1993; Yu et al., 1988).

The organisms are typically grown in a complex nutrient medium containing a carbon source (usually starch), a nitrogen source (organic/inorganic), phosphorus source (sodium or potassium phosphate) and mineral salts supplemented with micronutrients ($MgSO_4$ or $CaCl_2$). The pH of the medium is generally kept at neutral, or between 8-10 for alkalophillic bacteria (adjusted with Na_2CO_3).

Very few researchers have reported systematic media optimization and fermentation studies for CGTase production. Most of the fermentations are done in submerged, batch mode, although some workers have used solid state fermentations (Ramakrishna et al., 1994) for enzyme production. There are very few reports of fed-batch or continuous culture studies. Ramakrishna et al. (1994) have cultivated *Bacillus cereus* RJ-30 in solid state and slurry mode and have obtained higher CGTase activity compared to defined medium submerged fermentations. Bender (1981b, 1986) cultivated *Klebsiella pneumoniae* M 5 al in a chemostate culture. Continuous fermentations were also studied by Jamuna et al. (1993) for *Bacillus cereus* RJ-30. They have reported a decrease in the enzyme activity over the dilution rates of 0.6 to 1.6 h^{-1}. However, enzyme productivity increased with increase in the dilution rate. The maximum productivity (obtained at 1.6 h^{-1} dilution rate) was 3.1 times higher than that in the batch culture using the same

medium. Park et al. (1997) conducted fed-batch fermentations using a recombinant *Escherichia coli* strain containing the gene for *Bacillus macerans* CGTase. They reported 31-fold increase in CGTase activity and a 29-fold increase in cell mass compared to the control batch fermentation. Chen et al. (1994) obtained 50% more CGTase production in fed-batch operation as compared to that in batch operation using alkalophillic *Bacillus* sp (ATCC 31007).

3. CGTase assays

A number of CGTase assays have been developed based on various reactions catalysed by CGTase enzyme. Since all four reactions are performed simultaneously, assay based on any one reaction is usually presumed to be indicative of the overall CGTase activity.

a. Assays based on hydrolysis or dextrinization reaction

Hale and Rawlins (1951) developed the first CGTase assay, based on starch hydrolyzing or dextrinization activity. They measured the decrease in staining of starch with iodine. This assay does not distinguish between an amylase and a CGTase. It was further modified by Schwimmer and Garibaldi (1952), Fuwa (1954) and Nakamura and Horikoshi (1976a). In spite of the limitations, this assay is still used by many researchers in this field (Desai and Pai 1996; Ferrarotti et al., 1996; Kometani et al., 1994; Mori et al., 1994; Pongsawasdi and Yagisawa 1988; Salva et al., 1997; Sreenivasan et al., 1991; Tomita et al., 1993). The dextrinization activity can be also determined by measuring the increase in reducing power by dinitrosalicyclic method (DNSA) (Wind et al., 1995). The unit of enzyme activity is defined differently by different workers as the amount of enzyme that decreased the absorbance at 660 nm or 700 nm, by 1% or 10%, per min or per 10 min.

b. Assays based on coupling reaction

The coupling of CD or starch (donor) with specific acceptor molecule (intermolecular transglycosylation) results in the formation of a linear oligosaccharide. This can be followed by observing the disappearance of CD and related to the CGTase activity. The first CGTase assay based on transglycosylation reaction was developed by Thoma et al. (1965). Nakamura and Horikoshi (1976a) estimated CGTase activity by coupling α-CD

to a sucrose acceptor. The dextrins produced were hydrolysed to reducing sugars by addition of glucoamylase enzyme and estimated by DNSA method. Kometani et al. (1994) used salicin as an acceptor molecule to starch (donor) and estimated decrease in the amount of salicin by HPLC to measure transglycosylation activity. Wind et al. (1995) measured the disappearance of β-CD in presence of maltotriose (G_3) or maltotetraose (G_4) for estimation of coupling activity. Flaschel et al. (1996) used maltitol as an acceptor for coupling with α-CD. The unit of enzyme activity is defined as the amount of enzyme required for disappearance of one μmole of donor or acceptor molecule per min.

c. Assays based on disproportionation reaction

The change in the length of a linear oligosaccharide by CGTase action has been used to measure disproportionation activity, which is an intermolecular transglycosylation reaction. Specific acceptors or substrates are used to detect this activity. The first spectrophotometric CGTase assay based on this reaction was developed by Nakamura et al. (1994b). They used a derivatized maltooligosaccharide (attached with aglycon molecule at its reducing end and non-reducing end blocked) with a suitable acceptor. The CGTase can not use the modified reducing end of the maltooligosaccharide molecule as an acceptor and can only be disproportionated. Bovetto et al. (1992b) incubated 4-nitophenyl-maltooligosaccharides with varying chain lengths ($pNPG_1$ to $pNPG_8$) with CGTase enzyme and measured the degradation products of nitrophenyl-maltooligosaccharides by HPLC. Wind et al. (1995) observed conversion of maltohexaose (G_6) to other oligosaccharides by HPLC for determination of disproportionation activity. The unit of disporportionation activity is defined as the amount of enzyme able to convert one μmole of oligosaccharide substrate per minute.

d. Assays based on cyclization or intramolecular transglycosylation reaction

The quantification of CDs formed during the initial cyclization reaction of CGTase is arguably the most specific and accurate way of measuring CGTase activity. The CDs produced can be detected by their ability to form clathrates with a number of organic molecules or by chromatographic techniques. The first method, based on microscopic

examination of CD-iodine clathrate, was developed by Tilden and Hudson in 1942 (Tilden and Hudsom microscopic test). Alpha-CD forms a complex with trichloroethylene (TCE) and precipitates readily in the aqueous phase. The amount of precipitate can be quantified to determine CGTase activity (Nomoto et al., 1984). Bender (1981a) developed a spectrophotometric CGTase assay based on measurement of absorbance of an iodine solution in presence of α-CD in the ultraviolet range.

The capability of CDs to form inclusion complexes has been largely exploited for developing methods for their estimation. Many pH indicator dyes form inclusion complexes with CDs and result in concentration-dependent changes in their absorbance, which can be correlated to CGTase activity. Individual CDs are usually specific for a particular dye molecule because of their cavity size. Thus, different dyes have been used to estimate different CDs. Alpha-CD can be estimated by complexation with methyl orange (Landert et al., 1981; Lejeune et al., 1989; Makela and Korpela 1988). Beta-CD is estimated using phenolphthalein (Goel and Nene 1995b; Kaneko et al., 1987; Li et al., 1996; Makela et al., 1987; Vikmon 1981) or methyl orange (Makela and Korpela 1988) complexation. Estimation of γ-CD is usually performed by complexation with bromocresol green (Kato and Horikoshi 1984), calgamite (Hokse 1983) or alizarin yellow (Aoki et al., 1987). The unit of enzyme activity is generally expressed as μmoles of CD formed per min.

CDs can also be estimated by polarography (Laakso et al., 1984), spectrofluorometry (Kondo et al., 1976a and b) or by catalytic activity of CDs (hydrolysis of dimethyphenylacetate by α-CD, Lane and Pirt 1973). Assays based on various chromatographic techniques like thin layer chromatogarphy (TLC, Takeo et al., 1970; Sicard and Saniez 1987; Vrbaski and Lepojevic 1991), gas chromatogarphy (GC, Beadle 1969) and high performance liquid chromatogarphy (HPLC, Aoki et al., 1987; Bender 1983b; Brunt 1982; Frijlink et al., 1987; Hokse 1980; Kitahata et al., 1978; Sato et al., 1985a; Zsadon et al., 1979) have been reported. A number of non-chromatographic CDs assays have been reported by Makela et al., 1988a.

Rapid screening methods (plate based assays) for isolation of microorganisms producing β-CGTase and γ-CGTase from soil samples or recombinant clones having

CGTase gene insert have been developed and found useful for initial screening of hundreds of colonies (Hamaker and Tao 1993; Matioli et al., 1996; Park et al., 1989).

Rapid sensitive assays based on immunochemical techniques, like enzyme-linked immunosorbent assay or enzyme-linked immunofiltration assay, have also been reported for rapid identification of clinical samples containing CGTase producing organisms (Creminon et al., 1994; Han and Tao 1999a; Nogrady et al., 1998). Pocsi et al. (1998a) have developed an assay for simultaneous determination of β-CGTase and amylase activity in *Bacillus macerans* cultures.

4. CGTase purification

The enzyme purification involves many steps depending on the desired purity. In case of extracellular enzymes, the first step is removal of cells by centrifugation or filtration (for intracellular enzymes, an additional step of cell lysis is required). The crude enzyme preparation is concentrated by ultrafiltration, followed by optional alcohol, solvent or salt precipitation. The concentrated enzyme is generally purified by affinity chromatography. The matrices used for affinity adsorption include CDs attached to an inert support like Sepharose or modified starches.

Pongasawasdi and Yagisawa (1988) used a packed-bed starch column for affinity adsorption of the CGTase. The adsorbed enzyme was eluted with a solution of β-CD. In earlier reports, CGTase was adsorbed to starch by suspending the enzyme in a starch slurry in the presence or absence of ammonium sulphate (Kitahata and Okada 1982a; Nakamura and Horikoshi 1976b and d; Makela et al., 1988b). The simple technique of adsorption in packed-bed columns by binding at a low temperature and de-adsorption at a higher temperature (which is decided based on the enzyme stability) results in better purification, concentration and yield (Goel 1995).

The type of CD used as an affinity ligand will have affinity for a particular type of CGTase, i.e α-CGTase will bind more strongly to a Sepharose-α-CD matrix than Sepharose-β-CD or Sepharose-γ-CD matrix. Sepharose 6FF-α-CD matrix has been used for purification of α-CGTase from *Thermoanaerobacterium thermosulfurigens* EM1 (Wind et al., 1995). Sepharose 6B-β-CD has been used for purification of β-CGTase

from *Bacillus stearothermophilus* 239, *Paenibacillus* sp. F8 (Ahn et al., 1990, 1991; Larsen et al., 1998a), whereas sepharose 4B-γ-CD has been used for purification of γ-CGTase from *Brevibacterium* sp. No. 9605 (Mori et al., 1994).

The affinity purification step generally results in good enzyme recovery and fold-purification. In some cases a final gel-filtration step is necessary to get highly pure enzyme. This step can result in considerable loss of enzyme but usually results in very good fold purification. Gel filtration matrices in the Sephadex, Sepharose or Sephacryl series, having pore sizes in the range of more than twice the molecular weight of the particular CGTase, have been used in the past (Gawande et al., 1999; Kitahata and Okada 1982a; Makela et al., 1988b).

There are also reports of immunoaffinity matrices prepared by raising antibodies using a pure CGTase preparation. A novel method for single step purification of a recombinant CGTase by metal affinity chromatography has been reported by Berna et al. (1994). The cultivation conditions and purification of CGTases from some bacteria are summarised in Table 1.3.

5. Properties

a. Molecular weight

CGTases are monomeric or dimeric (homo) proteins having molecular weights in the range of 64-200 kDa (Table 1.4). When present as two identical subunits, the single subunit does not possess CGTase activity (Kobayashi et al., 1978). CGTases from *Bacillus licheniformis* IT 25 (Aoki et al., 1987) and *Bacillus* sp. 562 (Yan and Li 1995) contain two identical subunits. The molecular weights determined from the deduced amino acid sequences for the recombinant CGTases range between 70-80 kDa (Georganta et al., 1991; Kaneko et al., 1989; Kimura et al., 1987; Lee and Tao 1994; Paloheimo et al., 1992; Sin et al., 1991).

b. Temperature optimum and stability

The temperature optima of CGTases from mesophillic organisms is in the range of 45-60 °C. However, enzymes from thermophillic organisms usually have higher

Organism	Cultivation		Assay method	Purification scheme	Fold purifn	% Recovery	Refs
	Temp (°C)	Time (h)					
Bacillus macerans (ATCC 8514) Intracellular	40	8-10	Dextrinization	Cell extract → $MnCl_2$ ppt → $(NH_4)_2SO_4$ ppt → DEAE-Cellulose → $(NH_4)_2SO_4$ ppt → Preparative disc electrophoresis	140.1	12	2
Bacillus macerans (IAM 1243)	40	48	Dextrinization	Crude enzyme → Starch adsorption → DEAE-Cellulose (I) → DEAE-Cellulose (II)	157	37	6
Bacillus macerans	37	120	Dextrinization	Culture medium → Starch adsorption → $(NH_4)_2SO_4$ ppt → DEAE-Cellulose → Sephadex G-100	53	17	7
Bacillus macerans (IAM 1227)			Dextrinization	Crude enzyme → Starch adsorption → DEAE-cellulose (I) →DEAE-Cellulose (II) → Sephadex G-100	152	31	8
Bacillus macerans WKMW 506	37	72	Cyclization	Partial purification with $(NH_4)_2SO_4$ ppt	8	90	17
Bacillus macerans (NCAIM 00154) Intra*- and extracellular**	37	48-56	Cyclization	Cell-free supernate or crude enzyme extract → Starch adsorption → DEAE-Sephacel → Biogel P-100	41* 70**	13.2* 23.5**	13, 14
Bacillus macerans (NCIM 2281)	35	24	Dextrinization	Crude enzyme → Concentration → Acetone ppt → $(NH_4)_2SO_4$ ppt → Seralose 6B	9	74	15
Bacillus circulans (ATCC 21783) Acid-CGTase	37	48	Dextrinization Coupling	Culture fluid → Starch adsorption → Biogel P-100 → DEAE-Cellulose → Sephadex G-100	74	35	19
Bacillus circulans (ATCC 21783) Neutral-CGTase	37	50	Dextrinization Coupling	Culture filtrate → Starch adsorption → DEAE-Cellulose (I) → Sephadex G-150 → DEAE-Cellulose (II)	15	19	21
Bacillus circulans (ATCC 21783)	37	72	Cyclization	Culture fluid → Starch adsorption / affinity → Sephacryl S-300 → DEAE-Trisacryl → DEAE HPLC (four fractions)	170 (major)	69.7	22

Table 1.3 Cultivation and CGTase purification from various organisms (continued)

Organism	Cultivation		Assay method	Purification scheme	Fold purifn	% Recovery	Refs
	Temp (°C)	Time (h)					
Bacillus circulans (IFO 3329)	37	70	Dextrinization	Crude enzyme → Starch adsorption → UF conc. → $(NH_4)_2SO_4$ ppt → DEAE-Cellulose → Sephadex G-100 → IEF	2242	12.7	4,8
Bacillus circulans C31	37	96	Dextrinization	Crude enzyme → Starch affinity → Biogel A-500	91	30	24
Bacillus circulans E192	30	40	Cyclization	Crude extract → $(NH_4)_2SO_4$ ppt → DEAE-Trisacryl M → Q-Sepharose → Affinity and GF 05	42	62	25
Bacillus circulans DF 9	37	48	Dextrinization	Crude extract → Starch adsorption → $(NH_4)_2SO_4$ ppt → Sepharose 4B-α-CD	59.7	54	29
Bacillus circulans N° 76	37	120	Dextrinization	Cell-free extract → Starch adsorption → DEAE-Sephadex A-50	84.6	14	26
Bacillus megaterium	37	70	Dextrinization	Culture liquor → $(NH_4)_2SO_4$ ppt → Starch adsorption → DEAE-Cellulose → Sephadex G-75	135	50	4,5
Bacillus stearothermophilus TC-60			Dextrinization	Crude enzyme → Starch adsorption with $(NH_4)_2SO_4$ ppt → DEAE-Sephadex → Biogel P-150	18.4	27	31
Bacillus stearothermophilus 293	55	48	Dextrinization	Culture broth → $(NH_4)_2SO_4$ ppt → Sepharose 6B-β-CD	31	42	33
Bacillus stearothermophilus ET1	50	24	Cyclization	Culture broth → $(NH_4)_2SO_4$ ppt → Sepharose 6B-β-CD → DEAE-8HR column	90.8	31.6	35
Bacillus lentus	37	96	Dextrinization	Crude enzyme → $(NH_4)_2SO_4$ ppt → DEAE-Cellulose → CM-Cellulose			51, 52
Bacillus autolyticus 11149	30	24	Dextrinization	Crude enzyme → DEAE-Sephadex A-50 → Sepharose CL-6B (I) → Sepharose CL-6B (II) → Hydroxylapatite	53.6	31.1	30

Table 1.3 Cultivation and CGTase purification from various organisms (continued)

Organism	Cultivation		Assay method	Purification scheme	Fold purifn	% Recovery	Refs
	Temp (°C)	Time (h)					
Bacillus licheniformis IT25	37	48	Dextrinization Cyclization Coupling	Crude enzyme → Starch adsorption → $(NH_4)_2SO_4$ ppt → DEAE-Zeta prep → Crystallization	94.8	75	43
Bacillus subtilis No. 313	37	72-120	Cyclization	Culture filtrate → $(NH_4)_2SO_4$ ppt → DEAE-Sephadex A-50 → Chromatofocussing PBE 94 → Sephacryl S-200 → CM-Toyopearl	35.1	13.5	39
Bacillus firmus (NCIM 5119)	30	24	Cyclization	Cell-free supernate → UF → Starch affinity → Sephacryl S-200	400	61.2	55
Bacillus firmus No.324	40	48	Cyclization	Crude enzyme → UF → DEAE-Sephadex A-50 → DEAE-Sepharose CL-6B	89.8	26.6	53
Bacillus sp. A2-5a	33	72	Dextrinization Cyclization Coupling	Crude enzyme → Starch adsorption → $(NH_4)_2SO_4$ ppt → Q-Sepharose	22.7	51	46
Bacillus sp. HA3-3-2 (ATCC 39612)	37	96	Dextrinization Cyclization	Culture supernate → $(NH_4)_2SO_4$ ppt → Sephadex G-100 → DEAE-Cellulose (I) → DEAE-Cellulose (II) → DEAE-Cellulose (III)	83.3	7	45
Bacillus sp. C1	28	96	Dextrinization Cyclization	Crude enzyme → $(NH_4)_2SO_4$ pp → DEAE-Cellulose → Sephadex G-100	47.5	19.9	44
Bacillus sp. No. 562			Cyclization	Crude enzyme → $(NH_4)_2SO_4$ ppt → DEAE-Sephadex A-50 (I) → Sephadex A-50 (II) → Sephacryl S-300	64	32	49
Bacillus sp. AL-6	38	48	Dextrinization Cyclization	Crude enzyme → $(NH_4)_2SO_4$ ppt → Starch adsorption → DEAE-Sephadex A-50 (I) → DEAE-Sephadex A-50 (II)	231	14.4	48
Klebsiella pneumoniae M 5 al	28	12-24		Culture filtrate → $(NH_4)_2SO_4$ pp→ DEAE-Cellulose → Polyamine P → $(NH_4)_2SO_4$ ppt		61.2	63
Klebsiella oxytoca 19-1	37	9	Cyclization	Partial purification by ethanol ppt			64

Table 1.3 Cultivation and CGTase purification from various organisms (continued)

Organism	Cultivation		Assay method	Purification scheme	Fold purifn	% Recovery	Refs
	Temp (°C)	Time (h)					
Thermoanaerobacterium thermosulfurigene EM1 (Cloned in *E. coli*)	30	14-20	Dextrinization Cyclization Coupling Disproportion-ation	Cell-free culture broth → Sepharose 6FF-α-CD	35	79	68
Brevibacterium sp. No. 9605	37	48	Dextrinization	Crude enzyme → UF → Butyl-Toyopearl 650 M→ Sepharose 4B-γ-CD → Toyopearl HW-555	604.7	16	60
Paenibacillus sp. F8	27	24	Cyclization	Culture supernate → $(NH_4)_2SO_4$ ppt → Sepharose 6B-β-CD → Gel filtration (FPLC)	157	30	61

An empty box shows no data available, for references see Table 1.2

Table 1.3 Cultivation and CGTase purification from various organisms

temperature optima between 70-80 °C. The highest temperature optimum of 95 °C has been reported for the CGTase from *Thermoanaerobacter* sp. (Norman and Jorgensen 1992). Most CGTases are not stable at high temperatures at optimum temperatures (for activity) for longer times in the absence of substrate, product or Ca^{2+} ions. CGTases from mesophillic organisms lose their activity very rapidly above 50 °C, whereas CGTases from thermophillic organisms are stable up to 70-75 °C. CGTase from *Thermoanaerobacter* sp. is the most stable enzyme reported and retains almost 100% activity at 75 °C for 1 h at pH 5.5. The temperature stability of CGTases is greatly enhanced in the presence of its substrates, products or Ca^{2+} ions. CGTase inhibitors like moranoline and its derivates are also known to stabilize CGTase (Maruo et al., 1993).

c. pH stability and optimum

The pH optima of CGTases lie in a broad range from 4.5-10.5. However, most of them have an optimum around pH 6 (Table 1.4). *Bacillus circulans* (ATCC 21783) is reported to produce three CGTases possessing pH optima in acid, neutral and alkaline pH-range and are therefore named as acidic-CGTase, neutral-CGTase and alkaline-CGTase. Sometimes different reactions catalyzed by CGTases, such as cyclization and dextrinization, exhibit different pH optima. For different CGTases, the pH range for maximum stability varies between 5-11. Most CGTases are stable around neutral pH. However, CGTases from alkalophillic organisms are most stable at alkaline pH values.

d. Isoelectric point and kinetic parameters

The isoelectric point and kinetic parameters like K_m and V_{max} have not been reported for many CGTases (Table 1.4). The lowest pI of 2.8 is reported for *Brevibacterium* sp. No. 9605 CGTase (Mori et al., 1994), whereas the highest pI of 8.8 is reported for CGTase from *Bacillus circulans* IFO 3329 (Yagi et al., 1986). *Bacillus circulans* (ATCC 21783) CGTase is reported to have many isoforms, but all the isoforms are functionally identical (Makela et al., 1988b). Bovetto et al. (1992a) also observed two isoforms for CGTase from *Bacillus circulans* E192, the major isoform possessing 90% activity with a pI of 6.9 and the minor isoform possessing only 10% activity with a pI of 6.7. The kinetic parameters like K_m and V_{max} are usually determined for cyclisation or coupling reactions.

Organism		Molecular weight (kDa)	Optimum		Stability		pI	K_m	Ca^{+2} Effect	Predominant CD	Refs.
			Temp (°C)	pH	Temp (°C)	pH					
Bacillus macerans (ATCC 8514)		139[c]		6.1-6.2				3.33[*]		α-CD	2,3
Bacillus macerans (IFO 3490)		75[a] 65[c]	55	5.0-5.7	≤ 60	8-10	4.62	2.5[**]	+	α-CD	3-5,31
Bacillus macerans (IAM 1243)		74[a] 145[b]	60	6	≤ 50	5.5-9.5				α-CD	3,6
Bacillus macerans		75[a] 67[c]	60	5.4-5.8	≤ 50	6	5.4	5.7[*]		α-CD	7
Bacillus macerans (IAM 1227)		150	50	5.5	≤ 50	6.5-8.5	4.2			α-CD	8,9
Bacillus macerans WKMW 506		70[a]	51	6	56	6-7		3.8[*]	++	β-CD	17
Bacillus circulans (ATCC 21783)	Acid	88[a]	45	4.5-4.7	≤ 65	6-10	5.4	5.88[**]	+	β-CD	3,19,20
	Neutral	85-88[a]	50	7	≤ 60	6-9		10[**]	+	β-CD	3,21
	Alkaline	85-88[a]		8-9			5.4		+	β-CD	3
Bacillus circulans (IFO 3329)		200	55	6	≤ 55	6-9.5	8.5, 8.8			β-CD	8
Bacillus circulans C31		103[b]	60	5.5	≤ 50	5.5-9				β-CD	24

Table 1.4 Properties of some CGTases (continued)

Organism	Molecular weight (kDa)	Optimum		Stability		pI	K_m	Ca^{+2} Effect	Predominant CD	Refs.
		Temp (°C)	pH	Temp (°C)	pH					
Bacillus circulans E192	78[a]	60	5.5-5.8	45	7-8	6.9 6.7	5.7[*]	++	β-CD	25
Bacillus circulans DF 9R	78[a]	60	4.5-7.5	50	6.5-8.5	5.3		++	α-CD	28
Bacillus circulans N° 76	72[a]	62	6.5-8.5	35<55	8.5>5.7			+	β-CD	26
Bacillus megaterium	75[a] 66[c]	55	5.0-5.7	≤ 55	7-10	6.07 6.80	7.0[**]		β-CD	3-5
Bacillus stearothermophilus TC-60	68[a] 72[c]	70	6	≤ 50	7-9.2	4.5	3.1[**]		α-CD	5,31
Bacillus stearothermophilus 239	78[a]	60	6	≤ 55	5.5-9.5	4.8		++	β-CD	33,34
Bacillus stearothermophilus ET1	66.8[a] 79.3[c]	80	6	≥ 60		5.0		++	β-CD	35
Bacillus autolyticus 11149	70[a] 68[b]	60	5-6	≤ 40	5-9				β-CD	30
Bacillus licheniformis IT25	72[a] 145[b]	65-70	5.5	≤ 65	6-9.5	4.3		+	β-CD	43
Bacillus subtilis No. 313	64	65	8	≤ 50	6-8	7.1	6.67[**]	+	γ-CD	39
Bacillus firmus No. 324	75[a]	65	7.5-8.5	≤ 55	6.5-9				β-CD	53
Bacillus firmus (NCIM 5119)	78[a] 82[b]	65	5.5-8.5	≤ 40	7-11		1.21[*]		β-CD	55
Bacillus sp. A2-5a	80[a] 75[d]	50-55	5.5	≤ 60	6-10			+	β-CD	46
Bacillus sp. KC201	75	60-65	6	≤ 50	6-10				β-CD	47
Bacillus sp. C1	72[a]	45	9	50	7-10				β-CD	44

Table 1.4 Properties of some CGTases (continued)

Organism	Molecular weight (kDa)	Optimum		Stability		pI	K_m	Ca^{+2} Effect	Predominant CD	Refs.
		Temp (°C)	pH	Temp (°C)	pH					
Bacillus sp. AL-6	74[a,b]	55-60	7.5-10.5	≤ 40	5-8	3-4			γ-CD	48
Bacillus sp. HA3-3-2 (ATCC 39612)	68[a]	60	6.5-8	≤ 70	6-11				β-CD	45
Bacillus sp. No. 562	82[a] 170[b]	65	7	≤ 70	7-11	7.2		+	β-CD	49
Bacillus sp. 290-3	75[c]	60	6-8	≤ 50	6-8	4.1			γ-CD	50
Bacillus amyloliquefaciens AL35		70	6	60	8-10			+	α-CD	38
Bacillus ohbensis C-1400	80[a]	60	5.5	55	6.5-9.5	<4			β-CD	8,9,37
Bacillus lentus		55	6.5-7.5					+	β-CD	51,52
Bacillus coagulans A-147		65	6.5	≤ 50	6-9			++	β-CD	42
Klebsiella pneumoniae M 5 al	68 69[c]	50	5.2-7	≤ 45	6-7.5			+	α-CD	63
Klebsiella oxytoca 19-1		6	40						α-CD	64
Thermoanaerobacterium thermosulfuregens EM1	68[a] 75.1[e]	80-85	4.5-7	≥ 70		4.3, 4.4,4.6		++	α-CD	68
Thermoanaerobacter sp.	103[a]	90-95	5.8	≥ 75	5-6.7				α-CD	67
Brevibacterium sp. No.9605	75[a] 86[b]	45	10	≤ 30	6-8	2.8		++	γ-CD	60
Paenibacillus sp. F8	72[a]	50	7.5	40	6-8			++	β-CD	61

Molecular weight estimated by a: sodium dodesyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), b: gel permeation chromatography (GPC), c: ultra centrifugation, d: high performance liquid chromatography (HPLC) and e: calculated from deduced amino acid sequence; * indicates starch substrate, mg/mL and ** indicates CD substrate mM for K_m studies. An empty box shows no data available, for references see Table 1.2

Table 1.4 Properties of some CGTases

In the cyclization reaction, soluble starch or maltodextrin is generally used as the substrate, whereas for determining coupling activity, α-CD or β-CD is used along with specific acceptors. Using starch as the substrate, the reported K_m values vary from 1.21 to 5.7 mg/mL. Using CD as the substrate K_m values range between 2.5-10 mM.

6. Effect of metal ions and inhibitors

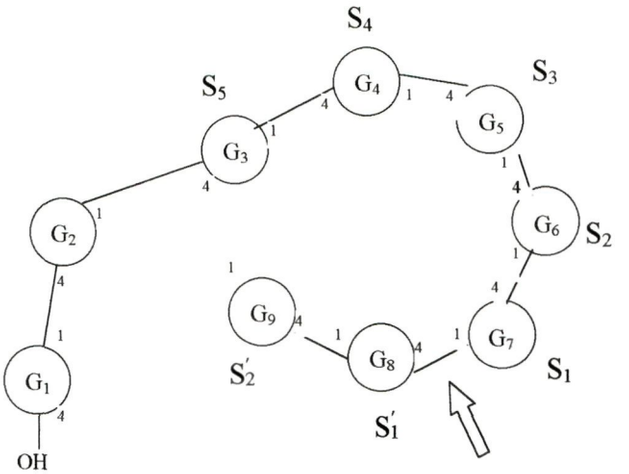
Most CGTases are strongly inhibited by metal ions like Cu^{2+}, Co^{2+}, Cd^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+}, Ag^{3+}, Mo^{6+}, Mg^{2+} and Zn^{2+} (Fujita et al., 1990; Kaneko et al., 1990; Mori et al., 1994; Nomoto et al., 1986; Tomita et al., 1993; Yan and Li 1995; Yim et al., 1998). However, the CGTase from *Bacillus licheniformis* is stabilized by Mg^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} at 1 mM concentratios (Aoki et al., 1987). The stability of most CGTases is greatly improved in the presence of Ca^{2+} ions. X-ray diffraction studies have shown that CGTases possess two Ca^{2+} ion binding sites (Klein and Schulz 1991; Lawson et al., 1994). One of these sites is near the active site and may play an important role in enzyme activity.

Simple sugars (or sugar analogues) and maltooligosaccharides are reported to be competitive inhibitors of CGTases (Bender 1985; Rendleman 1996a). Maltose and maltotriose are the most effective inhibitors for cyclization reaction, whereas they act as acceptors for intermolecular transglycosylation reactions. Bovetto et al. (1992b) have found glucose, maltose and salicin to be strong inhibitors. A pseudo-tetrasaccharide, acarbose, which has one normal maltose and one pseudomaltose (essential for the inhibitory properties), is a strong inhibitor of α-amylases and CGTases (Nakamura et al., 1993; Strokopytov et al., 1995). Recently, Hoxa, a pseudo-hexasaccharide, which is also an inhibitor of α-amylase and CGTase, was used for substrate binding and mutation studies for *Bacillus circulans* strain no. 8 CGTase (Parsiegla et al., 1998).

7. Structure and catalytic mechanism

Three dimensional structure of α-amylase family enzymes and CGTases by X-ray crystallographic data revealed presence of three common structural domains, named A, B and C, in α-amylase family enzymes and CGTases. CGTases possess two extra domains, D and E, which account for their higher molecular weight (70-75 kDa) than α-amylases

a



b

Figure 1.2 (a) Active site model of CGTase: S referes to the subsites for binding of a glucose residue (G) which are α-1,4-linked. The arrow indicates 'cleavage' and 'coupling' site located between subsites S_1 and S_1' (b) Schematic arrangement of protein domains of CGTase (Schmid 1996a)

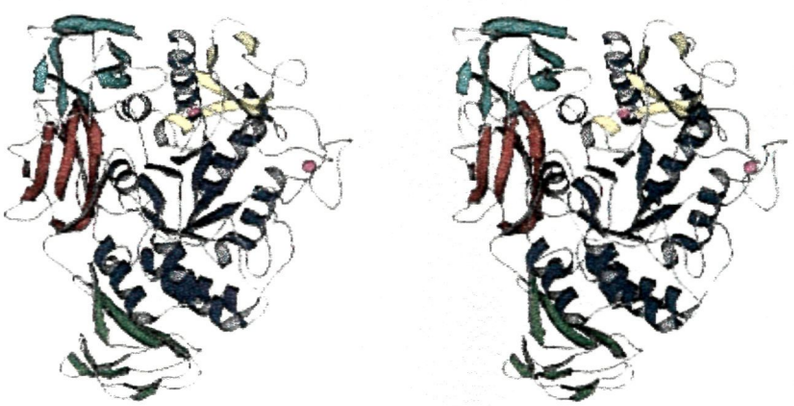


Figure 1.3 The backbone structure of CGTase. The five domains are shown with colours, blue (domain A), yellow (domain B), green (domain C), red (domain D) and light blue (domain E). Calcium ions are denoted by pink-coloured circles (Harata et al., 1996)

(45-55 kDa) (Figure 1.2b, 1.3). The X-ray crystallographic structures are available for five CGTases (Table 1.2). The structural domain A is about 300 to 400 amino acid residues long. It contains a highly conserved symmetrical fold of eight parallel β-strands surrounded by eight α-helices known as typical $(\beta/\alpha)_8$ barrel or TIM barrel structure. The structural domain B (44-33 amino acid residues) is an inserted small loop between β-strand 3 and α-helix 3 in domain A. The domain A contains the active site (catalytic center and substrate binding) in α-amylase family enzymes and CGTases. It contains four regions, which are highly conserved, indicating a common catalytic mechanism for α-amylase family enzymes and CGTases. The domain B is supposed to be responsible for functional diversity in α-amylase family enzymes. The C domain, which is about 100 amino acid residues long, folds as eight parallel β-sheets in a Greek key topology. The function of C domain is not properly understood. However, it is reported that it contains a binding site for maltose (Penninga et al., 1996). Some mutations in C domain of α-amylases inactivate the enzymes, which indicates that the domain is probably necessary for enzyme activity (Schmid 1996a). The domain D is approximately 90 amino acid residues long, with a structure similar to immunoglobulin topology. Its function is not known. The E-domain is about 110 amino acid residues long and is present in several glycosylase enzymes. It contains a putative raw starch-binding motif (Lawson et al., 1994; Svensson 1989). Dalmia et al. (1995) have reported domain E as an independent starch binding domain, which retains its starch binding activity even if separated from the other four domains. Domain E contains two maltose-binding sites (Penninga et al., 1996). CDs are known to bind at domain E and interfere with starch binding (Knegtel et al., 1995).

The active site forms a depression on the enzyme surface, which can accommodate 5-10 glucose residues of the substrate and may be regarded as consisting of corresponding number of subsites. The catalytic site is situated between two of the subsites and its location varies in different CGTases. *Klebsiella pneumoniae* M 5 al CGTase is supposed to possess 8 subsites (Bender 1990). Klein et al. proposed that the active center of CGTase contains 7 subsites for binding to the amylose chain. These subsites were numbered from the nonreducing end to the reducing end as S_5 to S_2'

(Figure 1.2a). The cleavage or coupling point is considered to be located between subsites S_1 and S_1'. The acidic amino acid residues Asp-229, Glu-257 and Asp-328 are located next to the cleavage site and presumably play a crucial role in the catalytic function. The proposed catalytic mechanism involves binding of CGTase to nine residues in the amylose chain, subsequent cleavage between glucose 7 and 8 at subsite S_1, S_1' releasing maltose while maltoheptaose (G_7) remains attached to the enzyme. There are two possibilities at this point. First, the nonreducing end of maltoheptaose can bind to the cleavage site and couple with the reducing end of G_7 resulting in formation of β-CD. Second, another nonreducing end of the linear dextrin can bind at the cleavage site and couple with G_7 to form a longer oligosaccharide chain (disproportionation reaction). The bond cleavage mechanism involves three carboxylates Asp-229, Glu-257 and Asp-328. To start with, Glu-257 is protonated forming a strong hydrogen bond with Asp-328, thus increasing the pK_a of Glu-257. The proton is then donated to the glycosidic O4-atom of G_8 at subsite S_1' resulting in cleavage of the oligosaccharide. The nonprotonated Asp-229 stabilizes the general oxocarbonium ion-like intermediate or a covalent intermediate and keeps the G_7 or transferred glucosyl chain tightly bound to the enzyme (Schmid 1996a).

Abe et al. (1991) have also investigated the nature of the active site of *Bacillus macerans* using modified maltooligosaccharides and found that S_1 and S_1' contain the catalytic site. Studies on CGTase from *Bacillus circulans* E 192 (Bovetto et al., 1992b) revealed the presence of acceptor and donor sites in the binding site with nine subsites. The cleavage occurs between subsite 2 and 3.

However, recently Terada et al. (1997) proposed a new hypothesis for cyclization reaction of CGTase. The new model says that instead of an exo-type attack (earlier mechanisms), CGTase probably attacks any α-1,4-linkage within the amylose chain. The newly formed reducing end of the substrate is transferred either to the non-reducing end of a separate linear acceptor molecule or glucose (intermolecular transglycosylation) or to its own non-reducing end (intramolecular transglycosylation). This random cyclization reaction produces wide range of cyclic α-1,4-glucans with degree of polymerization from

6 to more than 60. The equilibrium of the reaction favours the formation of α- or β-CD as the final major products.

8. Protein engineering

The amino acid residues near the active site or highly conserved residues have been replaced by other residues using site-directed mutagenesis to study the structure-function relationship (Mattsson et al., 1995; Nakamura et al., 1992; 1993, 1994a; Penninga et al., 1995; Sin et al., 1994). Nakamura et al. (1993) have substituted three highly conserved histidine residues (His-140, His-233 and His-327) in the α-amylase family enzymes and CGTases with asparagine. These changes strongly affected k_{cat} for cyclization, coupling and hydrolytic activity, whereas there was no effect on K_m values. This study further revealed that histidine residues participate in the stabilization of the transition state, with very little influence on substrate binding. Replacement of four histidine residues at 98, 140, 233 and 327 by aspartate also resulted in substantial decrease in cyclization and hydrolytic activities in *Bacillus circulans* (ATCC 21783) (Mattsson et al., 1995).

The four aromatic amino acid residues (Phe-183, Tyr-195, Phe-259 and Phe-283) located in the active center, which are highly conserved among the CGTases and are not found in α-amylase family enzymes, were replaced by site-directed mutagenesis in alkalophillic *Bacillus* sp. 1011 CGTase (Nakamura et al., 1994a). Replacement of Tyr-195 of resulted in production of considerably higher concentration of γ-CD and virtually no α-CD. The amino acid residues Phe-183 and Phe-259 are cooperatively involved in acceptor binding and play an important role in cyclization reaction. Phe-283 is involved in transition-state stabilization. Replacement of Tyr-188 in *Bacillus ohbensis* with Trp doubled γ-CD production (Sin et al., 1994). Penninga et al. (1995) replaced Tyr-195 in *Bacillus circulans* strain 251 CGTase with Phe, Trp, Leu and Gly. These substitutions significantly reduced cyclization and coupling activities, but did not affect disproportionation and hydrolytic activities.

9. *Chemical modification*

Many researchers have investigated effect of group specific chemical modifiers for carboxylate, histidine, tryptophan, tyrosine, cysteine and lysine residues. Even though it is clear from X-ray chrystallographic studies that carboxylates play an important role in catalytic activity of CGTases, chemical modification of carboxylates has not been well studied; there are only two reports of carboxylate modification of CGTase. Modification of carboxylates with 1-ethy-3-(3-dimethyl amino-propyl carbodiimide (EDC) in the presence of acarbose caused decrease in cyclization and disproportionation activities, whereas no change was observed in hydrolytic activity of *Thermoanaerobacter* sp. CGTase (Alcalde et al., 1998). Modification of β-CGTase from *Bacillus firmus* var. alkalophilus CGTase with Woodward's reagent, which is specific for modifying carboxylate groups, drastically reduced cyclization and hydrolytic activities (Shin et al., 1999).

Modification of histidine residues in CGTases has been reported by many researchers (Bender 1991; Mattsson et al., 1992; Jeang and Lin 1994; Shin et al., 1999; Villette et al., 1993). All these reports indicated that ethoxyformylation of histidine residues with diethyl pyrocarbonate (DEP) caused decrease in cyclization activity. The substrate, product or inhibitor could protect the enzyme from inactivation, which indicates that histidine residues are involved in substrate binding and transfer reactions. Modification of tryptophan residues with *N*-bromosuccinimide (NBS) caused inactivation of hydrolytic activity. Substrate protection studies with G_2-G_4 suggest that tryptophan is located in the substrate-binding site of CGTase in *Bacillus stearothermophilus* (Ohnishi et al., 1992, 1994). Shin et al. (1999) demonstrated that tryptophan residues are involved in catalytic activity and are located at the substrate-binding site of β-CGTase from *Bacillus firmus* var. alkalophilus. Results of nitration of tyrosine residues of *Bacillus circulans* E 192 CGTase with tetranitromethane (TNM) of *Bacillus circulans* E 192, indicated involvement of tyrosine residue in the catalytic site of CGTase (Villette et al., 1993). *Bacillus circulans* (ATCC 21783) CGTase was shown to contain two cysteine residues linked together by disulfide bridge, using chemical modification studies involving modification of cysteine residues with 5, 5′-dithio-(2-nitrobenzoic acid) (DTNB)

(Mattsson et al., 1992). Modification of lysine residues with acetic anhydride in *Thermoanaerobacter* sp. CGTase caused significant reduction in cyclization, coupling and disproportionation activities, whereas the hydrolytic activity was slightly enhanced (Alcalde et al., 1999).

10. Cloning and overexpression

Many CGTase genes have been cloned in either homologous or heterologous hosts, primarily to improve CGTase production (Schmid 1989, 1996a) (Table 1.2). The wild strains typically produce CGTase in mg/L quantities. Overexpression of CGTase genes is expected to yield g/L quantities of CGTases. Atttempts to express CGTases in *E. coli* resulted in low expression levels, mainly due to the inability to secrete the enzyme which accumulates in the periplasmic space (Georganta et al., 1991; Sin et al., 1991; Paloheimo et al., 1992; Kimura et al., 1990; Lee and Tao, 1994) or forms inclusion bodies (Lee and Tao, 1994). *Bacillus* should be a better host as it secretes proteins more efficiently. However, it produces high levels of proteases and development of protease-negative or protease-deficient strains is crucial (Sin et al., 1993). CGTase gene has been expressed under the control of inducible promoters (tac, trp, lac, λ-pL, α-amylase, cellulase or T_7 RNA polymerase) from different sources (Hellman et al., 1992; Kimura et al., 1990; Lee and Tao 1994; Paloheimo et al., 1992; Sin et al., 1993). In an interesting study by Oakes et al. (1991), CGTase gene from *Klebsiella* was expressed into potato plants to produce α- and β-CD in the tubers of transgenic potatoes. However, this resulted in very low levels of CDs, corresponding to 0.001-0.01% of the starch. Jorgensen et al. (1998) have integrated and amplified CGTase gene from *Thermoanaerobacter* sp. in the *Bacillus subtilis* chromosome. Recently, Han and Tao (1999b) observed 40% increase in the activity of soluble CGTase by inducing the enzyme production by lowering the temperature from 30 to 25 °C in the presence of 10 mM $CaCl_2$. The best results of overexpression are reported by a research group from BioTechnica International (Schimd 1989) for α-CGTase of *Klebsiella pneumoniae* M 5 al (1 g/L) and β-CGTase of *Bacillus* sp. No. 1-1 (6-7 g/L) when expressed in *Bacillus subtilis* host.

III. Cyclodextrin production

CGTases catalyze the conversion of starch or related materials like amylose, amylopectin, glycogen, dextrins and malto-oligosaccharides to cyclodextrins (CDs). Starch consists of two types of glucan polymers, amylose and amylopectin. Amylose is predominantly composed of linear chains of $\alpha(1\rightarrow4)$-linked glucose residues, each ~ 1000 residues long and is branched at a very low level by $\alpha(1\rightarrow6)$-linkages (one branch point approximately per 1000 residues) (Martin and Smith 1995). In solution, amylose forms hydrogen bonds between the molecules resulting in rigid gels. This solution, when heated, crystallizes and shrinks, in a process known as retrogradation. The amylose content of starches from different sources varies from 11 to 35% depending on the origin, plant species, variety within plants, plant organ, developmental age of that organ and growth conditions of the plant. Amylopectin, on the other hand, is a highly branched $\alpha(1\rightarrow4)$-linked glucan polymer, containing approximately one branch point $\alpha(1\rightarrow6)$-linkage per 20 glucose residues. Amylopectin possesses only one reducing end per molecule. In solution, amylopectin forms fewer hydrogen bonds than amylose and remains fluid with high viscosity and elasticity.

CGTase attacks a starch molecule from the non-reducing end to form CD. As there are very few non-reducing ends available in amylose, CD production from amylose gives low yield and requires longer reaction times (Schmid 1996b). However, CD yield from amylopectin is higher, simply because it provides more non-reducing ends than amylose. Further amylose chain retrogrades easily after shortening by enzyme reaction, resulting in precipitation. This also makes downstream processing more difficult because retrograded starch molecules cause turbidity in aqueous CD solutions. The starch used for CD production should contain very little lipid and protein impurities. Potato starch is the preferred substrate for industrial CD production because it contains 79% amylopectin and 21% amylose, with only minor amounts of lipids and proteins. Corn starch, in contrast contains 72% amylopectin, 28% of amylose, 10-fold more lipids and 5-fold more proteins and shows much higher tendency towards retrogradation. The amounts of amylose and amylopectin in wheat starch are similar to those in corn starch. Tapioca starch contains a higher fraction of amylopectin compared to potato starch and very few impurities.

However, it is relatively expensive and getting a consistent quality is often problematic. The use of waxy maize starch improves CD production yield because it contains nearly 100% amylopectin and the impurities are significantly lower than in ordinary corn starch. However, the cost of waxy corn starch is too high for large-scale production of CDs (Schmid 1996b). Other cheap sources like rice bran (Abelian et al., 1993), sago starch (Solichien 1995) have also been used for CD production.

Morita et al. (1996) synthesized β-CD using maltose in water-organic solvent system. They obtained a maximum of 13% (w/w) conversion to β-CD from 5% (w/v) maltose concentration in the presence of 44% (v/v) cyclohexane at 7 °C. Brunet et al. (1998) have studied CD production from malto-oligosaccharides (up to maltoheptaose). They observed that CGTase from *Thermoanaerobacter* sp. could sequentially disproportionate maltose to produce longer polymers and CDs, mainly α-CD.

CD production typically consists of the following main steps: Th. 8163

1. Cultivation of a microorganism which produces the CGTase
2. Downstream processing of the enzyme involving separation from fermentation broth, concentration and purification
3. Conversion of starch to a mixture of cyclic and non-cyclic dextrins using CGTase
4. Separation of CDs from the reaction mixture and crystallization

All known CGTases produce a mixture of CDs in varying ratios. CGTases, which can produce predominantly one type of CD, are useful for industrial CD production. Thus, microorganism which produce such CGTases are preferred in the first step.

In the second step, the enzyme is separated from the fermentation broth, either by centrifugation or microfiltration, and concentrated by ultrafiltration. Commercial processes for CD production do not use highly purified enzyme preparations, simply to reduce the production cost. The conversion of starch to CDs is a crucial step in CD production and depends on the following factors.

1. Effect of substrate concentration

The yield of CDs greatly depends on the initial substrate concentration and drastically decreases at higher concentrations. Nearly 80-90% conversion can be obtained with 1% (w/v) starch (Horikoshi 1979; Jamuna et al., 1993). The optimum starch concentration represents a compromise between several factors. Starch is a cheap raw material. Thus use of high concentrations is significantly cost-effective, primarily because of reduction in the reaction volume, which facilitates concentration of products. Generally, a starch concentration of 15-30% is found to be optimum for commercial production of CDs (Schmid 1996b). According to Szejtli (1982), no more than 5% (w/v) solutions of unmodified starch should be used because high substrate concentration causes retrogardation of starch, which impairs the CD yield. Moreover, viscosity of starch solutions, at concentrations above 5% (w/v), becomes very high. The viscosity of the starch solutions can be reduced by partial hydrolysis, using acid, enzymes or mechanical treatment (milling, extrusion or jet-cooking). However, excessive hydrolysis decreases CD yield. A 34% (w/v) starch solution, hydrolysed to a glucose equivalent (DE) of 1, resulted in 45% conversion to CDs, whereas the same concentration of starch solution hydrolysed to DE of 12, lead to only 17% conversion to CDs (Schmid 1996b).

By taking the advantage of its chain shortening reaction, CGTases are used to liquefy the native 30% (w/v) starch gels without any retrogradation (Bender 1986). However, stirring of the enzyme to large volumes of highly concentrated starch gels is technically difficult which can be overcome by simply heating the starch suspension with CGTase at 80 °C for both, solubilization and liquefaction. Prehydrolysis of starch is also performed by α-amylase treatment. The disadvantage of prehydrolysis in the conventional process is that during the substrate degradation by liquefying enzyme large amounts of glucose, maltose and low molecular weight dextrins are produced which reduce the CD yield. These products are used by the CGTase for coupling reaction resulting in reduction of the cyclization reaction.

Raw starch exits as a compact crystalline structure, which cannot be attacked by CGTases. Consequently the rate of CD production from raw starch is usually too low to

be useful for industrial production. Different researchers have developed improved pretreatment methods to overcome this problem.

Lee and Kim (1991a) developed a cost-effective process for the production of CDs from corn starch in an attrition bioreactor in which hydrolysis of starch and synthesis of CDs by CGTase occur simultaneously. It is known that CGTases are inhibited by the reaction products. The activity of *Bacillus* sp. BE101 CGTase decreased to 50% in presence of 6 g/L β-CD (Kim et al., 1992). Bergsma et al. (1988) also reported inhibition of *Bacillus circulans* CGTase activity by CDs. Kim et al. (1992, 1993) evaluated the production of CDs from milled corn starch in an ultrafiltration membrane bioreactor to increase the yield by reducing product inhibition. CGTase and unconverted starch are retained within the bioreactor by the membrane with appropriate molecular weight cut-off, whereas synthesized CDs pass through the membrane and are constantly removed. Cami and Majou (1992) also used ultrafiltration membranes for removal of CDs formed and obtained 29.9% β-CD yield after digestion of potato starch with *Bacillus ohbensis* CGTase for 7 h.

Kim et al. (1995) used moderately heat-treated corn starch for CD production. The optimum conditions were defined as heat-treating temperature 65 °C, heat-treating time of 1 h, corn starch 7.5% (w/v) and 48 U of enzyme per g of starch. When native starch is used CDs are produced in low yield, so controlled liquefaction like moderate heat-treatment has many merits. The low molecular weight dextrins are not produced hence do not decrease the CD yield due to coupling reaction of CGTase. The residual starch can be removed by simply filtration or centrifugation. The pretreatment method is simple and unconverted starch can be used as substrate for the production of other compounds like glucose, maltose, maltodextrins, etc.

Direct production of CDs from insoluble raw starch eliminates the gelatinization step leading to reduction in cost of production. It also has a distinct advantage of formation of high purity of CD without accumulation of undesirable oligosaccharides, thereby facilitating purification of CDs. Kim et al. (1997) used raw corn starch without pretreatent for the production of CDs and obtained 2.4 times higher conversion on the basis of a unit of CGTase used. Lee and Park (1991) used extruded corn starch for CD

production which yielded 54 g/L CDs as compared to that 45 g/L CDs obtained with liquefied corn starch and 6 g/L CDs with raw corn starch. Supplementation of α-amylase slightly enhanced initial CD production rate but later decomposed CDs. Han and Lee (1991) studied production of CDs from raw starch in the bead reaction system. They obtained 27% (w/w) conversion to CDs from 200 g/L raw corn starch concentration using 6 U of enzyme per g of starch.

2. Effect of enzyme concentration

The CD yield also depends on the enzyme concentration. The ratio of substrate to enzyme concentration greatly affects the overall CD yield the relative amounts of different CDs produced. The choice of a CGTase concentration suitable for industrial production of CDs depends on the price of the enzyme and length of the production process. There is always correlation between the attainable degree of conversion into CDs and the CGTase concentration. As the concentration of enzyme is increased conversion rate is greatly increased (Shiraishi et al., 1989a; Mattsson et al., 1991). However, excess enzyme above optimum concentration (at which maximum CDs are produced) resulted in rapid decrease in CD yield as CDs produced are utilized in coupling reaction. This indicates that enzyme concentration at which maximum CDs are produced is only recommended. Best results have been obtained with enzyme substrate ratios (w/v) of 1:1000 to 1:5000 (Bender 1986).

3. Effect of complexing agents

The equilibrium of the transfer reactions of CGTases can be shifted drastically towards cyclization reaction by addition of compounds capable of forming inclusion complexes (clathrates) with CDs. In presence of specific guest (complexant) the ratio of α : β : γ-CD is significantly altered with a yield higher than 90% for one of the major forms of CD depending upon the selectivity of the complexing agent. Conversion of starch to CDs is increased at least two-fold. McClenahan et al. (1942) pointed out that product ratio of β-CD can be influenced by addition of trichloroethylene from 22% to 54%.

Many researchers have used solvents like aliphatic alcohols and acetone for enhanced CD production (Lee and Kim 1991b; Mori et al., 1995; Shiraishi et al., 1989a and b;

Tomita et al., 1990). Ethanol is believed to decrease the water activity of the reaction medium thus decreasing the probability of an occasional hydrolysis reaction that might take place at the active site and also to inhibit the transglycosylation reaction (Mattisson et al., 1991; Tomita et al., 1990). Use of surfactants like sodium dodecyl sulphate (SDS, Kobayashi et al., 1977; Chiu 1987) and Triton X-100 (Tomita et al., 1993) and polyethylene glycols and other polyols (Delbourg et al., 1993) to achieve better conversion yields has also been studied. The surfactants those with straight carbon chain as hydrophobic moiety enhances the α-CD yield whereas those with bulky hydrophobic moiety than straight carbon chain selectively produce β-CD (Kobayashi et al., 1983). They obtained 1.6-fold more α-CD production from soluble starch in presence of SDS as compared to that without SDS addition.

4. Effect of debranching enzymes

The branching points in amylopectin i.e. α(1→6)-linkages, block the action of CGTase. Therefore, debranching enzymes like pullalanase and isoamylase, which break these bonds, are used prior to the conversion of starch to CDs to improve the yield (Rendleman 1997).

Industrial production of CDs

Industrial CD production processes can be classified into two main categories depending on the use of complexing agent (usually solvent) as solvent and non-solvent processes. A typical solvent process consists of liquefaction of starch, enzymatic conversion of liquefied starch to CDs, removal of complexing agent, concentration of CDs and crystallization or drying. In non-solvent process, removal of complexing agent is not required. However, there are additional crystallization steps for improved recovery of the final product. The major disadvantage of solvent processes is that CDs produced can not (or rarely) be used for food and pharmaceutical applications, as most of the complexing agents are extremely toxic even at low concentrations. However, in solvent process high product yields are obtained and therefore it is highly economical. In the non-solvent process maximum 20% conversion of starch can be obtained and the process is only suitable for production of β-CD (Schmid 1996b). The β-CD can be easily recovered by

crystallization because of low solubility in water, but the solubilities of α- and γ-CDs are very high. Therefore, isolation of α-CD or γ-CD from the filtrate after crystallization of β-CD involves large number of chromatographic steps. Thus non-solvent process is not economical and is only used in Japan owing to the specific food approval for β-CD (Schmid 1996b).

a. Production of α-CD

Alpha-CD is produced using α-CGTase produced by *Klebsiella pneumoniae* M 5 al or from β-CGTase of *Micrococcus varians* (Yagi et al., 1980), *Bacillus ohbensis* (Sato et al., 1994) or *Bacillus circulans* (Japan Maize Products Co., Ltd., Japan) by modifying the conversion conditions. Alpha-CD production was enhanced by addition of C_{1-8} aliphatic alcohols, aliphatic ethers, esters and C_{2-4} ketones with *Bacillus macerans* CGTase (Toyo Jozo Co., Ltd., Japan). In presence of complexing agent like ethanol, 35% yield (based on starch) can be obtained with liquefied starch (DE 5) using 5 U of *Klebsiella* α-CGTase per g of starch (Schmid 1996b). Ethanol forms soluble complex with α-CD; it can be separated from the reaction mixture by ultrafiltration or saccharification of nonconverted starch with amylase, followed by crystallization of α-CD. However, insoluble complexing agents like n-butanol (Abelian et al., 1993) or 1-decanol (Armbruster 1988; Armbruster and Jacaway 1972; Flaschel et al., 1984) are often used for α-CD production for better recovery. Shieh and Hedges (1993) used cyclohexane as complexing agent for enhanced production of α-CD. They obtained better reaction yields with waxy corn starch hydrolysate (DE 5) by reducing reaction temperature to 20-30 °C.

b. Production of β-CD

Industrial production of β-CD by a non-solvent process was developed by Horikoshi and coworkers (Matzuzawa et al., 1975; Horikoshi 1979). They obtained final conversion yield of 24% (w/w) to β-CD from 15% (w/v) potato starch. Since β-CD is produced more favourably than other two types of CDs, many workers studied its production on industrial scale using solvent-based processes for obtaining high conversion yields. The most commonly used additives for β-CD production are toluene (Armbruster and Kooi 1969; Cramer and Steinle 1955; Vakaliu et al., 1977) and trichloroethylene (McClenahan

et al., 1942; Okada and Tsujama 1973; Sato and Nakamura 1974; Yagi et al., 1986). The other complexing agents tried inclue cyclohexane (Morita et al., 1996) and bromobenzene (Raja et al., 1990; Raja and Ramakrishna 1994).

c. Production of γ-CD

Horikoshi et al. (1981) developed a process for isolation of γ-CD from the mixture of CDs with more than 98.5% pure γ-CD without using any complexing agent. A 15% (w/v) starch solution was acted upon *Bacillus* sp. No. 38-2 CGTase, residual starch was hydrolysed with α-amylase and digest was passed through activated carbon and ion exchangers. Beta-CD was separated from concentrated solution after crystallization and cooling. This mother liquor contained 8% γ-, 7% β- and 3% α-CD and 80% glucose and oligosaccharides. The mother liquor is used for isolation of γ-CD and from 1 t of mother liquor, 14 Kg of pure γ-CD was isolated with purity higher than 98.5%. The γ-CD production was enhanced by using combination of bromobenzene and sodium acetate with α-CGTase from *Klebsiella pneumoniae* M 5 al (Bender 1983a), 13-24 membered macrocyclic compounds (Schmid et al., 1988). Gamma-CD production was also enhanced by a mixture of two complexing agents methyl ethyl ketone and α-naphthol (Seres et al., 1985). Addition of tetra- or pentacyclic terpenoids like glycyrrhizic acid or stevioside (Sawaguchi et al., 1990; Sato et al., 1985b) and C_{12} cyclic compounds (Rendleman 1992; Schmid and Eberle 1988; Schmid 1996b) results in improved γ-CD production. An economical process for the production of γ-CD using CGTase of *Bacillus firmus* 290-3 produced by a recombinant strain and γ-CD specific complexing agent (cyclohexadec-8-en-1-one) is reported by Schmid (1996b).

The final step in the preparation of CDs is separation of individual CDs from the reaction mixture. A number of methods are used for the purification of CDs from the starch hydrolysate containing cyclic and acyclic dextrins. They are based on solubility differences, use of specific complexing agents, chromatographic techniques etc.

The solubility of β-CD in water is very low (18.5 g/L, at 25 °C). Therefore, it can be crystallized directly from concentrated solutions at low temperatures which can give almost 98% pure β-CD (Hedges 1992). CDs are concentrated and crystallized after

removal of complexing agent by steam distillation of solvent (Schmid 1996b). Beta-CD was purified by adding isopropanol or cyclohexane in the reaction mixture (Sophianopoulos and Warner 1992; Li et al., 1996). CDs are separated on specific adsorbents like strong cation-exchange resins (Fujita et al., 1989) and hydrophobic polymeric resins (Horikoshi et al., 1981). The affinity-chromatographic adsorbents, which are capable of selective adsorption of α-, β- or γ-CD are also used for CD purification (Mattsson et al., 1988; Makela et al., 1989; Tsuchiyama et al., 1991a,b).

IV. Scope of this thesis

Most CGTases produce a mixture of CDs from starch or related oligo-saccharides. Purification of individual CDs from the reaction mixture is a challenging problem. Therefore, novel CGTases, which can selectively produce only one type of CD from starch, have a great industrial potential. Beta-CD producing enzymes are the most common ones and have been studied extensively in the past. However, there have been very few systematic studies for optimization of media and growth conditions for CGTase production. The objectives of the present study are outlined below.

Objectives

1. Isolation of an organism that can produce a novel CGTase specific for conversion of starch to CDs other than β-CD
2. Purification to homogeneity and characterisation of this novel CGTase
3. Optimization of media for CGTase production
4. Study of cell growth and CGTase production in different fermentation modes
5. Optimization of conversion of starch to CD using this enzyme

Chapter 2 describes the isolation of CGTase producing organism and its taxonomical identification. The CGTase enzyme from this strain was purified to homogeneity. The purified enzyme was characterised in terms of its molecular weight, isoelectric point, pH optimum and stability, temperature optimum and stability, effect of calcium ions and kinetic parameters. The N-terminal sequence of the pure protein was determined. The action of CGTase on various gelatinized and raw starches was investigated. Preliminary results of chemical modification of carboxylate groups and tryptophan residues are also described.

Chapter 3 details the results of a two-step strategy for optimization of CGTase production from the selected strain in shake flasks and fermenters using a statistical experimental design approach. Effects of various components in the basal medium, like carbon, nitrogen, phosphorus, and mineral sources as well as temperature and initial pH on enzyme production were investigated. The optimum concentrations of the selected

media components were then determined using two level fractional factorial designs in five variables, namely, dextrin, peptone, yeast extract, ammonium dihydrogen orthophosphate, and magnesium sulphate concentrations. The CGTase activity obtained with optimized and basal media in shake flasks and fermenters was compared. The cell growth and CGTase production profiles were studied with the optimized medium in shake flask and 1 L fermenter.

In **Chapter 4**, the results of our studies on α-CD production using CGTase from selected strain are described. The conversion of raw wheat starch and dextrin to CDs in the presence of various additives or complexing agents like alcohols, organic solvents, polyethylene glycols, fatty acids and detergents was studied. The effect of n-butanol concentration and enzyme concentration was also investigated with the optimum concentration of raw wheat starch. Two level complete factorial experimental designs with three variables namely dextrin, n-hexanol and enzyme concentrations were used to optimize α-CD production from dextrin. The effectiveness of ultrafiltration membrane bioreactor for continuous removal of CDs was also studied.

Chapter 5 describes the experiments to study CGTase production in batch, fed-batch and continuous mode of operations in automated bioreactors. The effect of temperature, pH, agitation speed and substrate concentration on enzyme production was investigated The role of carbon sources like glucose, maltose, malto-oligosaccharides and CDs on CGTase induction was also investigated. High cell density fermentations, using controlled feeding of glucose or dextrin, were carried out with the objective of increasing CGTase production. Continuous culture experiments were also performed with tapioca starch or dextrin as carbon sources.

Finally, **Chapter 6** summarizes the conclusions of our work and offers suggestions for future studies in this area.

References

References are listed in Chapter 7

CHAPTER 2

A novel raw starch degrading α-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae pneumoniae* AS-22: purification, properties and use for cyclodextrin production

Summary

A novel raw starch degrading α-cyclodextrin glycosyltransferase producing organism (CGTase) was isolated from soil samples and taxonomically identified as *Klebsiella pneumoniae pneumoniae* AS-22. CGTase from this organism was purified to homogeneity by ultrafiltration, affinity and gel filtration chromatography. The specific cyclization activity of the pure enzyme preparation was 523 U/mg of protein. No hydrolysis activity was detected when soluble starch was used as the substrate. The molecular weight of the pure protein was estimated to be 75 kDa with SDS-PAGE and gel filtration. The isoelectric point of the pure protein was 7.3. The enzyme was most active in the pH range 5.5-9.0 whereas it was most stable in the pH range 6-9. The CGTase was most active in the temperature range 35-50 °C. This CGTase is inherently temperature labile and rapidly loses activity above 30 °C. However, presence of soluble starch and calcium chloride improved the temperature stability of the enzyme up to 40 °C. In the presence of 30% (v/v) glycerol, this CGTase was almost 100% stable at 30 °C for a month. The K_m and k_{cat} values for the pure enzyme were 1.35 mg/mL and 249.13 μM/mg/min, respectively, with soluble starch as the substrate. The N-terminal sequence of the protein shows some homology with CGTase from another organism of the same genus, but no homology with CGTases from other organisms. The preliminary chemical modification studies with carboxylate groups and tryptophan residues indicated involvement of these moieties in CGTase catalytic function. The enzyme predominantly produced α-cyclodextrin (α-CD) without using any complexing agents. The maximum production of α-CD was obtained with 100 and 125 g/L of gelatinized soluble starch and raw wheat starch, respectively, with 10 U of enzyme per g of starch. In 4 h, 21% (w/w) conversion of soluble starch to total CDs was obtained and the ratio of α : β : γ-CD was 81 : 12 : 7. With raw wheat starch as the substrate, 20% (w/w) conversion of raw wheat starch to total CDs was obtained in 4 h and ratio of α : β : γ-CD was 89 : 9 : 2.

Introduction

The enzyme cyclodextrin glycosyltransferase (CGTase; E.C. 2.4.1.19) converts starch to non-reducing cyclic malto-oligosaccharides called cyclodextrins (CDs). CDs are of three main types α-CD, β-CD and γ-CD, containing 6, 7 and 8 glucose residues, respectively, linked by α(1→4) glycosidic bonds. CD molecules have a unique structure with a hydrophobic cavity and a hydrophillic surface. Because of this feature they can form inclusion complexes with a variety of hydrophobic guest molecules (Bender 1986, Szejtli 1984). This molecular encapsulation changes the physical and chemical properties of the included molecules. Consequently, CDs are becoming increasingly popular in pharmaceutical, agricultural, chemical, cosmetic and food industries (Bender 1986, Hedges 1992, Horikoshi 1979, Szejtli 1984).

All known CGTases produce a mixture of α-, β- and γ-CDs in different ratios. Enzymes capable of predominantly producing a particular type of CD can decrease subsequent purification costs and hence are commercially valuable. However, most of the reported CGTases produce β-CD as the main product (Goel 1995, Schmid 1996a). Enzymes producing primarily α- or γ-CD are relatively rare. *Klebsiella pneumoniae* M 5 al (Bender 1977a), *Bacillus macerans* IAM 1243 (Kobayashi et al., 1978) and *Klebsiella oxytoca* 19-1 (Lee et al., 1992) CGTases are the representatives of α-CD producers. *Bacillus subtilis* No. 313 (Kato and Horikoshi 1986), alkalophillic *Bacillus* strain 290-3 (Englbrecht et al., 1990), *Bacillus* sp. AL-6 (Fujita et al., 1990) and *Brevibacterium* sp. No 9605 (Mori et al., 1994) CGTases are the only known γ-CD producers. Most known CGTases can only attack gelatinized (or physically modified) starches. If an efficient, raw starch-degrading CGTase is available, the energy-intensive gelatinization step can be avoided, with consequent reduction in cost of CD production.

We have isolated an organism which produces a novel CGTase that predominantly makes α-CD from raw starch. Although there are few raw starch degrading CGTases reported, they degrade gelatinized starch more efficiently than the raw starch. The present CGTase can convert gelatinized and raw starch with almost the same efficiency, with the additional advantage of producing primarily α-CD. We describe here the

purification and properties of this novel enzyme from *Klebsiella pneumoniae pneumoniae* AS-22 and its potential use in CD production.

Materials and Methods

Materials

Soluble starch and dextrin were purchased from E. Merck (Mumbai, India). Potato starch was obtained from Loba Chemie (Mumbai, India), whereas tapioca starch was from Laxmi Starch (Coimbature, India). Malto-oligosaccharides (G_1-G_7), α-, β- and γ-CDs, corn starch, wheat starch, rice starch, amylose, amylopectin, bovine serum albumin (fraction V), molecular weight calibration kits for electrophoresis and gel filtration, and standard pI markers were purchased from Sigma (USA). Sephacryl S-200 was procured from Pharmacia Fine Chemicals (Uppsala, Sweden). Magnafloc was a kind gift from Hindustan Antibiotics Ltd. (Pimpri, India). Yeast extract and peptone were purchased from HiMedia (Mumbai, India) while all other chemicals were of analytical grade from S. D. Fine Chemicals (Boisar, India).

Isolation of starch degrading organisms

Soil samples were collected from fields around two starch factories. The following three methods were employed to isolate starch degrading organisms from these samples.

Method 1:

Soil sample (10% w/v) was added to a sterile starch enrichment broth. The composition of enrichment medium was (in g/L) soluble starch 10, yeast extract 0.5, peptone 0.5, Na_2HPO_4 1.5, NaH_2PO_4 0.2, $MgSO_4.7H_2O$ 0.2, pH 7.0. The organisms were grown overnight at 30 °C and 200 rpm on a rotary shaker. The broth was serially diluted and 0.1 mL of each dilution was plated on selective plates. The composition of selective medium was (in g/L) soluble starch 10, yeast extract 5, peptone 5, Na_2HPO_4 1.5, NaH_2PO_4 0.2, $MgSO_4.7H_2O$ 0.2, congo red 0.01, xylene cynole FF 0.01, agar 20, pH 7.0 (Hamaker and Tao 1993). Plates were incubated at 30 °C for 24 h and starch-degrading colonies were picked (indicated by a halo around the colony) and streaked again on selective plates.

Method 2:

Soil sample (10 % w/v) was mixed in distilled water, diluted serially and 0.1 mL of each dilution was plated on selective plates and starch-degrading organisms were isolated.

Method 3:

Soil sample (10 % w/v) was mixed in distilled water, boiled at 80 °C for 10 min., cooled, diluted serially, plated on selective plates and starch-degrading organisms were isolated.

Screening for CGTase production

A single colony of culture isolate (starch-degrading) was inoculated in 10 mL of basal medium in 50 mL tube and incubated at 30 °C for 24-36 h on a rotary shaker. The composition of the basal medium was (in g/L), soluble starch 10, yeast extract 5, peptone 5, Na_2HPO_4 1.5, NaH_2PO_4 0.2, $MgSO_4.7H_2O$ 0.2, pH 7.0. Broth was centrifuged at 16,000 *g* and supernatant was used as enzyme preparation for further testing. A batch of 20 isolates was cultivated at one time and enzyme from all the isolates was stored at -20 °C and later on used for screening of CGTase activity. One milliliter of 1% (w/v) soluble starch, prepared in 50 mM phosphate buffer, pH 7.0, was mixed with 0.1 mL of enzyme and incubated at 40 °C for 20 min. The reaction was stopped by addition of 0.1 mL of 1.2 N HCl. Concentration of CDs in the reaction mixture was analyzed by HPLC (Sato et al., 1985a) as described below.

CGTase Assays

CGTases are known to perform four different enzymatic reactions (Penninga et al., 1995; Wind et al., 1995). The activities of these reactions were determined by following specific enzymatic assays. Unless stated otherwise, the enzyme activity refers to the cyclization activity.

Cyclization activity

Cyclization activity was determined using a modification of the method described by Lejuene et al. (1989). One milliliter of 1% (w/v) soluble starch, prepared in 50 mM phosphate buffer, pH 7.0, was mixed with 0.1 mL of appropriately diluted enzyme and incubated at 40 °C for 10 min. The reaction was stopped by immediately cooling the tubes in chilled water, followed by addition of 0.1 mL of 1.2 N HCl. Two milliliter

methyl orange solution (final concentration 0.035 mM) was then added to the reaction mixture and the tubes were maintained at 15 °C for 30 min. The absorbance of the mixture was measured at 507 nm and the decrease in absorbance (with respect to a control tube without the enzyme) was correlated with the amount of α-CD formed. One unit of cyclization activity is defined as the amount of enzyme that produced one μmole of α-CD per minute under standard conditions.

Coupling activity

The coupling activity was determined by measuring the disappearance of α-CD in the presence of maltotriose (G_3) (Wind et al., 1995). Appropriately diluted enzyme was incubated with a mixture of 1% (w/v) α-CD and 1% (w/v) G_3 in 50 mM phosphate buffer, pH 7.0 at 40 °C for 10 min. The concentration of α-CD was determined by HPLC Sato et al. (1985a). One unit of coupling activity is defined as the amount of enzyme that can convert one μmole of α-CD per minute under standard conditions.

Disproportionation activity

The disproportionation activity was determined by measuring conversion of maltohexose (G_6) to other products (Wind et al., 1995). Appropriately diluted enzyme was incubated with 1% (w/v) G_6 in 50 mM phosphate buffer, pH 7.0, at 40 °C for 10 min. The conversion of G_6 to other malto-oligosaccharides was estimated by HPLC as described by Sato et al. (1985a). One unit of disproportionation activity is defined as the amount of enzyme that can convert one μmole of G_6 per minute under standard conditions.

Hydrolysis activity

The hydrolysis activity was determined by measuring the increase in the reducing sugars during incubation with starch (Nakamura and Horikoshi 1976b; Wind et al., 1995). The pure enzyme (10-100 U/g of starch) was incubated with 10 g/L soluble starch in 50 mM phosphate buffer, pH 7.0, at 40 °C for 20 min. The concentrations of reducing sugars were determined by Di-nitro-salicylic-acid method (DNSA, Miller 1959) or by HPLC (Sato et al., 1985a) using glucose or maltose as standard. One unit of hydrolysis activity is defined as the amount of enzyme producing one μmole of either glucose or maltose per minute under standard conditions.

Protein estimation

Protein content was estimated according to the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

CGTase production

A loopful of culture was transferred from a slant to 50 mL of basal medium and incubated at 30 °C on a rotary shaker at 250 rpm for 24 h. Fifty milliliter of this preinoculum was then transferred to 500 mL basal medium, grown for 12 h and then used as an inoculum for a 14 L stirred fermenter (working volume 10 L, New Brunswick Scientific Co., USA). The fermenter medium consisted of (in g/L), soluble starch 20, yeast extract 20, peptone 20, $NH_4H_2PO_4$ 6.7, $MgSO_4.7H_2O$ 0.5, pH 7.0. Fermentation was performed with an airflow rate of 8 L/min and agitation speed 400 rpm at 30 °C for 18 h.

Preparation of affinity matrix

The affinity matrix was prepared using a method described by Hanus et al. (1974) with some modifications. Corn starch (100 g/L) was gelatinized, cooled to room temperature and kept at -20 °C for 24 h. It was thawed to room temperature and again kept at -20 °C for 24 h. This procedure was repeated thrice. The spongy starch thus formed was crushed and filtered through 0.5 mm sieve. This material was extensively washed with distilled water to remove fine particles of starch. The starch matrix was then stored in 20% ethanol at 4 °C.

CGTase purification

Crude enzyme

The bacterial cells in the fermentation broth were flocculated by addition of 5 mL/L of Magnafloc, allowed to settle overnight at 10 °C and centrifuged at 2300 *g* for 20 min. (Eltek, India, Model No. SC 7500). The cell free supernatant was used as a crude enzyme preparation and purified further as described below.

Ultrafiltration

The crude enzyme was concentrated by ultrafiltration using a hollow fibre polysulfone membrane module with a molecular weight cut off of 20 kDa at 4 °C (Nitto Denko Corporation, Japan, Model NTU 3250C, lumen flow rate 0.86 L/min, surface area 0.1 m^2).

Affinity chromatography

The concentrated enzyme was loaded at a flow rate of 0.4 mL/min on 50 mL of affinity matrix (prepared as described above), packed in a water-jacketed column (26 cm ID, Pharmacia XK 26) maintained at 4 °C. The unbound proteins were removed by washing the matrix with 50 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 7.5, at 4 °C till the absorbance of the eluent buffer at 280 nm reached zero. The column temperature was then raised to 25 °C and maintained there for 15 minutes. CGTase was eluted at the flow rate of 0.4 mL/min using the same buffer. The fractions with maximum activity were pooled together and dialysed against 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM $CaCl_2$ in a dialysis tubing (Sigma, USA).

Gel filtration chromatography

Above enzyme preparation was loaded on a Sephacryl S-200 (Pharmacia, Sweden) column (2 cm dia × 150 cm length) pre-equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM $CaCl_2$. The elution was carried out in the same buffer at the flow rate of 0.4 mL/min at room temperature and 3 mL fractions were collected using a fraction collector (Pharmacia Frac-100, Sweden). The enzyme activity was determined in the protein-containing fractions. The pure protein was lyophilized in a bench top lyophilizer and stored at -20 °C to be used for further studies.

Properties

Determination of protein molecular weight

Molecular weight of the protein was estimated by SDS-PAGE and gel filtration. SDS-PAGE was performed according to Laemmli (1970) on a vertical slab gel using 8% (w/v) acrylamide gel at a constant voltage of 200 V for 5 h at 25 °C. Carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b

(97.4 kDa), β-galactosidase (116 kDa) and myosin (205 kDa) were used as standard protein molecular weight markers. The gel was stained with the silver-staining method of Blum et al. (1987). Molecular weight of the native protein was also estimated by gel filtration on Sephacryl S-200. The column (1 cm dia × 150 cm length) was calibrated with cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa) standard molecular weight proteins with 50 mM Tris-HCl buffer, pH 7.5 at the flow rate of 0.25 mL/min.

Isoelectric focusing

Isoelectric focusing was carried out in a small vertical slab gel apparatus using 5% (w/v) acrylamide gel and carrier ampholyte (Ampholine, Sigma) with a pH range 3.5 to 10, as described by Robertson et al. (1987), at a constant voltage of 250 V for 2 h. The gel was loaded with standard pI markers in the pI range 3.6 to 9.3 (IEF-MIX 3.6-9.3, Sigma) and stained with Coomassie Brilliant Blue R-250.

N-terminal sequencing

The pure enzyme was electroblotted onto PVDF membrane according to LeGendre et al. (1993). The pure protein (75 μg) was first separated on an SDS-PAGE (10% w/v gel). After the electrophoresis, gel and PVDF membrane were sandwiched between Whatman paper and placed in a blotting cassette. The tank was filled with 10 mM CAPS buffer, pH 11, containing 10% methanol and electrotransfer was carried under a constant current of 250 mA for 60 min. PVDF membrane was then washed several times with Milli-Q water (Millipore, USA) and stained with Coomassie Brilliant Blue R-250. N-terminal amino acid sequence was determined by subjecting the blot to Edman degradation on an automated protein sequencer (Shimadzu model PQS-1) at National Facility for Photolabelling and Peptide Sequencing in Biomolecular Systems, Department of Chemistry, IIT, Bombay.

Chemical modification of carboxylate groups and tryptophan residues

Reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)

The enzyme solution (20 μg, 1 mL) was prepared in 50 mM MES/HEPES buffer (3:1), pH 6.0, and incubated with different concentrations of EDC (10-30 mM) at 30 °C (Means and Feeny 1971; Pho et al., 1977). Aliquots were removed at suitable time intervals and the reaction was terminated with 100 μL of 1 M acetate buffer, pH 4.5. The residual activity was determined by the standard methyl orange cyclization assay.

Reaction with EDC / Nitrotyroine ethylester (NTEE)

The enzyme solution (1.5 mg, 1 mL) was prepared in 50 mM MES/HEPES buffer (3:1) pH 6.0, and incubated with 20 mM EDC and NTEE at 30 °C for 10 min. The reaction was subsequently arrested by addition of 10% (w/v) TCA. The precipitated protein was collected by centrifugation at 10 °C, washed extensively with chilled acetone, air-dried and dissolved in 100 mM NaOH. The number of nitrotyrosyl groups incorporated was determined spectrophotometrically at 430 nm using a molar absorption coefficient of 4600 M^{-1} cm^{-1} (Pho et al., 1977).

Reaction with *N*-bromosuccinimide (NBS)

Reaction was carried out by titrating 1 mL of enzyme solution (20 μg) with freshly prepared NBS (1-2.5 μM) in 50 mM acetate buffer, pH 5.5. Samples were removed at suitable time interval and reaction was stopped by addition of 20 μL of 1 mM tryptophan (Means and Feeny 1971; Spande and Witkop 1967). The residual activity was determined by the standard methyl orange cyclization assay.

Optimum pH and stability

The effect of pH on enzyme activity was studied by incubating enzyme with substrate prepared in different buffers. One milliliter of 1% (w/v) soluble starch, prepared in 50 mM buffers having different pH values (pH 4.0-4.5 acetate buffer, pH 6.0-8.0 phosphate buffer and pH 8.5-10.5 glycine-NaOH buffer), was incubated with enzyme at 40 °C for 10 min. The CDs produced were estimated by methyl orange method. The effect of pH on enzyme stability was tested by incubating enzyme in above buffers at

different pH values at 30 °C for one hour and residual activity was measured by standard methyl orange method.

Optimum temperature and stability

The enzyme was assayed at different temperatures in 50 mM Tris-HCl buffer, pH 7.0, to see the effect of temperature on enzyme activity. For checking the enzyme stability at different temperatures, the enzyme was incubated at various temperatures for 20 min and residual activity measured by the standard methyl orange method.

Effect of starch and $CaCl_2$ on temperature stability

The enzyme was incubated with 1% (w/v) soluble starch and $CaCl_2$ (10 and 20 mM) at 40 °C. Aliquots were removed at different time intervals and the residual enzyme activity was measured. The enzyme was also incubated with only 20 mM $CaCl_2$ at 50 °C and aliquots removed to determine the residual activity.

Storage stability of CGTase

The crude (UF concentrated) enzyme was mixed with 50 mM Tris-HCl buffer, pH 7.5 containing 10 mM $CaCl_2$, with or without 30% glycerol, and kept at -20, 4 and 30 °C. Samples were taken after every five days and CGTase activity was determined.

Kinetic parameters

The K_m and V_{max} values for the pure enzyme were determined by incubating 0.08 μg of pure protein with 0-10 mg/mL soluble starch in 50 mM phosphate buffer, pH 7.0, at 40 °C for 10 min. The data obtained was fitted to a standard Michaels-Menten model using nonlinear least squares regression. The K_m and V_{max} values were estimated at different pH and temperature values.

Action on various substrates

Starches (1% w/v) from different sources was added to 50 mM Tris-HCl buffer, pH 7.5 containing 10 mM $CaCl_2$, gelatinized by heating in boiling water bath for 10 min. and cooled to room temperature. For testing raw starch utilization, 1% (w/v) starch from different sources was suspended as such in the above buffer. The starch solution (20 mL), gelatinized and raw, was added to 10 U of enzyme/g starch and incubated at

40 °C in a reciprocating water bath at 200 rpm for 1 h. The CDs formed were estimated by HPLC as described below.

CD production with gelatinized soluble starch and raw wheat starch

The gelatinized soluble starch and raw wheat solutions of different concentrations (25 to 150 g/L) were prepared in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$. The starch solution (20 mL) was added with 10 U of crude enzyme (UF concentrated) per g of starch and incubated at 40 °C in a reciprocating water bath at 200 rpm. Samples were removed at different time intervals from 2-24 h incubation time and analyzed for CDs by HPLC described as below.

Analysis of CDs by HPLC

The concentrations of CDs were determined by HPLC (Thermo Separation Products, USA), as described by Sato et al. (1985a), under the following conditions: Column, Shodex DC-630 (Showa Denko, Japan); mobile phase, acetonitrile:water (65:35); flow rate, 1.5 mL/min; column temperature, 60 °C; refractive index detector (Waters 410, USA or Shodex RI-71, Japan). Appropriately diluted reaction mixture (0.5 mL) was mixed with equal volume of acetonitrile, centrifuged at 20,000 *g* for 15 min at room temperature, filtered through a 0.45 μ filter (Millipore, USA) and injected through a Rheodyne 7725 (USA) manual injector with a 20 μL loop.

Results and discussion

Isolation of strain

A total of 217 starch degrading organisms were isolated from 32 soil samples. All these isolates were streaked to purity twice on plates and transferred to slants. The enzyme from each isolate was tested by HPLC for its capability to produce CDs from starch. Only 30 isolates were able to produce enzymes that can make CDs from starch, indicating that enzymes produced by these isolates were CGTases. The enzymes produced by these 30 CGTase positive organisms, converted starch to α-, β- and γ-CD in different ratios, except for the enzyme from one isolate, which converted starch to predominantly α-CD. This α-CD producer, isolate no. AS-22, had some desirable characteristics (outlined below) and therefore was selected for further studies.

- The enzyme secreted by this organism produced 92.8 % α-CD, 4.3 % β-CD and 2.7 % γ-CD from 10 g/L soluble starch in 20 min.
- The enzyme produced very negligible amounts of malto-oligosaccharides
- This culture grows very fast (doubling time of about 30 min).

It was later while working on CD production experiments with CGTase from this isolate, that we discovered one more important and novel characteristic of this enzyme, which is its ability to degrade raw starch.

Taxonomic identification of AS-22

This strain was identified as *Klebsiella pneumoniae pneumoniae* according to the Bergey's manual of Systematic Bacteriology (Orskov 1984). The results of identification tests are summarized below.

Morphological characteristics

Morphology on nutrient agar:	Circular, shiny, mucoid, opaque, convex
Motility:	Negative
Vegetative cell:	Single, short rods
Spore:	Not formed
Capsule:	Positive
Gram- staining:	Negative

Growth characteristics

Nutrient agar plate:	Good growth, circular white mucoidal colony
Nutrient agar slant:	Good growth, growing on whole of slant
MacConkey agar plate:	Good growth, red pink colony

Physiological characteristics

Behavior to oxygen:	Facultative anaerobic
pH for growth:	4-10
Temperature for growth:	20 °C - 45 °C
Hydrolysis of starch:	Positive
Liquefaction of gelatin:	Negative

Citrate utilization (Simmon's):	Positive
Indole production:	Negative
V P test:	Positive
M R test:	Negative
H_2S production (TSI):	Negative
Catalase:	Positive
Urease (Christensen's):	Positive
Nitrate reduction:	Negative
Lysine decarboxylase:	Positive
Ornithine decarboxylase:	Negative
Arginine dihydrolase:	Negative
Oxidase:	Negative
Malonate:	Positive
Gluconate:	Positive
Faecal coliform test:	Positive

Acid production from various sugars

Glucose:	Positive	Adonitol:	Positive
Lactose:	Positive	Sorbitol:	Positive
Sucrose:	Positive	Arabinose:	Positive
Fructose:	Positive	Raffinose:	Positive
Galactose:	Positive	Rhamnose:	Positive
Mannose:	Positive	Maltose:	Positive
Sorbose:	Positive	Xylose:	Positive
Mannitol:	Positive	Trehalose:	Positive
Dulcitol:	Negative	Cellobiose:	Positive
Salicin:	Positive	Xylitol:	Negative

The above data clearly indicates that isolate no. AS-22 belongs to *Klebsiella* genus and closely resembles the *pneumoniae* species and *pneumoniae* subspecies, except for the pectate degradation test, which is detected positive for this isolate.

Purification

The crude CGTase was purified to almost homogeneity in three steps. In the first step of ultrafiltration, the enzyme was concentrated 35-fold. In the next step of affinity adsorption, the CGTase bound strongly to the affinity matrix at 4 °C as no enzyme was detected in the column effluent even after washing with ten bed volumes of buffer (Figure 2.1). However, when temperature was raised to 30 °C, the enzyme immediately desorbed from the matrix giving a sharp peak. The affinity step resulted in 528-fold purification and about 11.5-fold concentration of the enzyme. In the gel filtration step, the fractions contained CGTase activity in one major peak whereas the other small peaks did not show any activity (Figure 2.2). The purification protocol resulted in a pure protein giving a single band on a silver-stained SDS-PAGE gel (Figure 2.3a) with overall 736-fold purification. The results of the purification scheme are summarized in Table 2.1. The different specific enzyme activities of the pure protein are listed in Table 2.2.

Adsorption of CGTase on starch (or modified starch) has been the most popular and commonly used technique of CGTase purification (Table 1.3). The modification to give more surface area increases the effective enzyme binding capacity of the matrix (Makela et al., 1988b). The binding of *Bacillus firmus* CGTase to various starches has been extensively studied in our laboratory (Goel 1995). He observed that the CGTase could bind more efficiently to freeze-thaw modified corn starch (Goel 1995). Similar results were obtained with this CGTase and therefore this method was used for preparing the affinity matrix. Affinity matrices, with CDs attached to inert Sepharose supports, have been used by others to achieve good purification of CGTase. Depending upon the type of CGTase, α-, β- or γ-CD can be attached to the Sepharose support (Ahn et al., 1990, 1991; Larsen et al., 1998a; Mori et al., 1994; Wind et al., 1995).

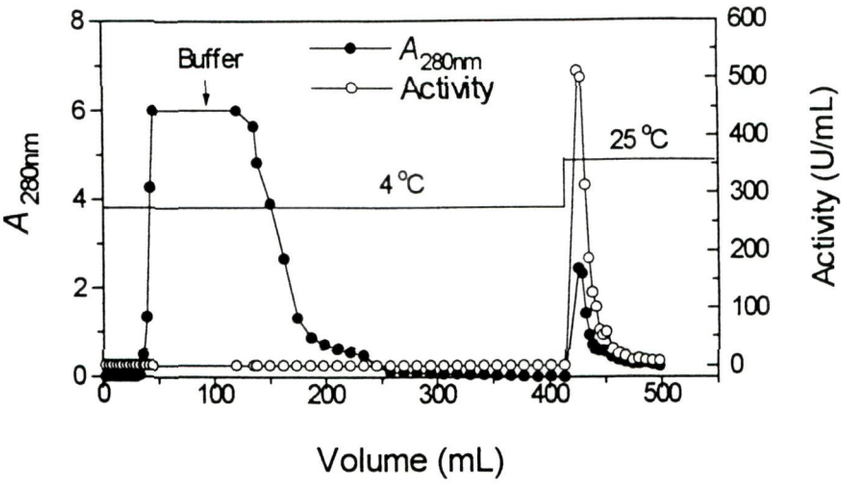


Figure 2.1 Binding and elution profile of CGTase on affinity matrix in 50 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 7.5

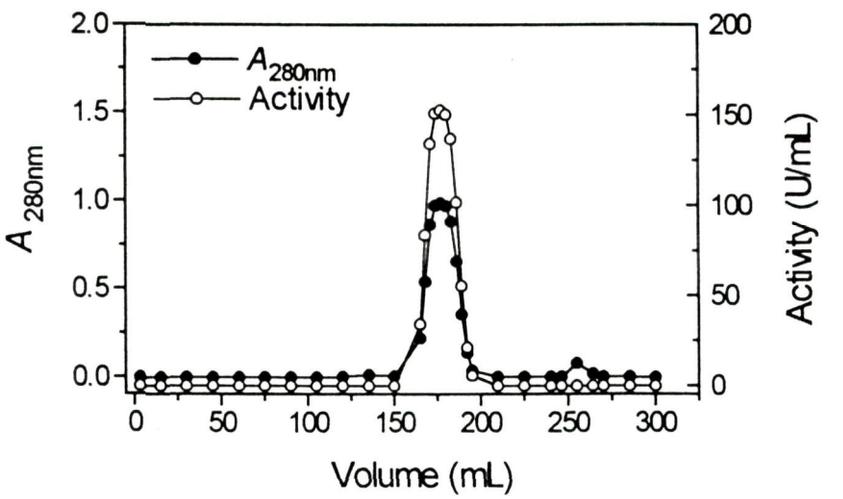


Figure 2.2 Elution profile of CGTase on Sephacryl S-200 in 10 mM Tris-HCl buffer containing 1 mM $CaCl_2$, pH 7.5

CGTases are known to catalyze four different reactions (Penninga et al., 1995; Wind et al., 1995). However, we have detected only three activities with this CGTase, which lacks hydrolysis activity (Table 2.2). When soluble starch was incubated with different concentrations of enzyme (10-100 U per g of starch), no reducing sugars were detected in the reaction mixture by DNSA as well as HPLC method. This clearly indicates that this enzyme is a true CGTase with no amylolytic activity. The CGTase primarily produces α-CD from starch with no detectable amounts of reducing sugars and linear malto-oligosaccharides (G_1 to G_7). Wind et al. (1995) and Penninga et al. (1995) have also reported different activities for *Thermoanaerobacterium thermosulfurigenes* EM1 and *Bacillus circulans* 251 CGTases, respectively. The disproportionation activity of our CGTase is comparable to these reported CGTases, whereas cyclization and coupling activities are higher for this enzyme.

Step	Total volume (mL)	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)[a]	Fold purification	Percent yield
Cell free supernate	8300	89890	63910	0.71	-	100
Ultrafiltration	240	4585	59436	13	18	93
Starch affinity chromatography	21	137	52406	382	528	82
Gel filtration on Sephacryl S-200	126	83	43458	523	736	68

[a] Cyclization activity

Table 2.1 Summary of purification results

Activity	Specific activity (U/mg)
Cyclization	523
Coupling	498
Disproportionation	764
Hydrolysis	ND

ND= Not detected

Table 2.2 Specific enzyme activities of CGTase.

Properties

Molecular weight and isoelectric point

The molecular weight of the enzyme was estimated to be 75 kDa by SDS-PAGE (Figure 2.3a). It was found to be same by gel filtration on Sephacryl S-200 (data not shown). Molecular weight estimation by SDS-PAGE and gel filtration yielded virtually identical results within experimental error, indicating that this CGTase is a monomer. Both monomeric and dimeric CGTases, with molecular weights ranging from 68-170 kDa, have been reported in literature (Table 1.4).

The isoelectric point of the pure enzyme was found to be 7.3 (Figure 2.3b). A highly acidic pI of 2.8 for CGTase from *Brevibacterium* sp. No. 9605 (Mori et al., 1994) as well as a basic pI of 8.8 for CGTase from *Bacillus circulans* IFO 3329 (Yagi et al., 1986) have been reported. CGTases from some organisms are also reported to exits as isozymes having different pI values (Bovetto et al., 1992a; Kitahata et al., 1974; Makela et al., 1988b). However, according to Makela et al. (1988b) various isoforms from *Bacillus circulans* CGTase are functionally identical.

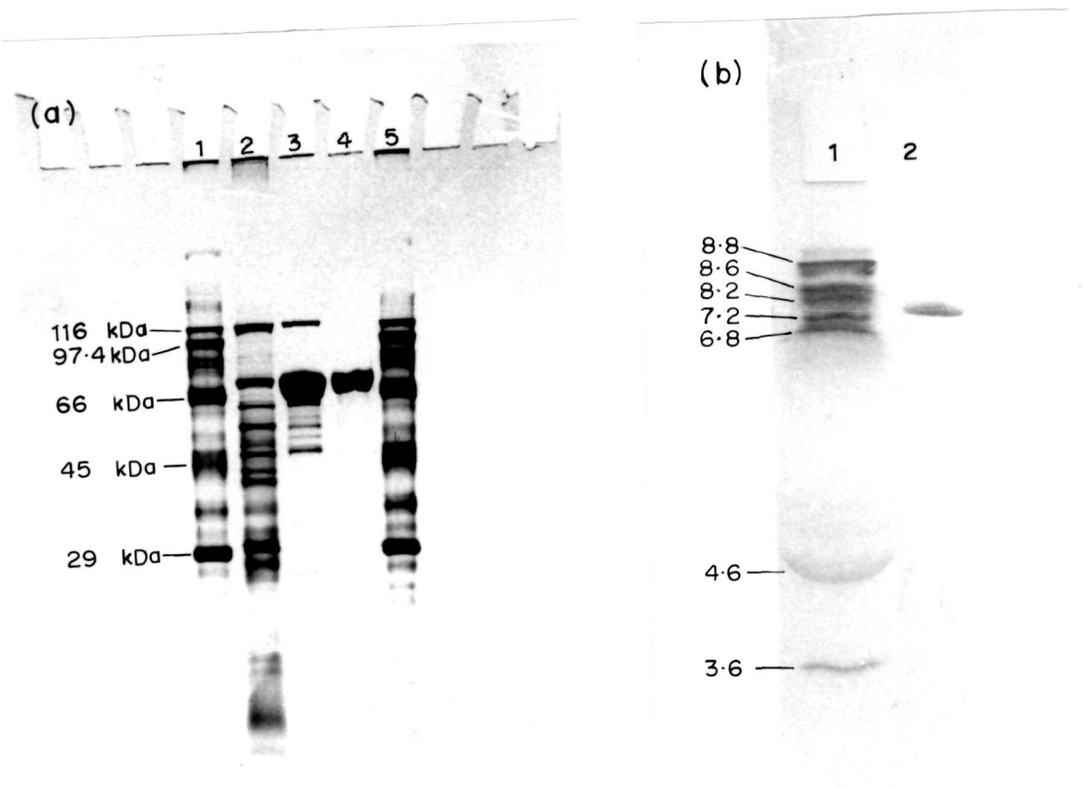


Figure 2.3 (a) Determination of molecular weight on SDS-PAGE. Lane 1, 5 molecular weight markers; Lane 2, crude enzyme (5 μg); Lane 3, affinity pure enzyme (2 μg) and Lane 4, gel filtered enzyme (1μg) (b) Determination of isoelectric point by IEF. Lane 1, standard pI markers and Lane 2, purified CGTase

N-terminal sequencing

The results of N-terminal sequence determination are summarised and compared with known N-terminal sequences of CGTase from other organisms in Table 2.3. Only the first three amino acids are identical to the N-terminal sequence of CGTase from *Klebsiella pneumoniae* M 5 al which belongs to the same genus and species of this organism. Homology with other CGTases is limited to only the first N-terminal amino acid. It should be noted that *Klebsiella pneumoniae* M 5 al has been renamed to *Klebsiella oxytoca* M 5 al recently (Feederle et al., 1996; Fiedler et al., 1996). This indicates that both these *Klebsiella* strains and their enzymes are certainly different. Makela et al. (1988b) have compared N-terminal amino acid sequences (32 amino acids) of CGTases from four *Bacillus* strains and that of *Klebsiella pneumoniae* M 5 al. They observed very close homology among the sequences of all *Bacillus* strains whereas little coherence between them and CGTase from *Klebsiella pneumoniae* M 5 al.

1.	Ala	Glu	Pro	His	?	Glu	Glu	Asp	His	Thr	Phe	Asp	Arg	Asp	Arg	Ile	His
2.	Ala	Glu	Pro	Glu	Glu	Thr	Tyr	Leu	Asp	Phe	Arg	Lys	Glu	Thr	Iie	Tyr	Phe
3.	Ala	Pro	Asp	Thr	Ser	Val	Ser	Asn	Lys	Gln	Asn	Phe	Ser	Thr	Asp	Val	Ile
4.	Ala	Pro	Asp	Thr	Ser	Val	Ser	Asn	Lys	Gln	Asn	Phe	Ser	Thr	Asp	Val	Ile
5.	Ser	Pro	Asp	Thr	Ser	Val	Asp	Asn	Lys	Val	Asn	Phe	Ser	Thr	Asp	Val	Ile

(1) *Klebsiella pneumoniae pneumoniae* AS-22 (2) *Klebsiella pneumoniae* M 5 al (3) Alkalophillic *Bacillus* sp. ATCC 21783 (4) Alkalophillic *Bacillus* sp. No. 38-2 (5) *Bacillus macerans* IAM 1243

Table 2.3 Comparison of N-terminal amino acid sequences of different CGTases

Chemical modification studies

The pure enzyme, when incubated with 10 mM EDC for 20 min at 30 °C, lost 95 % of its activity. However, there was no loss of activity in the control tube. When the different concentrations of EDC were tried for studies, it was not possible to control the reaction, as EDC was found to rapidly inactivate the enzyme. Therefore, the enzyme was treated with EDC / NTEE to calculate the number of carboxylate groups that could be modified with EDC. The results showed incorporation of 2.95 nitrotyrosyl residues per molecule of the enzyme suggesting that inactivation of the enzyme could be due to the modification of three carboxylate-containing residues. The reaction with 2.5 μM NBS resulted in more than 80% loss of activity in 20 min at 30 °C indicating that tryptophan residues play an important role in catalytic activity of this enzyme. Further detailed study on chemical modification of various residues in this CGTase needs to be done to know which amino acids are involved in the catalytic mechanism.

Effect of group-specific chemical modifiers for carboxylates, histidine, tryptophan, tyrosine, cysteine and lysine residues on CGTase activity has been studied by other researchers (Alcalde et al., 1998, 1999; Bender 1981; Ohnishi et al., 1992; Villete et al., 1993). Although, X-ray chrystallographic studies indicate that the residues containing carboxylate groups play an important role in catalytic activity of CGTases, chemical modification of these residues has not been studied in detail. Modification of carboxylates of *Thermoanaerobacter* sp. CGTase using 1-ethy-3-(3-dimethyl amino-propyl carbodiimide (EDC) in presence of acarbose resulted in decrease in cyclization and disproportionation activities, whereas the hydrolytic activity was not affected (Alcalde et al., 1998). Modification of β-CGTase from *Bacillus firmus* var. alkalophilus with Woodward's reagent, which is specific for modifying carboxylate groups, dramatically reduced cyclization and hydrolytic activities (Shin et al., 1999).

Modification of tryptophan residues with *N*-bromosuccinimide (NBS) caused inactivation of hydrolytic activity (Ohnishi et al., 1992; Shin et al., 1999). Substrate protection studies with G_2-G_4 indicated that tryptophan is located at the substrate-binding site of *Bacillus stearothermophilus* CGTase (Ohnishi et al., 1992, 1994; Shin et al., 1999).

Effect of pH on enzyme activity and stability

The enzyme was most active in the pH range of 5.5-9.0 (Figure 2.4a) with maximum activity at pH 7-7.5, whereas it was most stable in the pH range 6-9 (Figure 2.4b), with maximum stability at pH 8.5. The enzyme lost all its activity below pH 4 and above pH 10. CGTases are reported to have optimum pH for activity around 6.0, whereas the optimum pH for stability is usually in the range 6-9 (Table 1.4). This enzyme also shows similar results for pH activity and stability. The enzymes from alkalophillic organisms are usually more stable in the alkaline range, even up to pH 11.

Effect of temperature on enzyme activity and stability

With soluble starch as the substrate, CGTase was most active in the temperature range 35-50 °C (Figure 2.5a) and the optimum temperature for enzyme activity was 45 °C. The enzyme rapidly lost activity above 35 °C when incubated in the absence of substrate (Figure2.5b). The enzyme showed rapid decline in the activity above 50 °C. The temperature stability studies indicated that the CGTase is thermolabile and rapidly lost its activity above 35 °C. The temperature optima for CGTases are in the range of 40-70 °C (Table 1.4). Only a few CGTases from thermophillic organisms show optimum temperature above 80 °C and the highest temperature optimum of 95 °C has been reported for CGTase from *Thermoanaerobacter* sp. (Norman and Jorgensen 1992). CGTases are usually not stable above 60 °C and most of the enzymes are thermolabile (Table 1.4). Only CGTases from thermophillic organisms show stability above or at 70 °C

Effect of substrate and $CaCl_2$ on enzyme stability

The temperature stability of the enzyme was improved in the presence of soluble starch and $CaCl_2$ at 40 °C (Figure 2.6a). However, it was still not stable above 40 °C (Figure 2.6b). It is known that the presence of substrate, product or calcium ions enhances the stability of some CGTases (Aoki et al., 1987; Bovetto et al., 1992a). The temperature stability of CGTase from *Klebsiella pneumoniae pneumoniae* AS-22 also improved in the presence of substrate, soluble starch and calcium ions. However, addition of product, α-CD, did not improve stability of the enzyme.

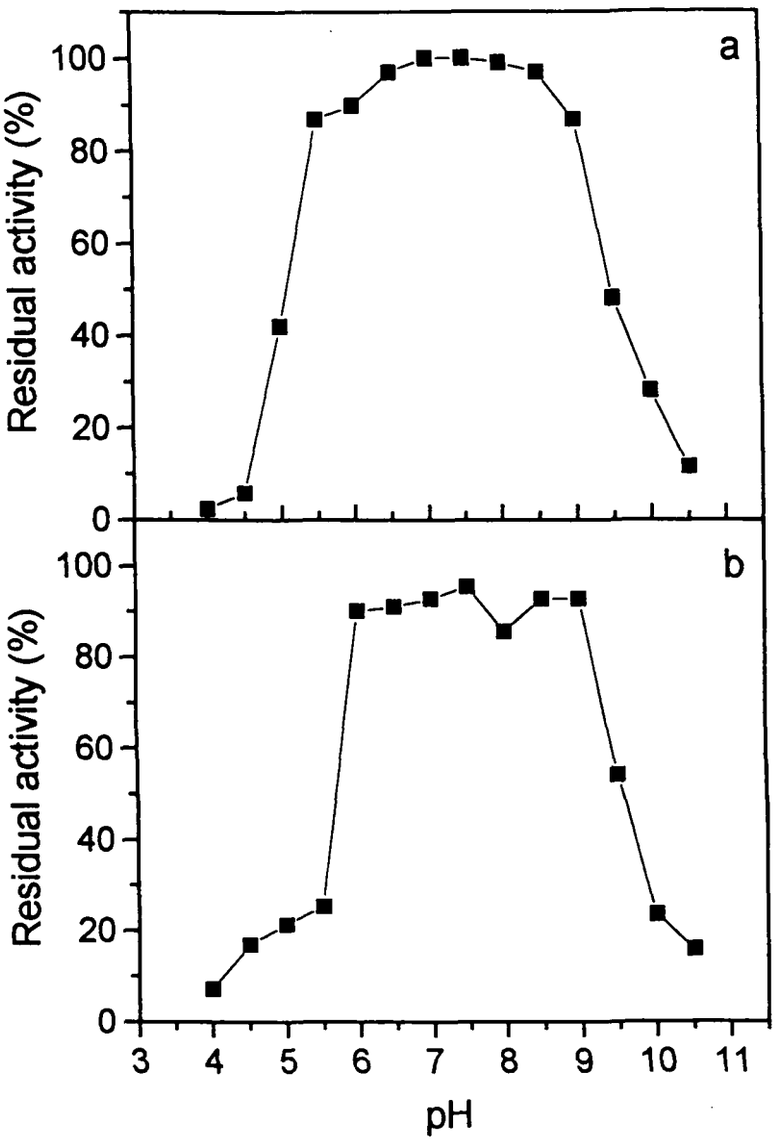


Figure 2.4 Effect of pH on (a) enzyme activity and (b) stability. CGTase was incubated at 40 °C for 10 min. for activity and at 30 °C for one hour for stability studies at different pH values.

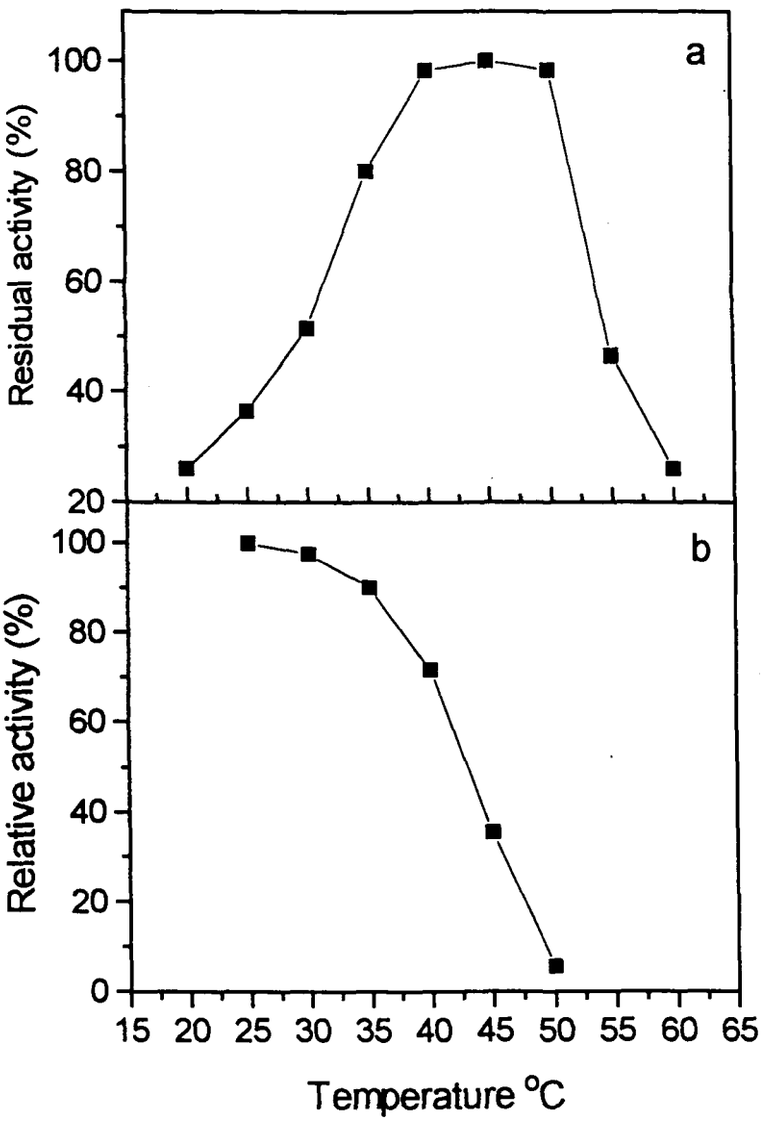


Figure 2.5 Effect of temperature on (a) enzyme activity and (b) stability. CGTase was incubated in 50 mM Tris-HCl buffer, pH 7.0 for 10 min. for activity and for 20 min for stability studies at various temperatures

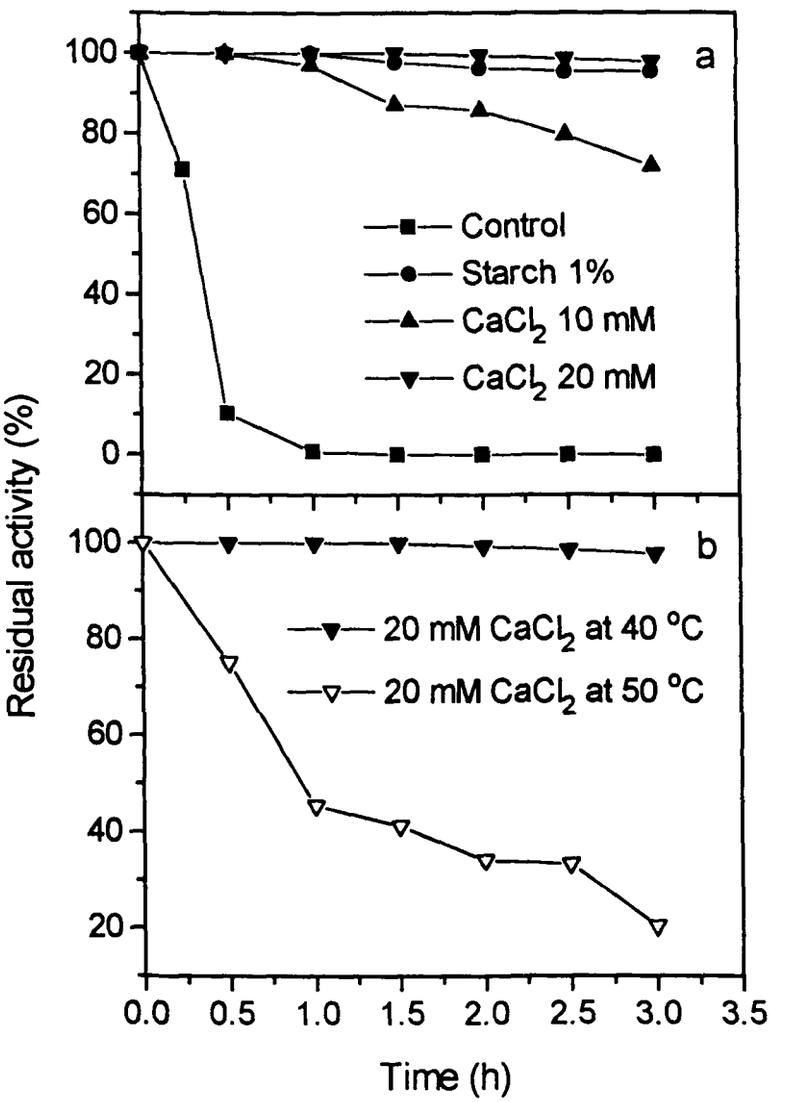


Figure 2.6 Effect of soluble starch and $CaCl_2$ on temperature stability of CGTase (a) in presence of 1% (w/v) soluble starch, 10 mM and 20 mM $CaCl_2$ and buffer (control) at 40 °C (b) in presence of 20 mM $CaCl_2$ at 40 and 50 °C

Storage stability of CGTase

It is important to study the shelf life of this commercially important enzyme. The storage stability of the enzyme was studied in presence of $CaCl_2$ and glycerol at -20, 4 and 30 °C (Figure 2.7). It was observed that enzyme almost 100% stable at low temperature, but it lost activity when incubated at 30 °C for longer time in absence of 30% (v/v) glycerol, even in presence of $CaCl_2$. No loss of activity was observed when pure CGTase was stored at -20 °C up to two years in 1mM $CaCl_2$.

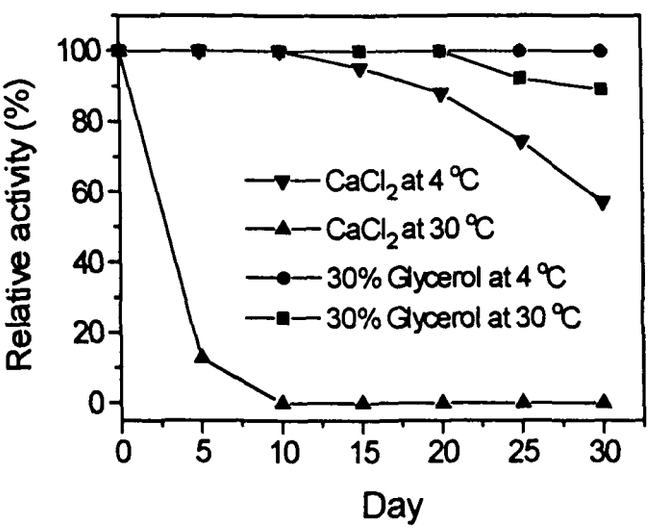


Figure 2.7 Storage stability of CGTase in presence and absence of 30% glycerol in 10 mM $CaCl_2$ at 4 °C and 30 °C

Kinetic parameters

For soluble starch as the substrate, the K_m and k_{cat} values were determined to be 1.35 mg/mL and 249.13 μM/mg/min. The low K_m value indicates that the enzyme has comparatively high affinity for the substrate, soluble starch. The reaction kinetics of few CGTases has been studied using starch (cyclization reaction) or CD (coupling or hydrolysis reaction) as substrate (Table 1.4). The lowest K_m using starch as substrate is reported to be 1.21 mg/mL (Gawande et al., 1999), whereas that using CD as the substrate is 2.5 mM (Kitahata and Okada 1982b). The pH activity profile indicates possible involvement of two ionisable groups in the catalytic activity of the CGTase, having pK_a's of 5.2 and 7.8 (Figure 2.8a). The Arrhenius plot for CD forming activity showed activation energy of 30.13 kcal/mol (Figure 2.8b).

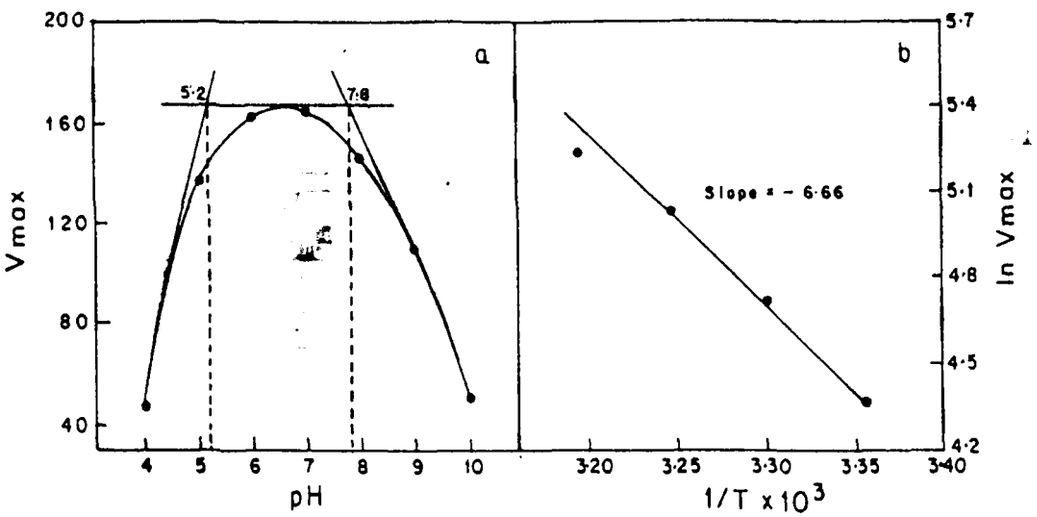


Figure 2.8 (a) pH activity profile (b) Arrhenius plot of CGTase. The K_m and V_{max} values were determined at different pH (4-10) and temperatures (20-60 °C) by using soluble starch as the substrate in the range 0-10 mg/mL and by fitting the data to a standard Michaels-Menten model using least squares nonlinear regression. The energy of activation was calculated by using the equation, E_a = -0.219 × slope where, E_a is the energy of activation in calories

Cyclodextrin production

The action of this CGTase on different gelatinized and raw substrates was investigated (Figure 2.9). Soluble starch was the best substrate when used gelatinized, whereas wheat starch was the best substrate when used raw. The enzyme could degrade all tested starches, amylose and amylopectin, although the yield of CDs with amylose was lower in the gelatinized form. However, when used raw, only wheat starch and dextrin could be converted CDs. Other substrates produced very little or no CDs. Gelatinization or physical treatment opens up the structure of starch granules, which become susceptible to CGTase action, whereas raw starch as such should be relatively inaccessible to the enzyme (Lee and Park 1991).

CD production was further studied using the best substrates obtained, *i.e.* gelatinized soluble starch and raw wheat starch. As this CGTase predominantly produces α-CD, we looked for substrate concentration, which will give maximum concentration of α-CD. The best substrate concentrations of gelatinized soluble starch and raw wheat starch were found to be 100 g/L and 125 g/L, respectively. The CGTase converted 16% of gelatinized soluble starch to CDs giving 88% of α-CD out of total CDs in 2 h (Figure 2.10d). In 4 h of incubation maximum α-CD concentration reached to 17.34 g/L converting 21% (w/w) starch to CDs, containing 81% of α-CD. Although total CD yield increased subsequently, concentration of α-CD decreased whereas those of β- and γ-CD increased. In 24 h of incubation, total conversion to CDs increased to 27.6%, containing only 50% α-CD out of total CDs (Figure 2.10d). This kind of product shift has been observed for enzymes primarily producing CDs other than β-CD (Schmid 1996a; Wind et al., 1995).

The enzyme produced higher percentage of α-CD from raw wheat starch as compared to that from gelatinized soluble starch. It produced 14.5% total CDs in 2 h of incubation, with 91% selectivity towards α-CD (Figure 2.11e). The maximum α-CD concentration reached 22.2 g/L in 4 h of incubation (20% w/w starch conversion with 89% of α-CD). Again, the α-CD concentration in the reaction mixture decreased to 63.4% of total CDs in 24 h of incubation. Figure 2.12 shows reaction products obtained with 100 and

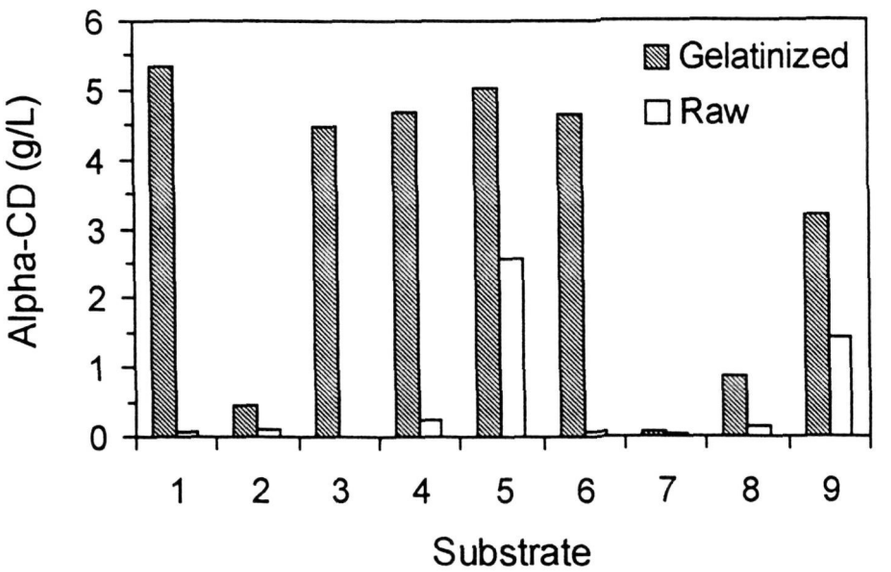


Figure 2.9 Action of CGTase on various gelatinized and raw substrates. The X-axis indicate substrates (10 g/L) in the following order, 1: soluble starch; 2: corn starch; 3: tapioca starch; 4: potato starch; 5: wheat starch; 6: rice starch; 7: amylose; 8: amylopectin and 9: dextrin.

125 g/L gelatinized soluble starch and raw wheat starch, respectively, in 4 h using 10 U of enzyme per g of starch. The effect of enzyme concentration on CD production was also studied with optimum substrate concentrations. The best results, however, were obtained with 10 U of the enzyme per g of starch and more enzyme addition did not result in higher CD yield (results not shown).

The use of raw starch for cyclodextrin production is commercially important as the cost involved in gelatinization process can be avoided. CD production from raw corn starch with *Thermoanaerbacter* sp. CGTase has been studied by Kim et al. (1997). They have obtained substrate conversion to total CDs and maltodextrin of 27.9% and 31.4%, respectively, from 75 g/L starch and 22 U of enzyme per g of starch. In some reports, gelatinization of starch has been avoided by the use of milled starch (Kim et al., 1993) or extruded starch (Ito et al., 1989; Lee and Park 1991). The physical treatment of starch usually increases the CD yield as compared to that from native starch. Penninga et al. (1996) have used site-directed mutagenesis to improve a raw starch degrading action of CGTase, although they have not mentioned the total yield of CDs obtained. We have also reported raw starch degrading β-CGTase produced by *Bacillus firmus* (Gawande et al., 1999).

Starch solutions, at concentrations higher than 150 g/L, become very viscous and rather difficult to handle. Some industrial processes use up to 300 g/L starch concentration, but in these processes the starch is liquefied with either α-amylase or CGTase (Schmid 1996b) before CD formation. Most of these processes also use complexing agents (trichloroethylene, cyclohexane, toluene, bromobenzene, long-chain aliphatic alcohols, etc.) to increase the CD production, as complexing agent can remove CDs from the reaction mixture. The selective complexing agents also drive the reaction towards the formation of one particular type of CD. The CDs used for food/pharmaceutical applications should be completely free of these toxic complexing agents. But complete removal of these chemicals is often difficult and expensive (Bender 1986, Schmid 1996b). Therefore production of CDs with economical conversion rate without any toxic complexing agents is commercially important.

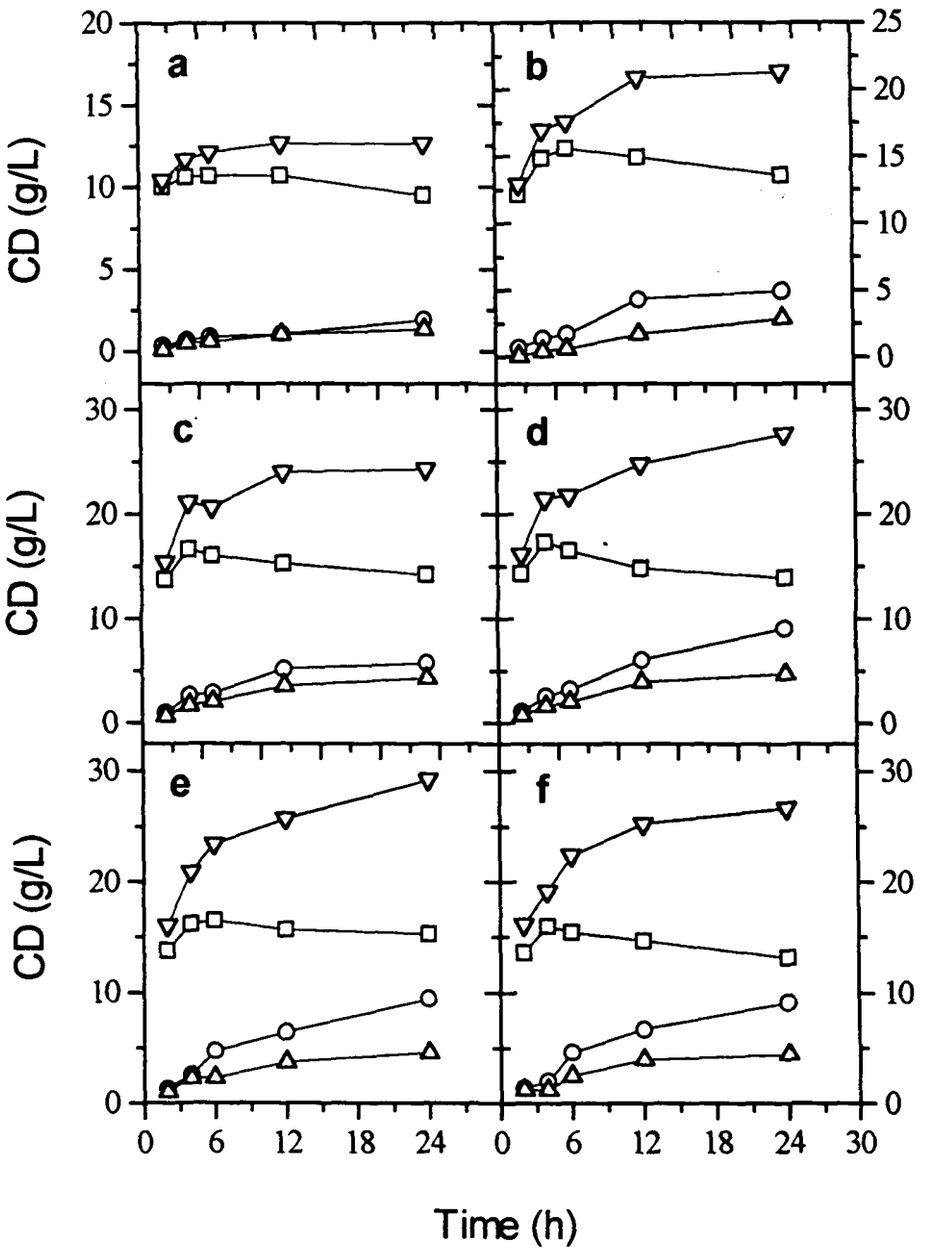


Figure 2.10 Effect of gelatinized soluble starch concentration on cyclodextrin production, (a) 25 g/L (b) 50 g/L (c) 75 g/L (d) 100 g/L (e) 125 g/L (f) 150 g/L, symbols represent -□-, α-CD; -O-, β-CD; -Δ-, γ-CD and -∇-, total-CD

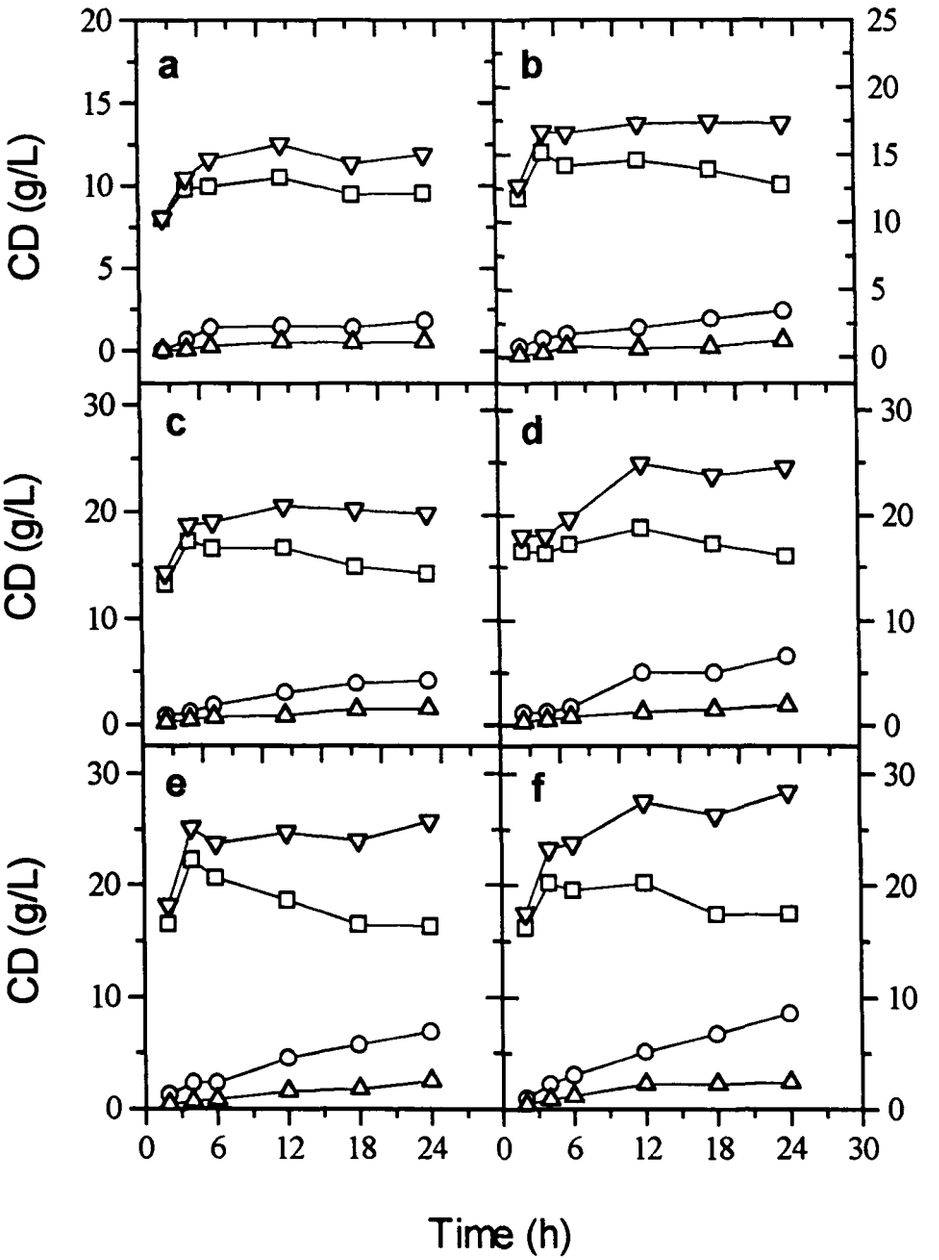


Figure 2.11 Effect of raw wheat starch concentration on cyclodextrin production, (a) 25 g/L (b) 50 g/L (c) 75 g/L (d) 100 g/L (e) 125 g/L (f) 150 g/L, symbols represent -□-, α-CD; -O-, β-CD; -Δ-, γ-CD and -∇-, total-CD

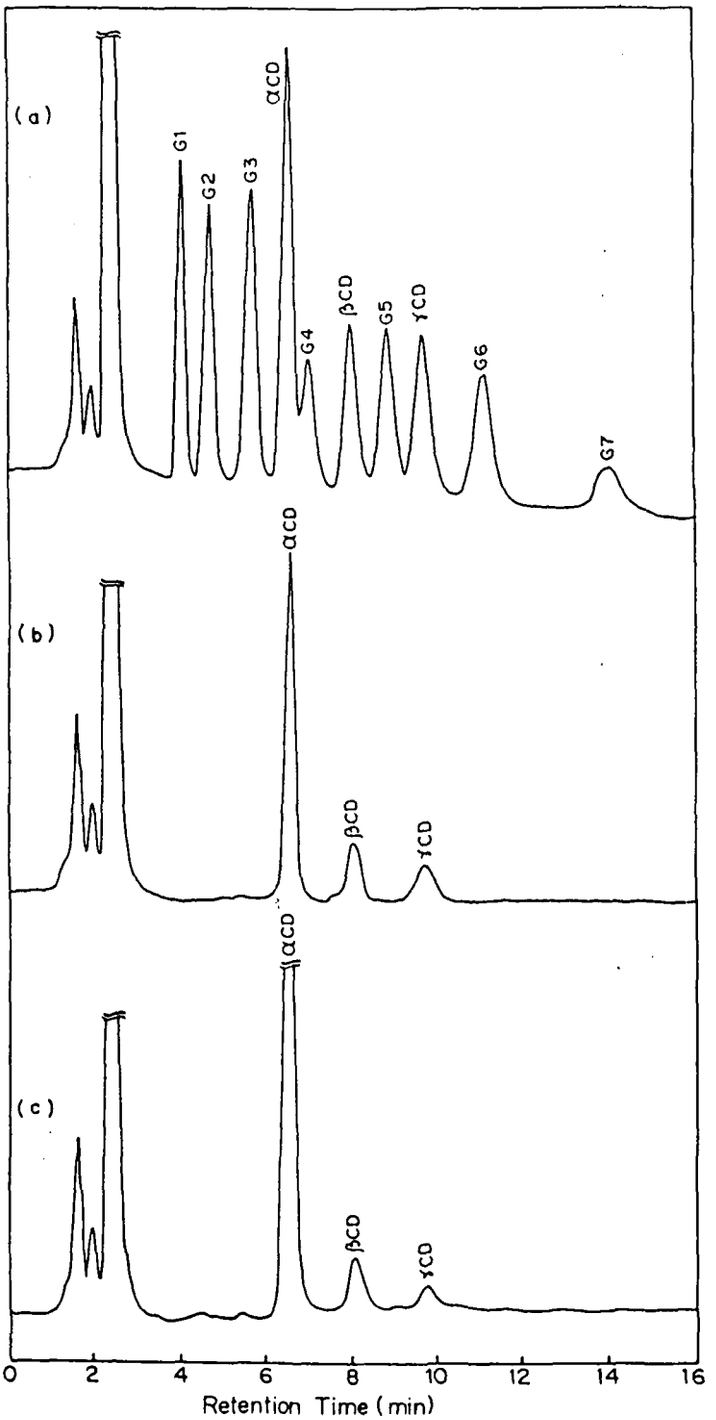


Figure 2.12 HPLC chromatographs of (a) standard sample containing CDs (α, β and γ) and malto-oligosaccharides (G_1-G_7) (b) gelatinized soluble starch (100 g/L) and (c) raw wheat starch (125 g/L), both treated with 10 U of the CGTase per g of starch for 4 h

Conclusions

A fast growing CGTase producing culture was isolated from soil samples and taxonomically identified. The CGTase produced by this organism had following novel characteristics. It rapidly and predominantly converted starch to α-CD. The pure enzyme preparation did not show any net hydrolytic activity. The enzyme is temperature labile but shelf life of the enzyme can be improved by addition of $CaCl_2$ at 4 °C or glycerol at room temperature. It can degrade raw wheat starch to α-CD with 91% selectivity, at 14.5% conversion to CDs from 125 g/L starch concentration, without addition of any complexing agent. This enzyme can thus have potential application for industrial production of α-CD.

References

References are listed in Chapter 7

CHAPTER 3

Application of factorial designs for optimization of cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae pneumoniae* AS-22

Summary

Production of cyclodextrin glycosyltransferase (CGTase) from *Klebsiella pneumoniae pneumoniae* AS-22 was optimized in shake flasks using a statistical experimental design approach. Effect of various components in the basal medium like carbon, nitrogen, phosphorus and mineral sources as well as initial pH and temperature were tested on enzyme production. The optimum concentrations of the selected media components were determined using statistical experimental designs. Two level factorial designs in five variables namely dextrin, peptone, yeast extract, ammonium dihydrogen orthophosphate and magnesium sulphate concentrations were constructed. The optimum medium composition thus found consisted of 49.3 g/L dextrin, 20.6 g/L peptone, 18.3 g/L yeast extract, 6.7 g/L ammonium dihydrogen orthophosphate and 0.5 g/L magnesium sulphate. The maximum CGTase activity obtained in optimized medium was 21.4 U/mL in 28 h of incubation as compared to 2.4 U/mL in the basal medium in 10 h of incubation in shake flasks. The cell growth and CGTase production profiles were studied with the optimized medium in shake flask and in 1 L fermenter. It was observed that the enzyme production was growth associated both in shake flask and in fermenter, although it was slower in shake flask. The maximum CGTase activity obtained in the fermenter was 32.5 U/mL in 16 h in optimized medium as compared to 3.75 U/mL in basal medium in 8 h of incubation. The optimized medium resulted in about 9-fold increase in the enzyme activity as compared to that obtained in the basal medium in shake flask as well as in fermenter.

Introduction

The enzyme cyclodextrin glycosyltransferase (CGTase; E.C. 2.4.1.19) synthesizes non-reducing cyclic malto-oligosaccharides called cyclodextrins (CDs) from starch or other alpha 1,4-glucans. CDs are of three major types and are named as α-CD, β-CD and γ-CD with six, seven and eight glucose molecules linked by α(1→4) glycosidic bond. CDs have torus shaped structure with hydrophobic interior cavity and hydrophilic exterior surface. Therefore they can form inclusion complexes with a variety of hydrophobic guest compounds or functional moieties (Bender 1986; Szejtli 1984) and thereby change their

physical and chemical properties. Because of this unique property CDs find increasing use in pharmaceutical, agricultural, chemical, cosmetic and food industries (Bender 1986; Hedges 1992; Szejtli 1984).

The conventional method of medium optimization (variation of one variable at a time) is time-consuming, expensive and inaccurate, especially when interactions between different components are present. Statistical experimental designs allow simultaneous, systematic and efficient variation of all components (Box et al., 1978; Davies 1993). The use of user-friendly software packages has made this technique increasingly popular for media optimization (Achary et al., 1997; Hounsa et al., 1996; Lee and Chen 1997; Zhu et al., 1996). We have also optimized CGTase production from *Bacillus firmus* in our earlier studies by simple two-level factorial designs (Gawande et al., 1998).

All known CGTases produce a mixture of α-, β- and γ-CDs in different ratios. Enzymes capable of predominantly producing a particular type of CD can reduce subsequent purification costs and hence are commercially desired. Majority of CGTases produce β-CD as the main product (Goel 1995; Schmid 1996a). Enzymes producing primarily α-CD or γ-CD are relatively rare. *Klebsiella pneumoniae* M5al, *Bacillus macerans* IAM 1243 and *Klebsiella oxytoca* 19-1 are the representatives of α-CD producers (Goel 1995). *Bacillus subtilis* No. 313, alkalophilic *Bacillus* strain 290-3, *Bacillus* sp. AL-6 and *Brevibacterium* sp. No 9605 are the only known γ-CD producers (Goel 1995; Schmid 1996a). We have isolated an organism producing a novel CGTase, which predominantly makes α-CD from starch (Gawande and Patkar 1998). In the present study, we used a two-step strategy to increase CGTase production in fermenters. In the first step, optimum medium composition was determined in shake flasks by using factorial designs. This optimum medium was subsequently used in fermenters to determine the extent of improvement in CGTase production.

Materials and Methods

Microorganism and media components

A novel α-CD specific CGTase producing strain *Klebsiella pneumoniae pneumoniae* AS-22 was isolated from soil samples and used in this study (ATCC 212075, NCIM 5121, Gawande and Patkar 1998).

Soluble starch and dextrin were purchased from E. Merck (Mumbai, India). Commercial grade potato starch was from Loba Chemie (Mumbai, India) and tapioca starch was obtained from Laxmi Starch (Coimbature, India) were obtained from local suppliers. Other starches, amylose, amylopectin and CDs were obtained from Sigma Chemical Co., USA. Yeast extract and peptone were purchased from HiMedia (Mumbai, India) while all other chemicals were of analytical grade from S. D. Fine Chemicals (Boisar, India).

Culture conditions

One mL of 12 h grown inoculum was added to a 500 mL Erlenmeyer flask (Borosil, India) containing 50 mL of basal medium: 10 g/L soluble starch, 5 g/L peptone, 5 g/L yeast extract, 1 g/L Na_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$ (pH 7.0) or modified basal medium and incubated at 28 °C or 30 °C on a rotary shaker at 300 rpm. Samples were removed at different time intervals for cell growth and CGTase activity measurements. For estimation of cell growth, each sample was appropriately diluted with 8.5 g/L NaCl solution and absorbance was measured at 660 nm. An aliquot was centrifuged at 20,000 *g* for 15 minutes and CGTase activity was measured in the supernatant.

CGTase assay

Lejuene et al's (1989) method (cyclization activity) was slightly modified for determination of CGTase activity. One mL of 1% soluble starch prepared in 50 mM phosphate buffer, pH 7.0 was added with 0.1 mL of appropriately diluted enzyme and incubated at 40 °C for 10 min. Enzyme reaction was stopped by immediately cooling the tubes in chilled water followed by addition of 0.1 mL of 1.2 N HCl. Then 2 mL of methyl

orange solution (final concentration 0.035 mM) was added to the reaction mixture and tubes were maintained at 15 °C for 30 min. The absorbance was measured at 507 nm and decrease in absorbance (with respect to a control tube without enzyme) was correlated with the amount of α-CD. One unit of enzyme activity was defined as the amount of enzyme that produced one μmole of α-CD per minute under standard conditions.

Factorial designs and analysis of results

Factorial experiments were designed using the approach given in standard texts on design of experiments (Box et al., 1978; Davies 1993; Montgomery 1991). To estimate standard error in enzyme activity measurement, an experiment was performed using six flasks containing identical medium. Effect of five variables namely dextrin, peptone, yeast extract, ammonium dihydrogen orthophosphate and magnesium sulphate concentrations on CGTase production were studied using two-level fractional factorial design (Table 3.6). The experimental data obtained from the factorial design was fitted to the following polynomial.

$$\text{Activity} = \alpha_o + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_4 x_4 + \alpha_5 x_5 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{14} x_1 x_4 + \alpha_{15} x_1 x_5 + \alpha_{23} x_2 x_3 + \alpha_{24} x_2 x_4 + \alpha_{25} x_2 x_5 + \alpha_{34} x_3 x_4 + \alpha_{35} x_3 x_5 + \alpha_{45} x_4 x_5$$

Here the α's are fitted constants, x_1, x_2, x_3, x_4 and x_5 are coded variables for dextrin, peptone, yeast extract, ammonium dihydrogen orthophosphate and magnesium sulphate, respectively, which are defined as,

$$x_i = \frac{2C_i - C_i^h - C_i^l}{C_i^h - C_i^l} \qquad i = 1,2, \ldots,5$$

where C_i^l and C_i^h are the low and high concentrations of coded variables, respectively.

Coefficients smaller than two times the standard error were presumed to be due to experimental error and were therefore neglected (Montgomery 1991). The direction of maximum increase in CGTase activity is given by the gradient of the above polynomial. The next set of experiments were conducted along this line of steepest increase. A new

two-level factorial design was then constructed with the optimum point thus found. This procedure was repeated till no further improvement in CGTase activity was obtained.

Time course of CGTase production

Time course of CGTase production was studied with the optimized medium in shake flask for 36 h. A 2% inoculum was added to 50 mL optimum medium in 500 mL Erlenmeyer flask and incubated at 28 °C at 300 rpm on a rotary shaker for 36 h. Samples were removed periodically and cell growth as well as CGTase activity was determined in each sample as stated earlier. Time course of CGTase production was also studied in 1 L magnetically driven bottom stirrer fermenter (Gallenkamp, U. K.) A 5% inoculum was added to 700 mL of optimized medium in the fermenter. The fermenter was operated at 28 °C with 1 L/min air flow rate and 400 rpm agitator speed. Samples were removed periodically and analysed for cell growth and CGTase activity measurement as described above.

Results and discussion

Effect of carbon source

Effect of different carbon sources on CGTase production was studied by substituting soluble starch in the basal medium with various carbon sources (Table 3.1). It was observed that enzyme could be produced only when starch, dextrin, amylopectin and CDs were used as carbon source. The maximum activity was obtained with dextrin as carbon source. When simple sugars were tested, there was no enzyme production suggesting that CGTase production from *K. pneumoniae pneumoniae* AS-22 is not a constitutive one. However, Jamuna et al. (1993) have reported constitutive CGTase production from *Bacillus cereus* when simple sugars like glucose and xylose were used as carbon source with maximum enzyme production in presence of xylose.

The starches (or their degradation products) may contain an inducer, which is essential for CGTase production. Surprisingly, in spite of high cell growth, no enzyme activity was obtained with amylose. Amylopectin, on the other hand, supported lower cell growth with higher enzyme activity. All the starches tested resulted in good cell growth as well as

enzyme production. The difference in activity obtained with different starches may be due to differences in their physical structure. Also, different starches contain varying amounts of amylose and amylopectin. As can be seen from the results, no CGTase activity is obtained with pure amylose while pure amylopectin resulted in 2.43 U/mL activity which is even better than soluble starch. Therefore, starches with more amylopectin contain should result in better enzyme production.

Gawande et al. (1998) have observed maximum CGTase production with corn starch for *Bacillus firmus* while Hwang et al (1990) reported maximum CGTase production with soluble starch for *Bacillus stearothermophilus*. Sreenivasan et al. (1991) have reported maximum CGTase activity when tapioca starch was used as carbon source for *Bacillus macerans*.

It was noted that *K. pneumoniae pneumoniae* AS-22 could produce CGTase very efficiently with CDs present in the medium. Selectivity to the various CDs as substrate for the CGTase production was in the order of γ-CD < α-CD < β-CD. This suggests that *K. pneumoniae pneumoniae* AS-22 may also produce either cyclodextrinase or α-amylase, the only enzymes capable of cleaving CDs. Chan et al. (1995) have also reported CGTase production from β-CD with *Bacillus firmus* var. *alkalophilus*.

Effect of nitrogen source

Influence of organic and inorganic nitrogen sources on enzyme production was tested (Table 3.2, 3.3). When the medium was not supplemented with yeast extract, cell growth as well as enzyme production was low (Table 3.2). However, maximum cell growth and enzyme production (1.75 U/mL) was obtained in yeast extract containing medium. This suggests that yeast extract contains some micronutrients essential for the production of the CGTase enzyme. Similar results have been obtained previously with *Bacillus firmus* (Gawande et al., 1998). Therefore effect of nitrogen sources was investigated using yeast extract as supplementary nutrient source (Table 3.3). The results indicated that CGTase production was good when an organic nitrogen source was present in the medium except for soyabean meal and malt extract (Table 3.3). Comparable enzyme production was also observed when inorganic nitrogen sources were present in the medium except for

Carbon source (10 g/L)	Cell growth (A_{660nm})	Activity (U/mL)
Soluble starch	13.11	2.40
Corn starch	13.17	1.37
Potato starch	12.18	2.56
Tapioca starch	12.51	2.72
Wheat starch	13.00	1.92
Rice starch	12.45	2.45
Arrowroot starch	12.81	1.59
Amylose	24.10	0.03
Amylopectin	12.66	2.43
Maltodextrin	13.86	1.40
Dextrin	13.14	2.99
Glucose	8.88	ND
Fructose	10.62	ND
Xylose	6.78	ND
Maltose	10.92	0.07
Lactose	12.00	0.06
Sucrose	11.64	0.04
Sorbitol	8.91	0.07
Mannitol	11.97	0.04
Glycerol	9.30	0.05
α-CD	12.21	2.59
β-CD	12.28	2.78
γ-CD	7.92	1.32

ND = Not detected

Table 3.1 Effect of carbon sources on enzyme production. Cultivated in basal medium containing yeast extract, 5 g/L; peptone, 5 g/L; Na_2HPO_4, 1 g/L; $MgSO_4.7H_2O$, 0.2 g/L (initial pH 7.0) at 30 °C.

Nitrogen source (5 g/L)	Cell growth (A_{660nm})	Activity (U/mL)
None	0.76	0.07
Peptone	5.88	0.83
Tryptone	5.20	0.59
Soyabean meal	2.89	0.04
Soyapeptone	7.92	1.14
Pharmamedia	3.42	0.27
Proflo	2.76	0.16
Cottonseedmeal	3.55	0.10
Casein	1.47	0.12
Cornsteepliquor	8.16	0.42
Yeast extract	9.24	1.75
Casein acid hydrolysate	6.00	0.40
Malt extract	0.70	ND
Urea	7.04	1.29
$(NH_4)_2SO_4$	0.89	ND
NH_4NO_3	0.94	0.15
NH_4Cl	0.98	ND
KNO_3	6.52	1.46
$NaNO_3$	4.79	0.94

ND = Not detected

Table 3.2 Effect of nitrogen sources on enzyme production when yeast extract is not used as supplementary nutrient source. Cultivated in basal medium containing dextrin, 10 g/L; Na_2HPO_4, 1 g/L; $MgSO_4.7H_2O$, 0.2 g/L (initial pH 7.0) at 30 °C

Nitrogen source (5 g/L)	Cell growth (A_{660nm})	Activity (U/mL)
None	8.84	1.53
Peptone	10.96	2.83
Tryptone	10.72	1.99
Soyapeptone	10.84	2.45
Soyabeanmeal	11.44	0.64
Pharmamedia	11.88	1.78
Proflo	12.00	1.94
Cottonseedmeal	10.48	1.94
Cornsteepliquor	11.84	2.07
Casein	9.44	2.26
Casein acid hydrolysate	11.08	2.48
Malt extract	8.76	0.37
Urea	9.84	2.26
$(NH_4)_2SO_4$	8.24	0.15
NH_4NO_3	7.00	2.56
NH_4Cl	8.76	0.10
KNO_3	10.17	2.33
$NaNO_3$	10.17	2.24

Table 3.3 Effect of nitrogen sources on enzyme production when yeast extract is used as supplementary nutrient source. Cultivated in basal medium containing dextrin, 10 g/L; yeast extract, 5 g/L; Na_2HPO_4, 1 g/L; $MgSO_4.7H_2O$, 0.2 g/L (initial pH 7.0) at 30 °C

$(NH_4)_2SO_4$ and NH_4Cl. Hwang et al. (1990) have, however, reported CGTase production with these two inorganic nitrogen sources for *Bacillus stearothermophilus*. Among the organic sources tested, highest enzyme production was observed with peptone, whereas among the inorganic sources tested NH_4NO_3 proved to be the best (Table 3.3).

Effect of phosphorus sources

Among the different phosphorous sources tested, ammonium dihydrogen orthophosphate was found to be the most effective (Table 3.4). When no phosphorus source is added enzyme activity is low (2.61 U/mL) as compared to addition of any phosphorus source which indicates that phosphorus source is required for the better cell growth and enzyme production. The small increase in the enzyme activity in ammonium dihydrogen orthophosphate (3.59 U/mL) as compared to di-sodium hydrogen phosphate (3.30 U/mL) is may be due to additional nitrogen present in the ammonium dihydrogen orthophosphate. Hwang et al. (1990) have observed better enzyme production with NaH_2PO_4 for *Bacillus stearothermophilus*.

Effect of mineral salts

Magnesium sulphate was found to be essential for enzyme production. In the absence of any mineral salt enzyme production was very low (Table 3.5). In other mineral salts tested, cell growth and enzyme production was very poor. The reason for low cell growth and enzyme activity with mineral salts other than magnesium sulphate is probably many salts like Cu, Fe, Zn and Ca are needed in very small concentrations and high concentrations are in fact inhibitory. Similar results have been reported by Hwang et al. (1990) for *Bacillus stearothermophilus*.

Phosphorus source (1 g/L)	Cell growth (A_{660nm})	CGTase (U/mL)
None	11.55	2.61
K_2HPO_4	12.51	3.36
Na_2HPO_4	12.21	3.30
$NH_4H_2PO_4$	12.51	3.59
NaH_2PO_4	12.27	3.27
KH_2PO_4	12.30	3.10

Table 3.4 Effect of phosphorus sources on enzyme production. Cultivated in basal medium containing dextrin, 10 g/L; peptone, 5 g/L; yeast extract, 5 g/L; $MgSO_4.7H_2O$, 0.2 g/L (initial pH 7.0) at 30 °C.

Mineral salt (0.2 g/L)	Cell growth (A_{660nm})	CGTase (U/mL)
None	8.19	0.49
$MgSO_4.7H_2O$	12.42	3.19
$CuSO_4.5H_2O$	7.98	0.23
$ZnSO_4.7H_2O$	7.47	ND
$MnSO_4.H_2O$	9.27	0.80
$FeSO_4.7H_2O$	8.97	0.26
Na_2SO_4	8.67	0.69
$CaCO_3$	8.88	0.80
K_2CO_3	8.79	0.47
$NaHCO_3$	8.64	0.47
KCl	8.79	0.46
$CaCl_2.2H_2O$	4.74	0.14
$CuCl_2.2H_2O$	7.59	0.18
$MnCl_2.4H_2O$	9.96	0.54

ND = Not detected.

Table 3.5 Effect of mineral salts on enzyme production. Cultivated in basal medium containing dextrin, 10 g/L; peptone, 5 g/L; yeast extract, 5 g/L; $NH_4H_2PO_4$, 1 g/L (initial pH 7.0) at 30 °C.

Effect of initial pH

The cell growth was maximum at an initial pH of 7.0 (Figure 3.1). However, for CGTase production two peaks were observed, a smaller peak at an initial pH of 7.5-8.0 and a bigger peak at an initial pH of 9.5. The reason for these two peaks is not clear. However, such peaks can arise if the broth contains a protease that is most active at pH 8.0-9.5.

Effect of incubation temperature

CGTase production was also carried out at different incubation temperatures (Figure 3.2). It was found that cell growth was better at 30 °C whereas CGTase production was maximum at 28 °C. When incubation temperature was increased, CGTase production was considerably decreased. This suggests that CGTase may be highly temperature labile.

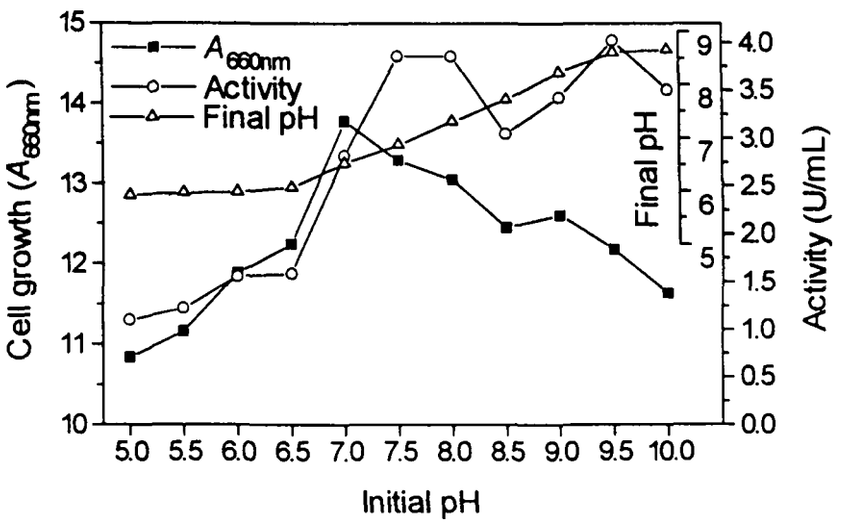


Figure 3.1 Effect of initial pH on cell growth and CGTase production in shake flasks. Cultivated in basal medium containing dextrin 10 g/L, peptone 5 g/L, yeast extract 5 g/L, $(NH_4)H_2PO_4$ 1 g/L and $MgSO_4$ 0.2 g/L at 30 °C

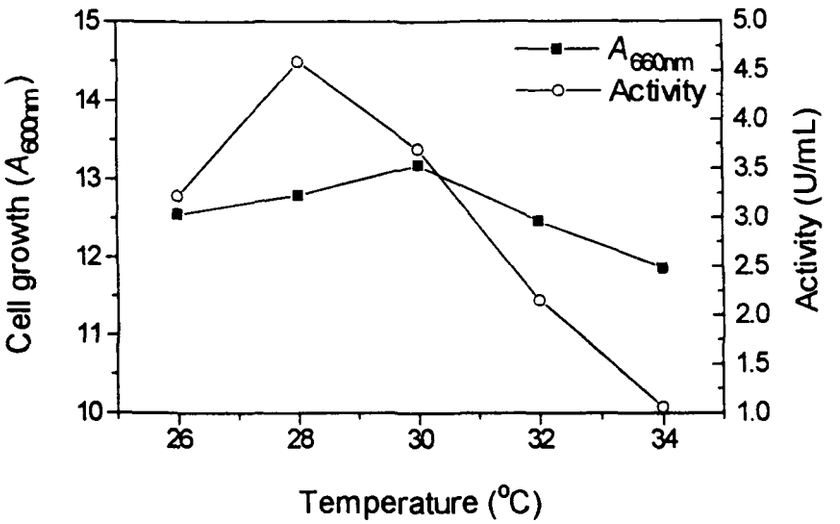


Figure 3.2 Effect of temperature on cell growth and CGTase production in shake flasks. Cultivated in basal medium containing dextrin 10 g/L, peptone 5 g/L, yeast extract 5 g/L, $(NH_4)H_2PO_4$ 1 g/L and $MgSO_4$ 0.2 g/L (initial pH 9.5)

Factorial design

The experiment was carried out to evaluate the standard error in enzyme production using six flasks containing identical medium having concentrations of all the variables at the center of the first fractional design. The standard error in enzyme production was calculated to be 0.144 U/mL. A fractional factorial experiment was designed with five significant variables namely dextrin, peptone, yeast extract, ammonium dihydrogen orthophosphate and magnesium sulphate (Table 3.6). The experimental results obtained from the first factorial design showed that all the variables had significant effect on enzyme production with peptone having the maximum effect. The effect of dextrin and yeast extract is shown graphically in Figure 3.3a, which is quite significant resulting in curved contours. The curved contours are observed when there is significant interaction between the variables. However, the effect of dextrin and peptone resulted in almost straight lines, which implies that there is no interaction between dextrin and peptone variables (Figure 3.3b).

When experiments were conducted in the direction of steepest ascent obtained from this factorial, there was further increase in the enzyme production (Table 3.7). A new fractional factorial design (2^{5-1}) was constructed around the optimum point obtained by the directional experiment (Table 3.8). The experimental results using this second fractional factorial design showed that dextrin had a significant but negative effect while magnesium sulphate had a negligible effect. Hence magnesium sulphate concentration was not changed when experiments were conducted along the direction of steepest ascent given by this factorial. There was no improvement in the enzyme production when experiments were conducted along this direction (Table 3.9). Thus, the optimum medium composition obtained from second fractional factorial was 49.3 g/L dextrin, 20.6 g/L peptone, 18.3 g/L yeast extract, 6.7 g/L ammonium dihydrogen orthophosphate and 0.5 g/L magnesium sulphate.

Flask No.	Dextrin (X_1)	Peptone (X_2)	Yeast extract (X_3)	$NH_4H_2PO_4$ (X_4)	Mg SO_4. $7H_2O$ (X_5)	Activity (U/mL)
1	-	-	-	-	-	3.97
2	-	-	-	+	+	5.99
3	-	-	+	-	+	4.13
4	-	-	+	+	-	5.59
5	-	+	-	-	+	5.18
6	-	+	-	+	-	6.47
7	-	+	+	-	-	5.12
8	-	+	+	+	+	6.53
9	+	-	-	-	-	5.39
10	+	-	-	+	+	5.25
11	+	-	+	-	+	5.39
12	+	-	+	+	-	6.06
13	+	+	-	-	+	4.98
14	+	+	-	+	-	6.74
15	+	+	+	-	-	5.66
16	+	+	+	+	+	8.42

Levels = Dextrin +: 20 g/L and -: 10 g/L, peptone +: 10 g/L and -: 5 g/L, yeast extract +: 10 g/L and -: 5 g/L, $NH_4H_2PO_4$ +: 2 g/L and -: 1 g/L, $MgSO_4$. $7H_2O$ +: 0.4 g/L and -: 0.2 g/L

Effects = α_1 0.591, α_2 0.894, α_3 0.389, α_4 0.574, α_5 0.304, α_{12} 0.034, α_{13} 0.404, α_{14} 0.424, α_{15} 0.149, α_{23} 0.201, α_{24} -0.119, α_{25} -0.084, α_{34} -0.084, α_{35} -0.119, α_{45} 0.201

Table 3.6 Experimental results of first fractional factorial design

Flask No.	Dextrin (X_1)	Peptone (X_2)	Yeast extract (X_3)	$NH_4H_2PO_4$ (X_4)	Mg SO_4. $7H_2O$ (X_5)	Activity (U/mL)
1	25.0	11.6	10.8	2.33	0.36	8.25
2	35.0	13.5	14.1	3.10	0.38	9.75
3	45.2	14.8	17.5	3.86	0.40	11.75
4	55.3	15.7	20.9	4.61	0.41	13.14
5	65.7	16.5	24.4	5.38	0.42	14.65
6	75.4	17.1	27.7	6.10	0.43	14.65

The concentrations of all the variables are given in g/L

Table 3.7 Experimental results of the linear direction of steepest ascent of first fractional factorial design

Flask No.	Dextrin (X_1)	Peptone (X_2)	Yeast extract (X_3)	$NH_4H_2PO_4$ (X_4)	Mg SO_4. $7H_2O$ (X_5)	Activity (U/mL)
1	-	-	-	-	+	18.79
2	-	-	-	+	-	20.67
3	-	-	+	-	-	18.03
4	-	-	+	+	+	17.66
5	-	+	-	-	-	19.54
6	-	+	-	+	+	21.42
7	-	+	+	-	+	19.54
8	-	+	+	+	-	20.29
9	+	-	-	-	-	17.66
10	+	-	-	+	+	18.03
11	+	-	+	-	+	18.79
12	+	-	+	+	-	16.53
13	+	+	-	-	+	16.15
14	+	+	-	+	-	17.66
15	+	+	+	-	-	17.66
16	+	+	+	+	+	16.91

Levels = Dextrin +: 82.1 g/L and -: 49.3 g/L, peptone +: 20.6 g/L and -: 12.4 g/L, yeast extract +: 30.5 g/L and -: 18.3 g/L, $NH_4H_2PO_4$ +: 6.7 g/L and -: 4 g/L, $MgSO_4$. $7H_2O$ +: 0.5 g/L and -: 0.3 g/L

Effects = α_1 -2.069, α_2 0.376, α_3 -0.564, α_4 0.376, α_5 -0.094, α_{12} -1.034, α_{13} 0.661, α_{14} -0.659, α_{15} 0.186, α_{23} 0.471, α_{24} 0.471, α_{25} -0.189, α_{34} -1.034, α_{35} 0.191, α_{45} -0.189

Table 3.8 Experimental results of second fractional factorial design (2^{5-1})

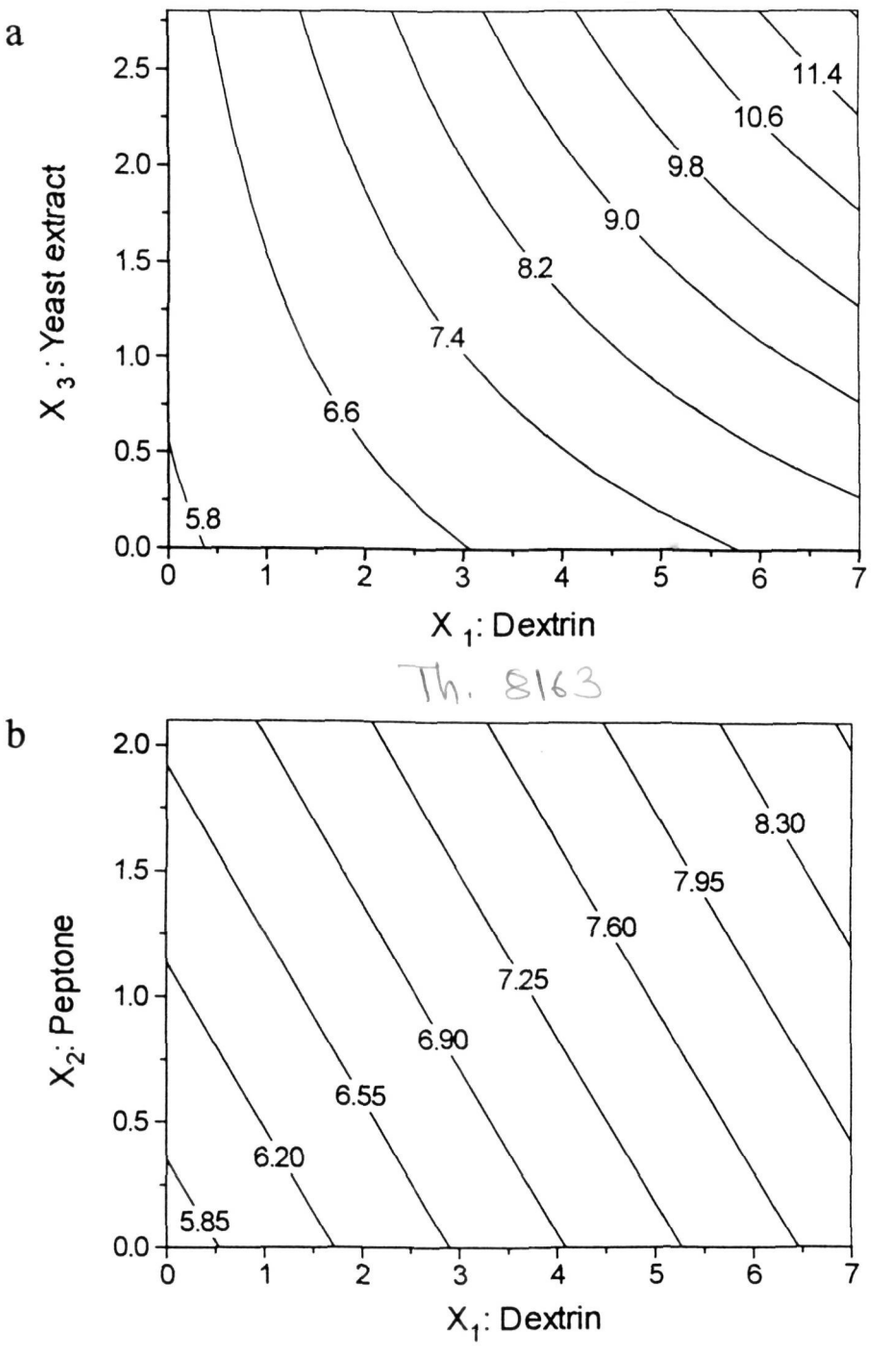


Figure 3.3 Response of the CGTase production to variation in (a) dextrin and yeast extract concentrations when peptone, $(NH_4)H_2PO_4$ and $MgSO_4$ were set at zero and (b) dextrin and peptone concentrations when yeast extract, $(NH_4)H_2PO_4$ and $MgSO_4$ were set at zero. Zero values refer to center of first fractional factorial design

Flask No.	Dextrin (X_1)	Peptone (X_2)	Yeast extract (X_3)	$NH_4H_2PO_4$ (X_4)	Activity (U/mL)
1	65.7	16.5	24.4	5.35	20.29
2	55.5	17.2	23.0	5.6	14.27
3	45.2	18.4	21.2	6.0	12.32
4	35.0	19.8	19.1	6.5	11.84
5	24.0	21.4	16.7	7.2	10.34

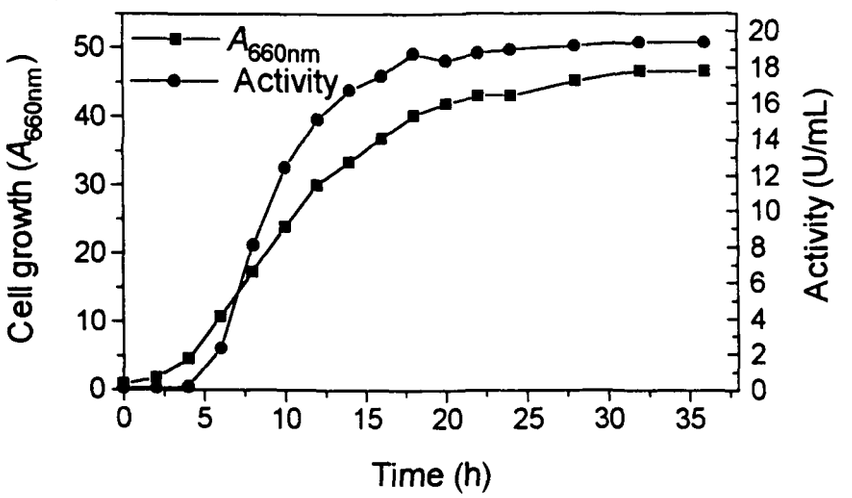
The concentrations of all the variables are given in g/L

Table 3.9 Experimental results of the linear direction of steepest ascent of second fractional factorial design

Cell growth and CGTase production profile

Time course of CGTase production from *K. pneumoniae pneumoniae* AS-22 was studied in shake flask and in 1 L fermenter with the optimized medium (Figure 3.4). It was found that CGTase production was growth associated both in shake flask and in fermenter. The CGTase activity obtained with optimized medium in shake flask was 21.4 U/mL as compared to 2.4 U/mL in the basal medium. Similarly, CGTase activity obtained with optimized medium in the fermenter was 32.5 U/mL as compared to 3.75 U/mL in the basal medium. Though the time of lag phase was same in shake flask and in fermenter, enzyme production was slow in shake flask as compared to fermenter indicating possible oxygen limitation in shake flask. Cell growth and CGTase production reached a maximum in 12 h in fermenter while in shake flask it took more than 28 h. Lee, et al. (1992) have also observed same kind of growth associated enzyme production with α-CD specific CGTase from *Klebsiella oxytoca* 19-1. However, CGTase production was reported to be biphasic with *Bacillus circulans* var. *alkalophilus* (Makela et al., 1990) and during late log phase in *Bacillus firmus* (Goel and Nene 1995).

a



b

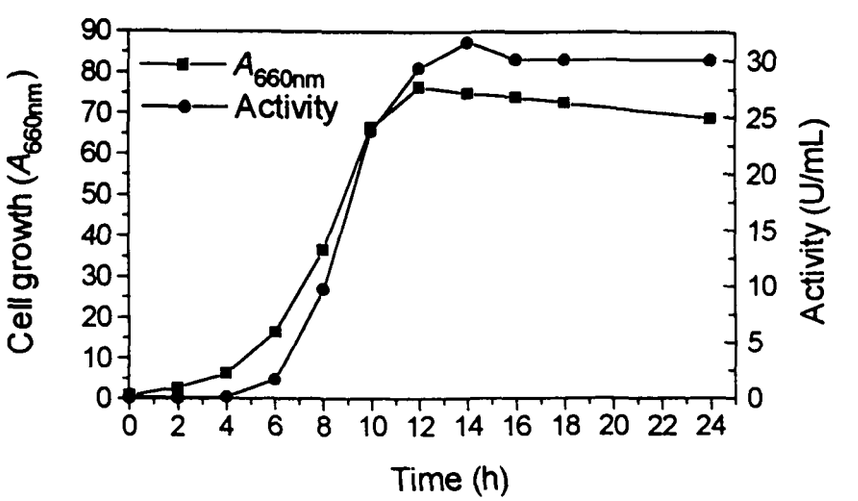


Figure 3.4 Time course of CGTase production with optimized medium (a) in shake flask and (b) in 1 L fermenter

Conclusions

Two level fractional factorial designs in five variables were used for optimization of medium composition for production of α-CD specific CGTase from *K. pneumoniae pneumoniae* AS-22. The optimized medium resulted in nine-fold higher production of CGTase enzyme as compared to that in the basal medium.

Although fermenters are used for commercial production of enzymes, media optimization in fermenters is time-consuming and expensive. This study demonstrates that one can take a convenient and efficient two-step approach consisting of media optimization in shake flasks followed by translation of the results to fermenters. We have found that application of factorial designs for optimization of media components in the shake flask and fermenter showed the same degree of improvement.

References

References are listed in Chapter 7

CHAPTER 4

Alpha-cyclodextrin production using cyclodextrin glycosyltransferase from *Klebsiella pneumoniae pneumoniae* AS-22

Summary

Alpha-cyclodextrin (α-CD) production using cyclodextrin glycosyltransferase (CGTase) from *Klebsiella pneumoniae pneumoniae* AS-22, was investigated in presence of various complexing agents using raw wheat starch and dextrin. The addition of n-butanol resulted in maximum conversion of raw wheat starch to α-CD, whereas presence of n-hexanol lead to maximum conversion of dextrin to α-CD. In presence of 2% (v/v) n-butanol, with 125 g/L raw wheat starch and 20 U of the enzyme per g of starch, maximum of 42.5% (w/w) conversion of CDs was obtained. The ratio of α : β : γ-CD at this stage was 97 : 3 : 0 with negligible amounts of malto-oligosaccharides. The production of α-CD was optimized using two-level factorial designs in three variables: dextrin, n-hexanol and enzyme concentrations. The optimum concentrations for α-CD production were found to be: dextrin 500 g/L, n-hexanol 3% (v/v), enzyme 15 U per g of dextrin. At this optimum condition, maximum conversion of 12.1% (w/w) of dextrin to total-CDs was achieved and the ratio of α : β : γ-CD was 91 : 3 : 6 with negligible amounts of malto-oligosaccharides. This CGTase was strongly inhibited by α-CD, 50% inhibition was observed at 4 g/L initial concentration of α-CD. The effectiveness of ultrafiltration membrane bioreactor for continuous removal of product was also tested.

Introduction

Cyclodextrin glycosyltransferase (CGTase, E. C. 2.4.1.19) catalyzes formation of non-reducing cyclic malto-oligosaccharides known as cyclodextrins (CDs) from starch and related materials. The common type of CDs contain six, seven and eight glucose units linked together by α-1,4-glycosidic bonds and are named as α-, β- and γ-CD respectively. A CD molecule has a unique torus-shaped structure with hydrophobic internal cavity and hydrophilic external surface. As a result CDs can form inclusion complex with number of hydrophobic guest molecules and thereby change their physical and chemical properties. Therefore, CDs are becoming popular in various industries like pharmaceutical, agricultural, chemical, cosmetics, food, etc (Bender 1986; Hedges 1992; Szejtli 1984).

Most CGTases produce a mixture of α-, β- and γ-CDs in varying ratios in rather low yields. The ratio of CDs can be significantly altered and shifted towards formation of only one type of CD by addition of compounds those form inclusion complex with CDs. These compounds are known as complexing agents and are highly specific for particular CD. They can improve the selectivity to more than 90% purity for individual CDs. The production of CDs can be increased at least two-fold in presence of complexing agent. Armbruster and coworkers (Armbruster 1970; Armbruster and Jacaway 1972; Armbruster and Kooi 1969) were the first to report the use of complexing agents for industrial production of CDs. The complexing agent is usually a solvent and hence this method of CD production is also known as solvent method.

Enhanced production of α-CD was observed in the presence of complexing agents like C_{1-8} aliphatic alcohols, aliphatic ethers, esters, C_{2-4} ketones (Toyo Jozo Co., Ltd., Japan), n-butanol (Abelian et al., 1993), 1-decanol (Armbruster 1988; Armbruster and Jacaway 1972; Flaschel et al., 1984) or cyclohexane (Shieh and Hedges 1993). The production of β-CD was improved by addition of toluene (Armbruster and Kooi 1969; Cramer and Steinle 1955; Vakaliu et al., 1977), trichloroethylene (McClenahan et al., 1942; Okada and Tsujama 1973; Sato and Nakamura 1974; Yagi et al., 1986), cyclohexane (Morita et al., 1996) and bromobenzene (Raja et al., 1990; Raja and Ramakrishna 1994). For improving γ-CD production, combination of bromobenzene and sodium acetate (Bender 1983b), methyl ethyl ketone and α-naphthol (Seres et al., 1985), glycyrrhizic acid or stevioside (Sato et al., 1985b; Sawaguchi et al., 1990), C_{12} cyclic compounds (Rendleman 1992; Schmid 1996b; Schmid and Eberle 1988) or cyclohexadec-8-en-1-one (Schmid 1996b), has been reported.

The great advantage of using complexing agent for CD production is the high product yield. Usually, when a selective complexing agent is used for production of particular CD, negligible levels of other CDs are produced. Therefore, isolation and purification of individual CD is greatly simplified. The use of complexing agent like alcohol is especially beneficial because it is practically harmless, highly evaporative and can be completely removed by distillation and reused. If, the given CGTase can produce CDs from raw starch without any pretreatment then further improves economics of the

production process, because energy-intensive gelatinization step prior to CD production can be avoided. It is well known that ratio of substrate to enzyme and complexing agent to substrate affect CD production. The exact ratio of components for optimum production of CDs is difficult to study by conventional method of varying one component at a time. Therefore, we used simple two-level factorial design approach for optimization of α-CD production with three variables namely dextrin, n-hexanol and enzyme concentration for the first time.

In the present study, we report an efficient method for production of α-CD from raw wheat starch and dextrin using CGTase from *Klebsiella pneumoniae pneumoniae* AS-22 in presence of n-butanol and n-hexanol respectively. Also production of α-CD with dextrin in ultrafiltration membrane bioreactor is reported.

Materials and methods

Materials

Fatty acids, wheat starch, malto-oligosaccharides (G_1-G_7), CDs were purchased from Sigma (USA). Iso-octane, n-dodecane, undecan-1-ol, tri-, tetra- and octadecan-1-ol were purchased from Aldrich (USA). Dextrin (yellow) was obtained from Anil Starch (Ahmedabad, India). Tapioca starch was obtained from Laxmi starch (Coimbature, India). The n-butanol, n-hexanol was procured from E. Merck (Mumbai, India) while all other alcohols, solvents, polyethylene glycols, detergents were from S. D. Fine-chemicals (Boisar, India). The media components were from Hi-Media (Mumbai, India). Magnafloc was a kind gift from Hindustan Antibiotics Ltd. (Pimpri, India).

Strain

A novel CGTase producing strain *Klebsiella pneumoniae pneumoniae* AS-22 (ATCC 212075, NCIM 5121, Gawande and Patkar 1998) was isolated from soil samples and used in this study.

CGTase assay

CGTase activity was determined using a modification of the method described by Lejuene et al. (1989). One milliliter of 1% (w/v) soluble starch prepared in 50 mM

phosphate buffer, pH 7.0, was mixed with 0.1 ml of appropriately diluted enzyme and incubated at 40 °C for 10 min. The reaction was stopped by immediately cooling the tubes in chilled water, followed by addition of 0.1 ml of 1.2 N HCl. Two milliliter methyl orange solution (final concentration 0.035 mM) was then added to the reaction mixture and the tubes were maintained at 15 °C for 30 min. The absorbance of the mixture was measured at 507 nm and decrease in absorbance (with respect to a control tube without the enzyme) was correlated with the amount of α-CD formed. One unit of the enzyme is defined as the amount of the enzyme that produced one μmole of α-CD per minute under standard conditions.

Enzyme production and preparation

A loopful of culture was transferred from a slant to 50 mL of basal medium containing (in g/L) tapioca starch 10, yeast extract 5, urea 5, Na_2HPO_4 1, $MgSO_4$ 0.2 pH 7.0 and incubated at 30 °C on a rotary shaker at 250 rpm for 12 h. A 25 mL of this was further transferred to 500 mL basal medium, cultivated as above for 12 h and then used as inoculum (10% v/v) for a 12 L stirred fermenter (New Brunswick Scientific Co., USA). The fermenter medium consisted of (in g/L) tapioca starch 20, yeast extract 5, urea 5, Na_2HPO_4 1, $MgSO_4$ 0.2 pH 7.0. The fermentation was performed with airflow rate of 12 L/min, agitation speed of 500 rpm at 30 °C for 12 h. After harvesting the fermentation broth, the cells were flocculated by addition of 5 mL/L of Magnafloc, allowed to settle overnight at 10 °C and centrifuged at 2300 *g* for 20 min. The cell-free supernate was concentrated to 20-fold by ultrafiltration using a hollow fibre membrane module with a 20 kDa molecular weight cut off (Nitto Denko Corporation, Japan) and was used for CD production experiments. The concentrated enzyme had an activity of 90 U/mL.

Analysis of CDs by HPLC

The concentrations of malto-oligosaccharides, α-, β- and γ-CDs were determined by HPLC (Thermo Separation Products, USA), as described by Sato et al. (1985a) under the following conditions: Column, Shodex DC-630 (Showa Denko, Japan); mobile phase, acetonitrile:water (65:35); flow rate, 1.5 ml min^{-1}; column temperature, 60 °C; refractive index detector (Waters 410, USA or Shodex RI-71, Japan). Appropriately diluted

reaction mixture (0.5 ml) was mixed with equal volume of acetonitrile, centrifuged at 20,000 *g* for 15 min at room temperature, filtered through a 0.45 μ filter (Millipore, USA) and injected through a Rheodyne 7725 (USA) manual injector with a 20 μL loop.

Factorial design

Factorial experiments were designed using the approach given in standard texts on design of experiments (Box et al., 1978; Davies 1993; Montgomery 1991). The standard error in CD production measurement was estimated from an experiment was performed using five flasks containing identical medium (medium composition at the center of the first factorial). The effect of three variables, namely dextrin, n-hexanol and enzyme, on α-CD production was studied using simple two-level factorial designs. The data obtained from the factorial experiments was fitted to the following polynomial.

$$\alpha\text{-CD yield} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3$$

Here the β's are fitted constants and x_1, x_2 and x_3 are coded variables for dextrin concentration, n-hexanol concentration and enzyme amount per g of dextrin, respectively, which are defined as,

$$x_i = \frac{2C_i - C_i^h - C_i^l}{C_i^h - C_i^l} \qquad i = 1,2,3$$

where C_i^l and C_i^h are the low and high concentrations of coded variables, respectively.

The direction of maximum increase in α-CD (in the x_1, x_2, x_3 space) is given by the gradient of the above polynomial. The next set of experiments was conducted along this direction. Coefficients smaller than two times the standard error were presumed to be due to experimental error and were therefore neglected (Montgomery 1991).

CD production in bioreactor

The slurry of raw wheat starch (300 g/L) becomes highly viscous and can not be mixed well in shake flasks. Therefore, a water-jacketed glass bioreactor (100 mL), equipped with an overhead stirrer (Bright Stars Electronics, India), was used for CD production experiments at high starch concentrations. The samples were removal at regular time intervals from the side arm of the reactor vessel and analysed using HPLC.

CD production in membrane bioreactor

A schematic of the membrane bioreactor setup is given in Figure 4.9. The reaction was carried out in a stirred, water-jacketed stainless steel bioreactor maintained at 40 °C. The contents of the reactor were circulated through the lumen of a tubular ceramic ultrafiltration membrane module with 10 kDa molecular cut off (Carbosep™ 40, France; 6 mm ID and 40 cm length) at a flow rate of 600 L/min and a back-pressure of 1 kg/cm^2 A gear pump (Cole Parmer, USA) magnetically coupled to a DC motor (Baldor, USA) was used for recirculation. The pump flow rate was adjusted with a Saftronics (India) controller. The permeate was collected at regular time intervals and analysed using HPLC

Results and discussion

Effect of alcohols

For α-CD production without using any complexing agents, the optimum raw wheat starch concentration was found to be 125 g/L (Figure 2.11). The effect of various alcohols (primary, secondary and substituted) on CD production was studied at this optimum concentration (Table 4.1). Among all the tested alcohols, addition of n-butanol resulted in maximum (42.5 % w/w) conversion of raw wheat starch to total CDs in 6 h with α : β : γ-CD ratio of 95.6 : 3.1 : 1.3. The presence of n-butanol resulted in about 2.4-fold increase in α-CD yield. However, addition of ethanol resulted in only 1.2-fold increase in α-CD.

No	Alcohol 2% (v/v)	Incubation 2 h		Incubation 4 h		Incubation 6 h		
		α-CD (g/L)	β-CD (g/L)	α-CD (g/L)	β-CD (g/L)	α-CD (g/L)	β-CD (g/L)	γ-CD (g/L)
1	None	16.9	ND	21.1	2.4	17.8	2.6	1.0
2	Methanol	15.8	1.2	16.0	2.1	17.7	2.8	1.0
3	Ethanol	21.2	1.0	22.8	1.2	26.2	1.9	0.8
4	Propan-1-ol	32.9	ND	41.8	2.3	47.5	2.5	ND
5	Propan-2-ol	18.9	0.9	21.1	2.5	24.2	2.5	0.9
6	2-methyl-propan-2-ol	19.4	0.7	21.6	1.7	27.4	2.9	0.9
7	2-methyl-propan-1-ol	28.4	0.4	39.1	1.3	40.1	1.1	0.7
8	Butan-1-ol	35.5	0.8	40.6	1.5	50.5	1.6	0.7
9	Butan-2-ol	40.7	1.3	41.5	1.3	40.0	1.9	ND
10	Pentan-1-ol	34.0	0.7	39.0	0.9	46.9	0.9	ND
11	3-methyl-butan-1-ol	30.3	1.0	33.7	1.1	44.4	1.6	0.4
12	Hexan-1-ol	36.2	ND	47.7	ND	48.1	1.1	ND
13	Heptan-1-ol	37.6	ND	49.3	ND	46.6	1.3	ND
14	Octan-1-ol	36.8	0.8	34.3	1.9	39.9	1.4	ND
15	Octan-2-ol	36.4	0.8	42.7	1.2	45.8	1.9	0.8
16	Decan-1-ol	36.6	0.8	37.0	1.0	45.6	1.3	ND
17	Undecan-1-ol	33.5	1.5	46.1	0.9	47.2	1.7	1.1
18	Dodecan-1-ol	35.0	1.0	43.8	0.4	44.3	1.5	ND
19	Tridecan-1-ol	31.1	0.9	39.2	0.9	42.9	1.8	ND
20	Tetradecan-1-ol	27.7	ND	37.5	1.3	27.8	1.9	ND
21	Cyclohexanol	27.5	1.0	28.5	1.2	31.3	1.5	ND
22	2-ethyl-hexanol	30.8	0.6	37.0	2.4	43.7	2.0	ND
23	Homo allyl alcohol	25.2	0.9	27.6	2.0	32.6	2.3	0.7
24	Allyl alcohol	27.1	0.6	25.9	2.2	29.5	2.2	0.8
25	3-methyl-butan-2-ol	27.8	1.0	29.2	1.8	32.9	1.9	0.0
26	Benzyl alcohol	18.3	0.6	22.4	1.3	26.8	1.5	ND
27	Phenyl alcohol	15.2	ND	21.4	ND	25.3	0.4	ND

ND = Not detected

Table 4.1 Effect of alcohols on cyclodextrin formation with 125 g/L raw wheat starch. The reaction mixture (10 mL total volume) also contains 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 2% (v/v) alcohol and 10 U of CGTase per g of starch. The flasks were kept in a reciprocating water-bath at 200 rpm and 40 °C.

The small increase in CD yield obtained with 2% (v/v) ethanol might be because ethanol concentration used here is less. Other researchers have used 15-30% (v/v) ethanol concentration for enhanced CD production (Mattsson et al., 1991; Tomita et al., 1990; Mori et al., 1995). Since this was the screening experiment using various alcohols, the concentration of all the alcohols was kept same. Addition of n-hexanol and n-heptanol also resulted in very good conversion of raw starch to α-CD. The maximum of 48.1 g/L concentration of α-CD was obtained with n-hexanol in 6 h of incubation with 38.5 w/w conversion of starch to CDs and ratio of α : β : γ-CD was 98 : 2 : 0. The results obtained with n-heptanol were still interesting. There were no other CDs produced in presence of n-heptanol when maximum concentration of α-CD reached to 49.3 g/L in 4 h of incubation. Further experiments need to be performed to study the effect of high ethanol concentration and n-hexanol and n-heptanol alcohols on α-CD yield.

The effect of alcohol addition was also investigated with 50 g/L dextrin (Table 4.2). The maximum conversion (22.7% w/w) of dextrin to α-CD was obtained in presence of n-hexanol without detection of other CDs and malto-oligosaccharides. The α-CD yield increased to 2.5-fold more as compared to no addition of complexing agent. The results obtained with n-pentanol were marginally low as compared to that with n-hexanol. The addition of n-propanol to n-dodecanol (linear chain aliphatic alcohols) resulted in enhanced conversion of both the substrates to α-CD while substituted and cyclic alcohol resulted in low conversion yields. For selecting complexing agent for enhanced conversion of substrate to CDs, price of the complexing agent, whether can be removed completely and reused should be considered.

No	Alcohol (2% v/v)	α-CD in 6h (g/L)
1	None	4.6
2	Methanol	4.2
3	Ethanol	6.1
4	Propan-1-ol	8.7
5	Propan-2-ol	5.5
6	2-methyl-propan-2-ol	5.5
7	Butan-1-ol	10.2
8	Butan-2-ol	8.5
9	3-methyl-butan-1-ol	8.8
10	Pentan-1-ol	11.2
11	Hexan-1-ol	11.3
12	Heptan-1-ol	10.8
13	Octan-1-ol	10.7
14	Octan-2-ol	10.5
15	Decan-1-ol	9.4
16	Undecan-1-ol	8.6
17	Dodecan-1-ol	8.4
18	Tridecan-1-ol	7.0
19	Tetradecan-1-ol	5.8
20	Octadecan-1-ol	4.6
21	Cyclohexanol	8.7
22	2-ethyl-hexanol	9.5
23	Homo allyl alcohol	7.4
24	Allyl alcohol	7.2
25	3-methyl-butan-2-ol	7.9
26	Benzyl alcohol	6.3
27	Phenyl alcohol	5.3

Table 4.2 Effect of alcohols on α-CD production with 5% (w/v) dextrin. The reaction mixture (10 mL total volume) also contains 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 2% (v/v) alcohol and 10 U of CGTase per g of dextrin. The flasks were kept in a reciprocating water-bath at 200 rpm and 40 °C. No other than α-CD was detected in any alcohol.

Shiraishi et al. (1989a) have obtained 1.8-fold increase in α-CD yield with 10% (w/v) soluble starch from *Bacillus macerans* CGTase in presence of ethanol. They further reported that presence of ethanol decreased formation of β- and γ-CD as compared to absence of ethanol. Flaschel et al. (1984) observed addition of n-decanol resulted in high yield (50% w/w) of α-CD. Mattsson et al. (1991) obtained 2-fold increase in total CD production with 150 g/L potato starch in presence of 15% (v/v) ethanol. The increase in total CDs was mainly due to the increase in production of β-CD, which is the main product of CGTase from *Bacillus circulans*. Tomita et al., (1990) have also observed 2.5-fold increase in production of γ-CD from 25 g/L potato starch in presence of 30% (v/v) ethanol. Lee and Kim (1991b) have obtained two-fold increase in total CD yield with 10% (v/v) ethanol from 50 g/L soluble starch. Mori et al. (1995) observed about 2.5-fold increase in γ-CD yield with 50 g/L soluble starch in presence of 20% (v/v) ethanol with CGTase from *Brevibacterium* sp. No. 9605.

There are two possible explanations why alcohol enhances CD production. First, alcohols are found to reduce decrease in concentration of CDs by transglycosylation and coupling reactions (Shiraishi et al., 1989b; Tomita et al., 1990). When CGTase was incubated with α-CD in absence of alcohol, α-CD concentration decreased and the amounts of β- and γ-CDs increased to the same level of decrease in α-CD after prolonged incubation. This implies that α-CD gets converted to these CDs by coupling reaction of CGTase followed by cyclization (Shiraishi et al., 1989b). However, when alcohols were added, the extent of decrease in α-CD was smaller for ethanol and n-decanol. No decrease was observed in presence of n-butanol. Further in presence of n-butanol no other CD was detected. We have obtained similar results here i.e. less formation of β- and γ-CDs in presence of n-butanol with 125 g/L raw wheat starch and no detection of these CDs with 50 g/L dextrin in presence of n-hexanol. Second, according to Mattsson et al. (1991) enhanced conversion of CDs in presence of alcohols results from decreased water activity in the reaction mixture, which in turn decreases hydrolysis reaction of CGTase. However, Lee and Kim (1991b) have observed an increase in hydrolysis activity of CGTase in presence of ethanol. They attributed higher CD production to enhancement in starch hydrolysis.

Effect of enzyme concentration

Effect of enzyme concentration (10-40 U per g of starch) on CD production was studied using 125 g/L raw wheat starch in presence of 2% (v/v) n-butanol (Figure 4.1). As expected, increase in enzyme concentrations increased the initial rate of CD production. However, the final (at 6 h) α-CD yield was almost same. Because of higher rate of CD production at 20 U of enzyme per g of starch, this enzyme concentration was used for further experiments. Shiraishi et al. (1989a) studied formation of α-CD with enzyme concentrations ranging from 3.63 to 242.4 U per g of starch using 10 g/L soluble starch in presence and absence of 4% (v/v) ethanol. When concentration of the enzyme was increased conversion rate greatly increased. The α-CD concentration reached to maximum within half an hour above enzyme concentrations of 40.4 U per g of starch with or without addition of alcohol. Moreover, at 242.4 U of the enzyme per g of starch, CDs were rapidly degraded to malto-oligosaccharides and degradation was almost completed in 75h. This clearly indicates that optimum enzyme concentration giving maximum conversion to desired CD should always be used, because excess enzyme favours coupling reaction producing large amounts of malto-oligosaccharides. Mattsson et al. (1991) have observed an increase in β-CD formation with increase in the enzyme concentration up to 300 U of enzyme per g of starch using 100 g/L starch in presence of 10% (v/v) ethanol. However, in absence of ethanol 100 U the enzyme per g of starch produced maximum amounts of β-CD.

Effect of n-butanol concentration

The concentrations of n-butanol were varied in the range of 2-8% (v/v), with 125 g/L raw wheat starch and 20 U of the enzyme per g of (Figure 4.2). Maximum conversion of raw wheat starch was obtained at 2% (v/v) n-butanol concentration. Further increase in n-butanol concentration decreased CD yield. When 2% (v/v) n-butanol was used small amount of β-CD was formed. However, at n-butanol concentrations 4% and above, only α-CD was produced. Mori et al. (1995) also found that an increase in ethanol concentration from 20 to 30% (v/v) resulted in production of only γ-CD but to low conversion yields.

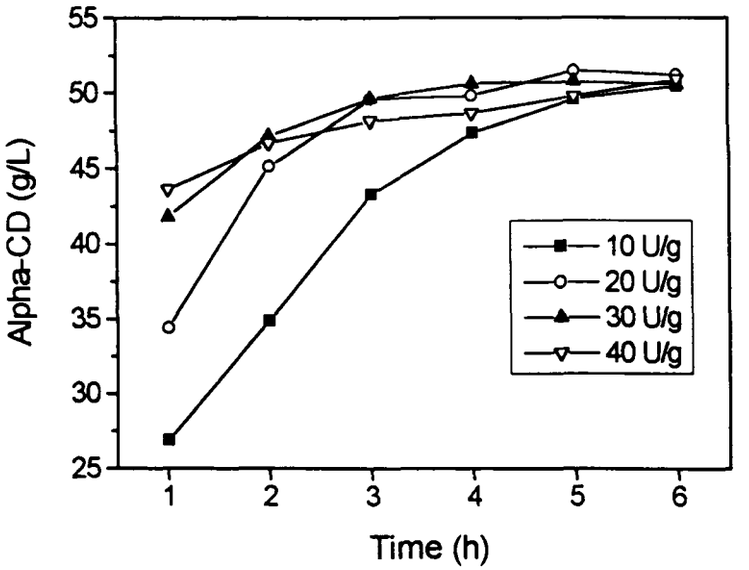


Figure 4.1 Effect of enzyme concentration on α-CD production with 125 g/L raw wheat starch in presence of 2% (v/v) n-butanol

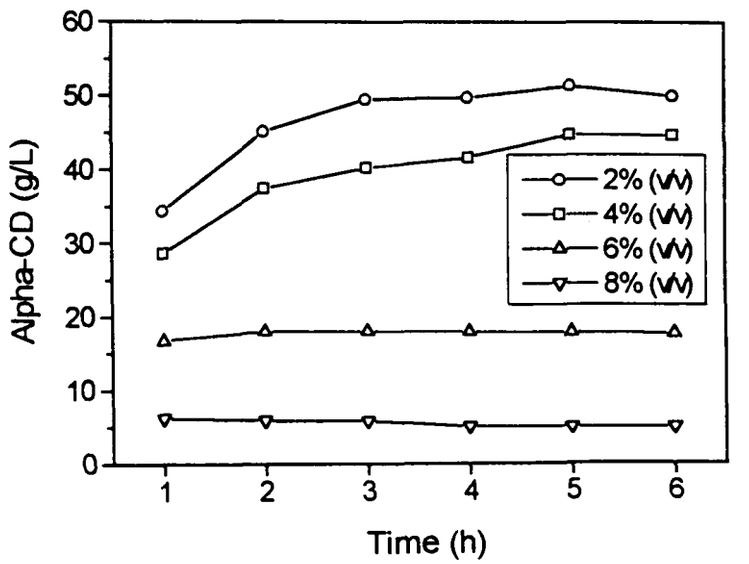


Figure 4.2 Effect of n-butanol concentration on α-CD production with 125 g/L raw wheat starch and 20 U of the enzyme per g of starch

Surprisingly, at 8% (v/v) n-butanol addition, maltohexaose (G_6) concentration increased with time and reached to 3.46 g/L in 6 h. This indicates that in presence of high concentration of n-butanol CGTase probably could disproportionate the amylose chain to the exact size of its main product but could not cyclize it.

Shiraishi et al. (1989b) observed that the optimum concentration of n-butanol, for α-CD production from 10 g/L soluble starch using *Bacillus macerans* CGTase, was 5% (v/v). When ethanol was used as complexing agent, the optimum concentration was usually in the range of 10-20% (v/v) (Lee and Kim 1991b; Mattsson et al., 1991; Mori et al., 1995; Shiraishi et al., 1989b; Tomita et al., 1990). The higher optimum concentration of ethanol as compared to n-butanol is possibly because the inclusion complex of CD with ethanol is soluble whereas that of n-butanol is insoluble. Therefore, at low concentration, n-butanol can remove α-CD out of solution and is more effective at driving the reaction towards α-CD production. The presence of ethanol decreases conversion of α-CD to other types of CDs, but could not prevent it completely (Shiraishi et al., 1989a). However, when n-butanol is used with optimum starch concentration, the concentration of α-CD stayed almost constant after reaching a maximum (Figure 4.4).

CD production profile

Production of CDs was investigated using 125 g/L raw wheat starch and 20 U of enzyme per g of starch in absence (Figure 4.3) and presence of 2% (v/v) n-butanol (Figure 4.4) for 24 h in bioreactor. It was observed that α-CD production reached to maximum in 4 h and then declined when complexing agent is not used. Also concentrations of β- and γ-CD increased as α-CD concentration declined. However, in presence of complexing agent α-CD concentration reached to maximum in 6h and thereafter remained almost constant. The concentration of β-CD was increased slowly and reached to maximum of 2.75 g/L (6.9 g/L in absence of n-butanol) and no γ-CD was detected. The maximum (42.5% w/w) conversion of raw wheat starch to total CDs was achieved in 6 h containing 97% of α-CD out of total CDs.

CD production was also studied with very high raw wheat starch concentration (300 g/L) in a bioreactor using 2% (v/v) n-butanol and 20 U of the enzyme per g of starch

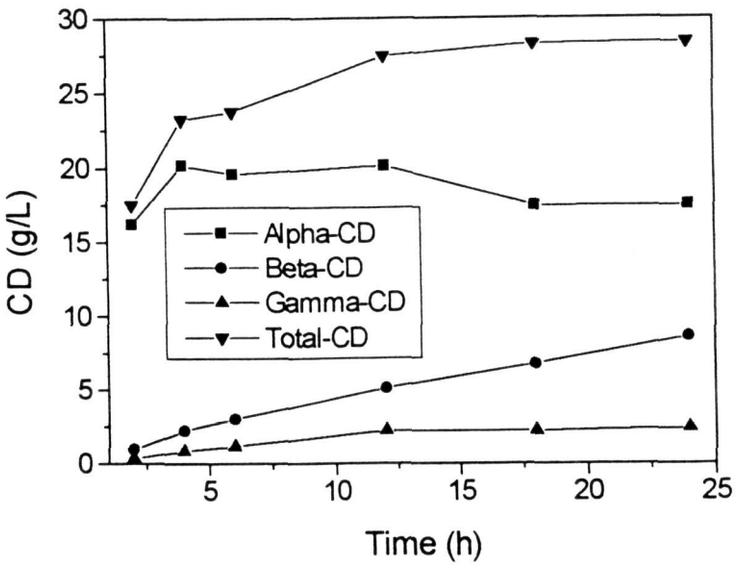


Figure 4.3 Cyclodextrin production using 125 g/L raw wheat starch and 20 U of enzyme per g of starch in absence of n-butanol in bioreactor.

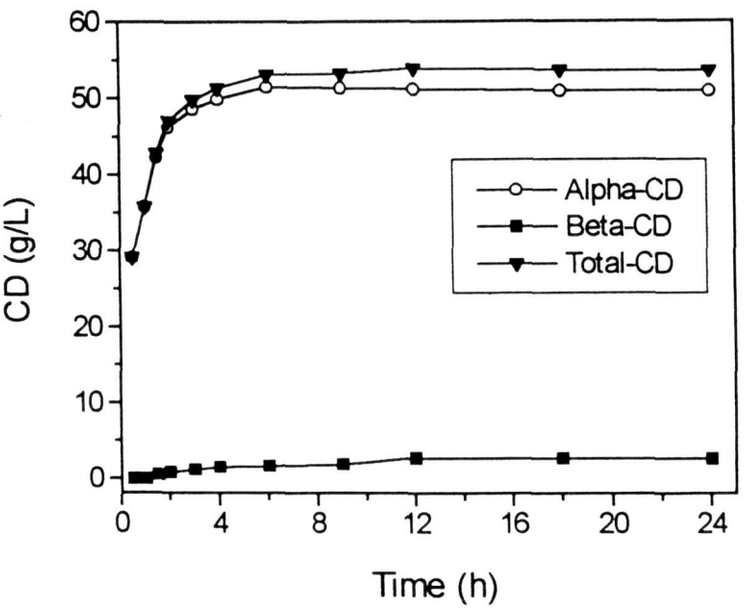


Figure 4.4 Cyclodextrin production using 125 g/L raw wheat starch and 20 U of enzyme per g of starch in presence of 2% (v/v) n-butanol in bioreactor

(Figure 4.5). The maximum α-CD concentration of 59.63 g/L was obtained in 5 h. The ratio of α : β : γ-CD at this point was found out to be 95.1 : 2.9 : 2 with 21% (w/w) conversion to CDs. It is known that conversion yield of CDs decreases at higher starch concentrations (Mori et al., 1995; Shiraishi et al., 1989a; Tomita et al., 1990). Further, at higher starch concentration, even in the presence of complexing agent, β- and γ-CDs appeared in the reaction mixture.

Effect of fatty acids, polyethylene glycols and detergents

Effect of complexing agents, like fatty acids, polyethylene glycols (PEGs), detergents (1% w/v), on α-CD production was tested using 50 g/L dextrin and 10 U of enzyme per g of dextrin (Table 4.3). The fatty acids containing 4 to 18 carbon atoms were used. The highest production of α-CD was obtained with lauric acid, a fatty acid containing 12 carbon atoms, with 2.1-fold increase in α-CD concentration than the control. Sakai and Horie (1987) have observed enhanced CD production using sucrose fatty acid esters.

Addition of polyethylene glycols with different molecular weights did not improve CD yield. This is in contrast to some results reported in literature where PEG is reported to enhance CD production 1.5 to 2-fold (Delbourg et al., 1993). However, these workers used very high PEG concentrations (20% w/v). Addition of SDS decreased α-CD formation by almost 50%. However, Chiu (1987) has observed 51-60% improvement in CD production in presence of SDS. Similar results were obtained by Kobayashi et al. (1977), who used *Bacillus macerans* CGTase for α-CD production. Among the various detergents tested, Tween-20 was found to increase α-CD yield by a factor of 1.5 with this CGTase (Table 4.3). Nakanishi et al. (1993) have observed enhanced CD production in presence of Triton X-100, however, our results do not agree with their findings.

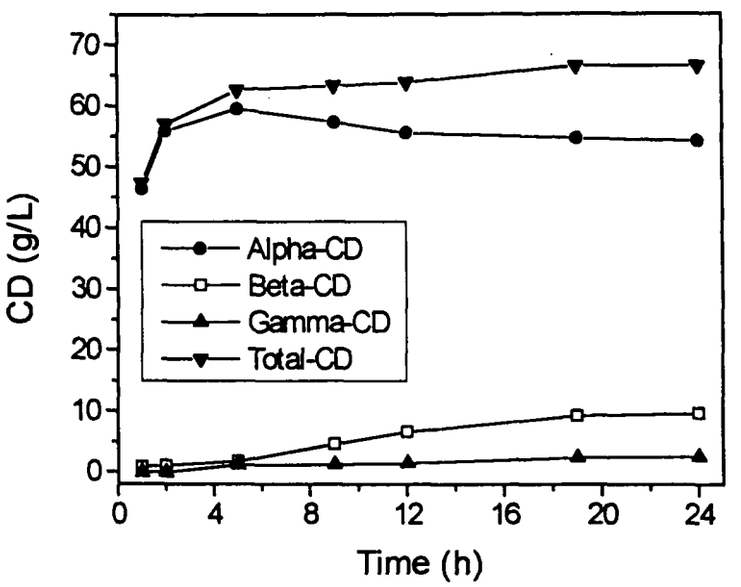


Figure 4.5 Cyclodextrin production using 300 g/L raw wheat starch and 20 U of the enzyme per g of starch in presence of 2% (v/v) n-butanol in bioreactor

No	Additive (1% w/v)	α-CD in 6h (g/L)
1	None	4.6
2	n-butyric acid	ND
3	n-caproic acid	ND
4	Heptanoic acid	ND
5	Palmitic acid	4.5
6	Stearic acid	4.2
7	Oleic acid	6.4
8	Lauric acid	9.8
9	PEG 400	4.5
10	PEG 4000	4.5
11	PEG 6000	4.5
12	PEG 8000	4.6
13	PEG 9000	4.2
14	SDS	2.4
15	Tween-20	7.0
16	Tween-80	5.4
17	Triton X-100	4.3

ND = Not detected

Table 4.3 Effect of some additives on α-CD formation with 5% (w/v) dextrin. The dextrin solution (20 mL total volume) contains 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, and 1% (w/v) additive (pH adjusted) and 20 U of the enzyme per g of dextrin. The reaction was started by keeping flasks at 40 °C on a reciprocating water-bath at 200 rpm. Levels of β- and γ-CDs were below detectable limits.

Effect of solvents

Effect of solvents other than alcohols on α-CD production was also investigated with 50 g/L dextrin and 20 U of the enzyme per g of starch (Table 4.4). Among the solvents tested only toluene and chloroform slightly increased α-CD yield. Toluene has been successfully used as a complexing agent for industrial production of β-CD at a concentration of 5% (v/v) with 280 g/L maize starch (Vakaliu et al., 1977). They have obtained 48.3% (w/w) conversion to β-CD. Raja et al. (1990) obtained 10% (w/w) conversion to β-CD from 5-10% (w/v) cassava starch in presence of bromobenzene and chloroform. Later on Raja and Ramakrishna (1994) improved conversion to 28% (w/w) from 5% (w/v) cassava starch using bromobenzene. Armbruster (1988) used cyclohexane (5% v/v) for the enhanced production of CDs from 30% (w/w) of potato starch. He isolated 42% (w/w) CDs containing 22% (w/w) of pure β-CD and 14 % (w/w)

No	Solvent (2% v/v)	α-CD in 6h (g/L)
1	None	4.6
2	Hexane	4.3
3	Cyclohexane	4.8
4	Toluene	5.1
5	Benzene	4.3
6	Chloroform	5.2
7	Iso-octane	4.2
8	n-Dodecane	4.5

Table 4.4 Effect of some solvents on α-CD formation with 5% (w/v) dextrin. The dextrin solution (20 mL total volume) contains 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, and 2% (v/v) solvent and 10 U of the enzyme per g of dextrin. The reaction was started by keeping flasks at 40 °C on a reciprocating water-bath at 200 rpm. No other than α-CD was detected.

of pure α-CD. Rendleman (1992) used C_{12} cyclic compounds as complexing agents for enhanced production of γ-CD and obtained 50% yield from 5% (w/v) potato starch using cyclodocanone (0.18 mmol) when CGTase from *Bacillus macerans* was added in increments for long period of time (10 days).

Effect of dextrin concentration

The effect of concentration of dextrin on α-CD production was investigated in presence of 2% n-hexanol (Figure 4.6). Higher dextrin concentrations increased the final α-CD concentration, but the conversion decreased substantially from 25.8-10.9% (w/w). Dextrin can be dissolved to a very high concentration (up to 600 g/L). As the concentration of α-CD was increased with increase in dextrin concentration, factorial design approach was used to find out optimum dextrin concentration.

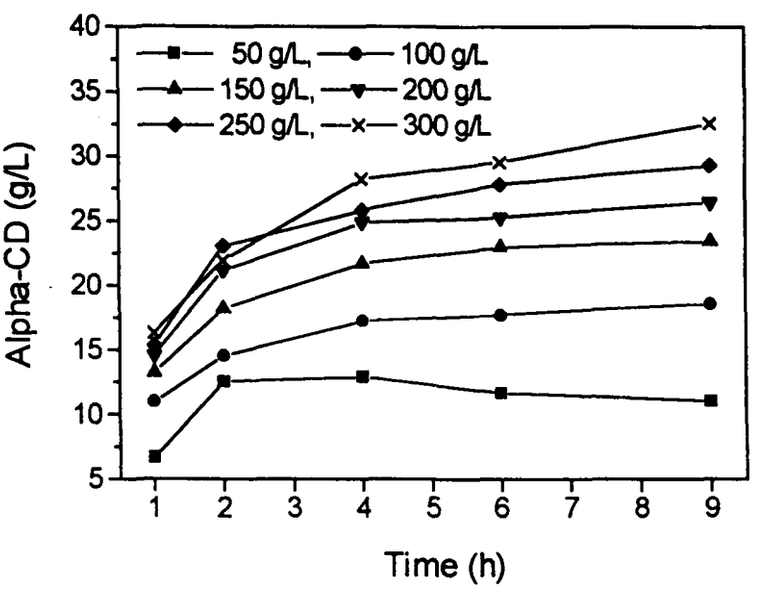


Figure 4.6 Effect of dextrin concentration on CD production in presence of 2% (v/v) n-hexanol. The dextrin solution (20 mL total volume) contains 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 2% (v/v) n-hexanol and 10 U of the enzyme per g of dextrin. The reaction was started by keeping flasks at 40 °C on a reciprocating water-bath at 200 rpm.

Optimization of α-CD production by factorial design

The production α-CD was optimized using simple two-level factorial designs in three variables, namely dextrin, n-hexanol and CGTase concentration. The standard error in final α-CD production was calculated to be 0.128 g/L using five parallel flasks containing identical concentrations of three variables, corresponding to the center of the first factorial design. The results of the first factorial design are shown in Table 4.5. The effects of n-hexanol and enzyme concentration were found out to be smaller than two times standard error. Therefore both these variables were kept constant when experiment carried out in the direction of steepest ascent (Montgomery 1991). The results of this experiment are shown in Table 4.6. As there was no increase in α-CD concentration when more than 500 g/L dextrin concentration was used, no further experiment was carried out. The maximum 12.1% (w/w) conversion was obtained and the ration of α : β : γ-CD was 91 : 3 : 6 with negligible amounts of malto-oligosaccharides formed.

Product inhibition effect

The optimization study described in the previous section showed that percentage conversion of dextrin to α-CD drastically decreased when concentration of dextrin was above 300 g/L (Table 4.6). Such reduction in the conversion can be possibly be due to inhibition of the enzyme by the products of the reaction. Hence, product inhibition of CGTase with its predominant product, α-CD, was investigated (Figure 4.7). Effect of initial concentration of α-CD on enzyme inhibition was studied by adding 0-10 g/L of α-CD using 10 g/L soluble starch and 6.5 U of the enzyme per g of starch to the reaction mixture. CGTase was found to be severely inhibited by α-CD. At an initial α-CD concentration of 4 g/L, about 50% inhibition was observed. When 10 g/L initial concentration of α-CD was used, inhibition was nearly 90%.

Bergsma et al. (1988) have reported low yield of CDs at high starch concentration due to strong but reversible inhibition of *Bacillus circulans* CGTase by β-CD. They also found that removal of β-CD from the reaction mixture by formation of an inclusion complex or by ultrafiltration increased the CD yield. Tomita et al. (1990) have studied inhibition of *Bacillus* sp. AL-6 CGTase by β- and γ-CDs in presence of 30% ethanol.

Flask No.	Dextrin (X_1)	n-Hexanol (X_2)	Enzyme (X_3)	α-CD (g/L)	Fitted 'β' values
1	–	–	–	13.4	β_0: 17.99
2	–	–	+	13.4	β_1: 9.03
3	–	+	–	13.4	β_2: -0.05
4	–	+	+	13.6	β_3: -0.04
5	+	–	–	22.5	β_{12}: -0.06
6	+	–	+	22.6	β_{13}: -0.08
7	+	+	–	22.6	β_{23}: -0.04
8	+	+	+	22.2	β_{123}: -0.19

Levels = Dextrin +: 100 g/L and -: 50 g/L, n-hexanol +: 4 (% v/v) and -: 2 (% v/v), enzyme concentration +: 20 (U/g) and -: 10 (U/g)

Table 4.5 Results of first full factorial design

Flask No.	Dextrin (g/L)	α-CD (g/L)	β-CD (g/L)	γ-CD (g/L)	Total CDs (g/L)	% conversion total-CDs (w/w)
1	100	27.0	1.3	ND	28.3	28.3
2	150	32.8	1.4	ND	34.2	22.8
3	200	39.5	1.5	ND	41.0	20.5
4	250	43.1	1.5	ND	44.6	17.8
5	300	46.7	1.6	1.8	50.1	16.7
6	350	49.3	1.7	2.4	53.4	15.2
7	400	51.8	1.8	3.2	56.8	14.2
8	450	54.7	1.8	3.5	60.0	13.3
9	500	55.0	1.8	3.6	60.4	12.1
10	550	54.8	1.8	4.0	60.6	11.0

Table 4.6 Results of experiment carried out along the direction of steepest ascent from the center of the first factorial. The concentrations of X_2 and X_3 were kept constant at the center of the first factorial design. ND = Not detected

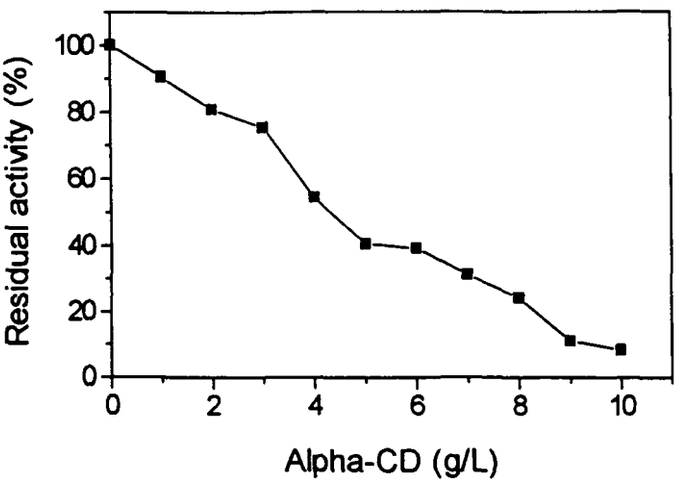


Figure 4.7 Effect of initial concentration of α-CD on the enzyme activity. To 10 g/L soluble starch solution (1 mL) containing varying concentrations of α-CD (0-10 g/L), 0.065 U of CGTase was added and incubated at 40 °C for 10 min. CDs produced were estimated by HPLC method.

They observed that at an initial concentration of 11.3 g/L of γ-CD, formation of γ-CD decreased to 90% in 6 h while that of β-CD decreased to 40%. However, addition of 11.3 g/L of β-CD at the beginning, decreased formation of β-CD to 19% and that of γ-CD to 42%. CGTase from *Bacillus* sp. BE101 was also found to be severely inhibited by its main product, β-CD (Lee and Kim 1992). A 50% inhibition was observed at 6 g/L initial concentration of β-CD and presence of complexing agent decreased the extent of inhibition effect. Kim et al. (1993) have reported 50% inhibition of *Bacillus* sp. BE101 CGTase activity from in presence of 2 g/L of α- or γ-CD. Production of each CD was severely inhibited by the addition of corresponding CD to the reaction mixture at the beginning.

Alpha-CD production in membrane bioreactor

An ultrafiltration membrane module (Figure 4.9) was used for continuous removal of CDs produced by the action of CGTase (15 U per g of dextrin) on 200 g/L dextrin concentration in presence of 3% v/v n-hexanol in a membrane bioreactor (Figure 4.8). A total of 33.24 g/L of α-CD was obtained without formation of other CDs and malto-oligosaccharides (G_1-G_7). However, under the same conditions, without using ultrafiltration membrane, 39.50 g/L of α-CD was produced with 1.5 g/L of β-CD (Table 4.6). About 15% less production in α-CD, even after continuous removal of α-CD may have obtained because of two reasons. First, the stability of the CGTase through out the run is a question resulting in low conversion. Second, adsorption of CGTase to membrane and possible lose of activity.

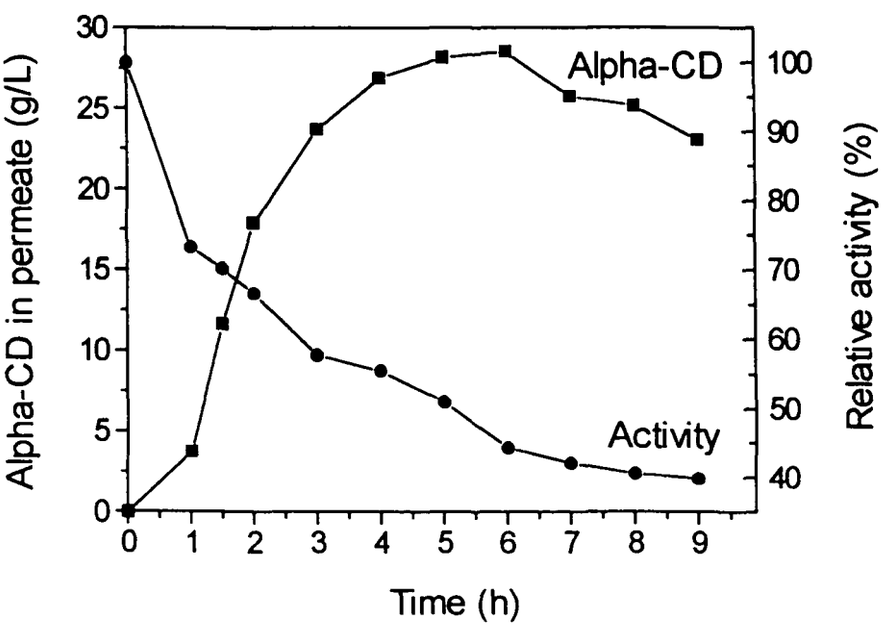


Figure 4.8 Production of α-CD and CGTase stability in ultrafiltration membrane bioreactor with 200 g/L dextrin, 20 U of enzyme per g of dextrin and 3 % (v/v) n-hexanol

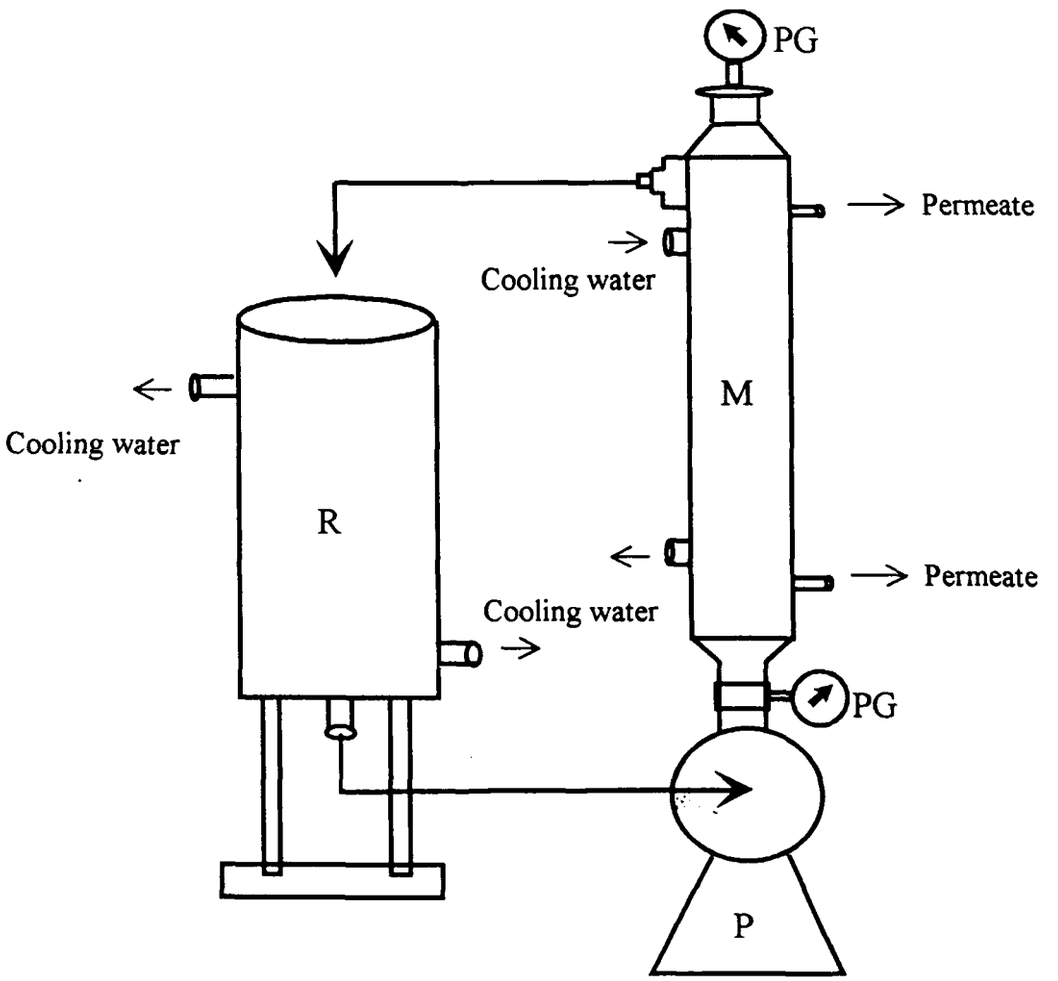


Figure 4.9 Schematic diagram of ultrafiltration membrane bioreactor. M: membrane module, R: bioreactor, P: magnetically coupled gear pump, PG: inlet and outlet pressure gauge

When samples were removed from the reactor and CGTase stability was measured, it was observed that 73% activity was obtained at first hour of circulation, which decreased to 50% at 5 h of circulation (Figure 4.8). This implies that low conversion obtained in membrane bioreactor is possibly due to reduced CGTase activity. Further studies need to be carried out on incremental addition of CGTase in membrane bioreactor operation.

Kim et al. (1993) have reported production of CDs from milled corn starch using in a ultrafiltration membrane bioreactor in batch and continuous operation. They used 70 g/L starch concentration and high enzyme concentration of 350 U per g of starch. The yield of CDs was 57% higher than that without ultrafiltration membrane in a batch operation. While there was no improvement in CD production yield in the continuous operation which required double time for production of same amount of CD as compared to conventional batch operation. They further observed that stability of the CGTase decreased exponentially for 20 h and only 30% of initial activity was maintained up to 60 h in membrane bioreactor in continuos operation and presence of starch protected enzyme to some extent.

Conclusions

Alpha-cyclodextrin production was investigated with raw starch and dextrin using *Klebsiella pneumoniae pneumoniae* AS-22 CGTase. The complexing agent, n-butanol and n-hexanol, enhanced α-CD production from raw wheat starch and dextrin, respectively. A maximum of 42.5% (w/w) conversion to total CDs was obtained with 97% selectivity to α-CD from raw wheat starch with negligible amounts of malto-oligosaccharides. Optimization of α-CD production using two-level factorial designs in three variables resulted in 12.1% (w/w) conversion to CDs with 91% selectivity to α-CD from high dextrin concentrations (500 g/L). This CGTase was strongly inhibited by its main product, α-CD. The use of ultrafiltration membrane bioreactor for production of α-CD from dextrin is presented

References

References are listed in Chapter 7

CHAPTER 5

Fermentation studies on cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae pneumoniae* AS-22

Summary

Cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae pneumoniae* AS-22 was studied in batch, fed-batch and continuous mode of operations in automated bioreactors. Effect of various fermentation parameters on cell growth and enzyme production was studied. The specific growth rate was highest at 37.5 °C, whereas the largest CGTase activity was observed at 30 °C. In a fermentation run without pH control and an initial pH of 7.0, more enzyme was produced than that in the run where pH was controlled at 7.0. At higher substrate concentration, controlled pH of 7.0 resulted in higher CGTase production. Low agitation speed was found to cause dissolved oxygen limitation resulting less cell growth and enzyme production. Experiments at different tapioca starch and dextrin concentrations indicated that optimum substrate concentration is required for maximum enzyme production. It was also found that CDs induced CGTase production in this organism. In the presence of glucose (G_1), CGTase was not produced at all. Glucose strongly repressed CGTase production even in the presence of starch or α-CD. CGTase was produced when maltotriose (G_3) was used as substrate, although at a very low level. However, in the presence of malto-oligosccharide mixture (G_4-G_{10}), the level of CGTase produced was 65% of that produced in the starch-containing medium. Comparable amount of enzyme was produced when α-CD was used as the carbon source. However, very low levels of enzyme was produced when β- or γ-CDs were used instead. High cell density (76 g/L dry cell weight) was achieved by controlled addition of a glucose-based feed. Controlled feeding of dextrin-based feed produced 1.7-fold higher enzyme level and 1.73-fold more dry cell mass than that observed in typical batch fermentations. Continuous culture studies were also performed using tapioca starch and dextrin as carbon sources. Compared to the batch culture, the volumetric enzyme productivity was substantially higher. The volumetric productivity in continuous culture was increased to 1.66-fold with tapioca starch and 3.52-fold that with dextrin.

Introduction

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) synthesizes cyclic non-reducing malto-oligosaccharides known as cyclodextrins (CDs), from starch and related carbohydrates. CDs are made up of glucose molecules linked together by α-1,4-glycosidic bonds and are commonly found to contain six (α-CD), seven (β-CD) and eight (γ-CD) glucose residues in the ring. They have a typical torus-shaped structure, with an internal hydrophobic cavity and an external hydrophillic surface. Thus, CDs can form inclusion complex with a number of guest molecules or functional groups, thereby changing their physical and chemical properties. As a result, CDs find increasing uses in pharmaceutical, chemical, agricultural, cosmetic and food industries (Bender 1986; Hedges 1992; Horikoshi 1979; Szejtli 1984).

CGTases are usually secreted out in the culture medium. However, some intracellular CGTases have been reported (Bender 1977b; DePinto and Campbell 1968; Nogardy et al., 1996a and b; Vandamme et al., 1984). In most organisms, CGTase production is growth associated and most of the enzyme is produced during the exponential phase and secreted out in the stationary phase of growth cycle.

CGTases are inducible enzymes and are produced in media containing starch or related carbohydrates, the only known exception being a CGTase produced constitutively even in presence of either glucose or xylose (Jamuna et al., 1993). Simple sugars, either monosaccharides (glucose, fructose etc.) or disaccharides (maltose, lactose, sucrose etc.) are usually not able to induce CGTase production. However, in few organisms some basal activity is reported in presence of these simple sugars (Nakamura and Horikoshi 1976a; Nogrady et al., 1995; Pocsi et al., 1998b). Recently, Pocsi et al. (1998b) have studied CGTase production in presence of glucose, maltose, maltotriose, maltoheptaose, CDs and starch as carbon sources.

The starch utilization pathway in *Klebsiella oxytoca* involves product of ten genes (*cym A* to *J*) located immediately upstream of the *cgt* gene coding for CGTase (Fiedler et al., 1996). Four of these genes code for proteins required for maltose and linear maltodextrin uptake and another one codes for a putative cytoplasmic cyclodextrinase

(CDase). CDase hydrolyses CDs and linear maltodextrins much faster than starch, but can not synthesize CDs from starch. The later characteristic distinguishes it from CGTases, which can degrade as well as synthesize CDs. According to Fiedler et al. (1996), extracellular CGTase converts starch into CDs which are taken inside the cell by a special uptake system, linearised by CDase and the malto-oligosaccharides produced are channeled into the maltodextrin-degradation route. Feederle et al. (1996) have purified and characterised cytoplasmically located CDase from *Klebsiella oxytoca* and proved that CDs transported by specific transport system inside the cell and then degraded inside the cell.

CGTase production can be increased by optimization of various fermentation parameters. There are relatively few reports of detailed fermentation studies for CGTase production (Tonkova 1998). Ramakrishna and coworkers studied CGTase production using different modes of fermentations for *Bacillus cereus* RJ-30 (Ramakrishna et al., 1994; Jamuna et al., 1993). Others have investigated CGTase production from *Bacillus circulans* (Makela et al., 1990; Vandamme et al., 1984). The fed-batch fermentation for CGTase production have been studied by Chen et al. (1994) and Park et al. (1997). Chen et al. (1994) have obtained 50% more enzyme production in fed-batch culture as compared to batch culture. Park et al. (1997) reported 31-fold increase in CGTase activity and a 29-fold increase in cell mass compared to the control batch fermentation. The continuous culture studies have been reported for some CGTase producing organisms namely *Klebsiella pneumoniae* M 5 al (Bender 1981b and 1986), *Bacillus cereus* RJ-30 (Jamuna et al., 1993) and *Bacillus macerans* (Lane and Pirt 1973). Jamuna et al. (1993) obtained maximum enzyme productivity at 1.6 h^{-1} dilution rate, which was 3.1 times higher than that in the batch culture using the same medium.

In this study, we report optimization of various fermentation parameters like temperature, pH, agitation speed and substrate concentration for CGTase production from *Klebsiella pneumoniae pneumoniae* AS-22. We have investigated possible induction of CGTase by different malto-oligosaccharides (G_1-G_{10}), CDs, dextrin and starch. High cell density cultivation with controlled addition of glucose/dextrin-based feeds and continuous culture studies in presence of tapioca starch and dextrin are also presented.

Materials and methods

Chemicals and media components

Soluble starch was purchased from E. Merck (Mumbai, India). Glucose to maltoheptaose (G_1-G_7), malto-oligosaccharide mixture (G_4-G_{10}, Product No. M 3639) and CDs as HPLC standards were purchased from Sigma (USA). Dextrin (yellow) was obtained from Anil Starch (Ahmedabad, India). Tapioca starch was obtained from Laxmi starch (Coimbature, India). All other media components, such as yeast extract, peptone, agar were from Hi-Media (Mumbai, India). All other chemicals were of analytical grade from S. D. Fine Chemicals (Boisar, India).

Strain and composition of basal medium

A novel CGTase producing strain *Klebsiella pneumoniae pneumoniae* AS-22 (ATCC 212075, NCIM 5121, Gawande and Patkar 1998) was isolated from soil samples and used in this study. Experiments to assess the effect of temperature and pH was carried out in basal medium I. In later experiments, basal medium II was used due to unavailability and high cost of media components. The composition of basal medium I was (in g/L) soluble starch 10, peptone 5, yeast extract 5, Na_2HPO_4 1.5, NaH_2PO_4 0.2, $MgSO_4$ 0.2 pH 7.0. Basal medium II consists of (in g/L) tapioca starch 10, urea 5, yeast extract 5, $NH_4H_2PO_4$ 1.5, $MgSO_4$ 0.2 pH 7.0.

CGTase assay

CGTase activity was determined using a modification of the method described by Lejuene et al. (1989). One milliliter of 1% (w/v) soluble starch prepared in 50 mM phosphate buffer, pH 7.0, was mixed with 0.1 ml of appropriately diluted enzyme and incubated at 40 °C for 10 min. The reaction was stopped by immediately cooling the tubes in chilled water, followed by addition of 0.1 ml of 1.2 N HCl. Two milliliter methyl orange solution (final concentration 0.035 mM) was then added to the reaction mixture and the tubes were maintained at 15 °C for 30 min. The absorbance of the mixture was measured at 507 nm and decrease in absorbance (with respect to a control tube without the enzyme) was correlated with the amount of α-CD formed. One unit of

enzyme is defined as the amount of enzyme that produced one μmole of α-CD per minute under standard conditions.

Estimation of protein and sugars

Total protein in the broth samples was estimated by Lowry's method (Lowry et al., 1951) using bovine serum albumin (Fraction V, Sigma) as the standard. Total carbohydrate content of samples was estimated by Anthrone method (Jermyn 1971) using glucose as the standard.

Estimation of dextrin

Residual dextrin was measured by ICUMSA method (1998) of sugar analysis as outlined below. To 1 mL of sample, 1.2 mL of 2N acetic acid, 0.25 mL of 10% (w/v) KI and 2.5 mL of 1.66 mM KIO_3 solutions were added and tubes were mixed vigorously. Absorbance of this solution was measured at 570 nm against a reagent blank. The dextrin concentration in the sample was determined using a standard calibration curve for dextrin (30-300 μg/mL).

Analysis of malto-oligosaccharides and CDs by HPLC

The concentrations of malto-oligosaccharides (G_1-G_7) and CDs in the fermentation broth were determined by HPLC (Thermo Separation Products, USA), as described by Sato et al. (1985a) under the following conditions: Column, Shodex DC-630 (Showa Denko, Japan); mobile phase, acetonitrile:water (65:35); flow rate, 1.5 mL/min; column temperature, 60 °C; refractive index detector (Waters 410, USA or Shodex RI-71, Japan). The fermentation broth was centrifuged at 20,000 *g* at 4 °C for 20 min. To 0.1 mL of cell free supernatant, 0.5 mL of acetonitrile and 0.4 mL of deionized water were added. This solution was centrifuged at 20,000 *g* for 15 min at room temperature, filtered through a 0.45 μ filter (Millipore, USA) and injected through a Rheodyne 7725 (USA) manual injector with a 20 μL loop.

Cell growth measurement

Cell growth was monitored by measurement of turbidity at 660 nm on UV-Visible spectrophotometer (Shimadzu UV-240, Japan). Each sample was diluted with NaCl (8.5 g/L) and immediately read at 660 nm. For dry cell weight (DCW) estimation, the

sample (usually 1 or 10 mL) was centrifuged at 16,000 *g* for 20-30 min., supernatant was discarded and cell pellet was kept for drying in a vaccum oven at 60 °C till constant weight. If tight cell pellet is not obtained, then it was washed with saline solution and centrifuged again.

Shake flask studies

Preinoculum was started by adding loopful of culture from a slant to 50 mL of basal medium II in 500 mL shake flask. The flasks were kept on a rotary shaker at 250 rpm for 12 h at 30 °C. This was further transferred (1mL) to 50 mL basal medium II, cultivated as above for 12 h and then used as inoculum (2% v/v) for shake flasks (500 mL capacity, Erlenmeyer, Borosil, India) containing 20 mL of appropriate medium. The carbon source in the basal medium was replaced with G_3, malto-oligosaccharide mixture (G_4-G_{10}), dextrin and tapioca starch (10 g/L each) to evaluate the extent of induction of CGTase production. Samples (1.5 mL) were removed aseptically every two hours till 14 h incubation. Each sample was analysed for cell growth, CGTase activity, presence of malto-oligosaccharides (G_1-G_7) and CDs by HPLC.

CGTase production in fermenters

CGTase production was studied in a 1 L bioreactor (working volume 700 mL, magnetically driven bottom stirrer, Gallenkamp, U. K.) or a 14 L bioreactor (working volume 10 L, New Brunswick Scientific, USA). The New Brunswick bioreactor was equipped with three six-bladed Rushton-disk impellers fitted on a top-driven shaft. The inoculum was grown in basal medium I or II as described above and added 5% (v/v) to the fermenter. UCN (Hico Products Ltd., India) was used as antifoam. Antifoam was added at a concentration of 0.2 ppm in the fermenter medium. A 10% (v/v) solution of antifoam in water was sterilized and added dropwise whenever excess foaming occurred. Samples were withdrawn from the fermenters using a sampling device as described by Jogdand and Karanth (1983). The sample size for 1 L and 14 L fermenters was 15 and 25 mL, respectively.

Batch experiments

The effect of temperature on CGTase production was studied by growing AS-22 in basal medium I (pH 7.0) at temperatures ranging from 25 to 40 °C in a 1 L Gallenkamp (UK) fermenter. The effect of pH on enzyme production was investigated at different controlled pH's of 5.5 to 7.0 (pH controlled with 5 N NaOH or HCl) and at an uncontrolled initial pH of 7.0 in 1 L Gallenkamp (UK) fermenter in basal medium I (temperature 30 °C). The substrate-dependent effect of pH was studied at 10 and 20 g/L tapioca starch concentrations at a controlled pH of 7.0 and uncontrolled initial pH of 7.0 in 14 L fermenter in basal medium II. Effect of agitation speed was also determined in a 14 L fermenter by running fermentations in basal medium II at 400-600 rpm. The effect of tapioca starch concentration on enzyme production was determined in 1 L and 14 L fermenters, whereas that of dextrin concentration was studied in a 1 L fermenter. Induction of CGTase production with glucose, maltose, tapioca starch or CDs was investigated in 1 L or 14 L fermenter. The cell growth and CGTase production profile was studied with dextrin medium containing (in g/L) dextrin 49.3, urea 6.2, yeast extract 18.3, $NH_4H_2PO_4$ 6.7 and $MgSO_4$ 0.5, pH 7.0 in a 1 L fermenter. Cell growth and CGTase activity were determined for all the experiments. For the induction experiments, the concentrations of malto-oligosaccharide (G_1-G_7) and CDs in the fermentation medium were analysed by HPLC.

Fed-batch experiments

The fed-batch experiments to achieve high cell densities were performed in 14 L automated fermenter (New Brunswick Scientific, USA). Two different carbon sources were used in these experiments.

The batch run was started with 6 L medium containing (in g/L) tapioca starch 20, urea 5, yeast extract 5, $NH_4H_2PO_4$ 1.5 and $MgSO_4$ 0.2, pH 7.0. Two different feeds were used: 2 L of glucose feed (1) and 2 L of starch feed (2). The composition of feed 1 was (in g/L) glucose 400, urea 40, yeast extract 40, $NH_4H_2PO_4$ 6, $MgSO_4$ 0.8, pH 7.0 and composition of feed 2 was (in g/L) tapioca starch 75, urea 5, yeast extract 5, $NH_4H_2PO_4$ 1.5 and $MgSO_4$ 0.2, pH 7.0. After 6.5 h of batch cultivation, feed 1 was started. The feed rate

was controlled using a feed-back control based on dissolved oxygen (DO), which was maintained between 0-25%. The addition of feed 1 was complete at 52 h, after which feed 2 was started. The addition of feed 2 was complete at 69 h. The fermentation was continued further till DO reached almost 100%.

The batch run was started with 6 L of medium containing (in g/L) dextrin 49.3, urea 6.2, yeast extract 18.3, $NH_4H_2PO_4$ 6.7 and $MgSO_4$ 0.5, pH 7.0. The composition of feed (4 L) was (in g/L) dextrin 172.5, urea 26.5, yeast extract 18.3, $NH_4H_2PO_4$ 6.7, $MgSO_4$ 0.5, pH 7.0, which is 2X (final concentration) of the optimum medium with respect to carbon and nitrogen sources. Feeding was started at 7 h. The DO-based feeding strategy was same as described above. Additional pure oxygen was supplied at the rate of 0.5 L/min when DO dropped to 40% (2 h) during the entire feeding period. Feed addition was stopped at 30 h, because absorbance at 660 nm started decreasing, indicating stoppage of cell growth and possible cell lysis. Fermentation was continued till DO reached 100%.

Continuous culture experiments

Continuous culture experiments were performed with media based on tapioca starch or dextrin in a 1 L fermenter. The composition of the tapioca starch medium was (in g/L), tapioca starch 20, urea 5, yeast extract 5, $NH_4H_2PO_4$ 1.5, $MgSO_4$ 0.2, pH 7.0. The optimized dextrin medium was described above. AS-22 was grown in batch mode for 10 h in starch-based medium (for 14 h in dextrin medium) and feed was started. The fermenter vessel was fitted with a side arm for liquid to overflow and fermenter volume could be maintained at 700 mL (Figure 5.27). The dilution rate was varied from 0.1 to 0.6 h^{-1} with a calibrated peristaltic pump. At each dilution rate, the feed was added for at least five reactor volumes to ensure steady state. Samples were withdrawn at specific time intervals and analyzed for cell growth and CGTase activity as described earlier. Total sugar or residual dextrin and total proteins were also determined at steady state as described earlier.

Results and discussion

Effect of temperature

AS-22 was cultivated in basal medium (I) in a 1 L bioreactor at various temperatures ranging from 25 to 40 °C at 2.5 °C intervals (Figure 5.1). The maximum specific growth rate (μ_{max}) was almost constant in the temperature range of 25-30 °C. However, it increased rapidly at temperatures above 30 °C and was highest (2.12 h^{-1} μ) at 37.5 °C. This corresponds to a doubling time of about 20 min, comparable to that of *Escherichia coli* cultures in rich medium. Such high cell growth rate, leading to short fermentation times, is a commercially desirable characteristic. CGTase production, on the other hand, was optimum at 30 °C. At 40 °C specific growth rate decreased abruptly to 0.49 h^{-1} and enzyme production was very slow. AS-22 is a mesophillic organism, which produces maximum CGTase at 30 °C. Although the growth is maximum at 37.5 °C, the enzyme production at this temperature is only 20% of that produced at 30 °C. This CGTase is relatively unstable at high temperatures and this could be possible reason for lower enzyme production observed at higher temperatures. Further experiments were carried out at 30 °C.

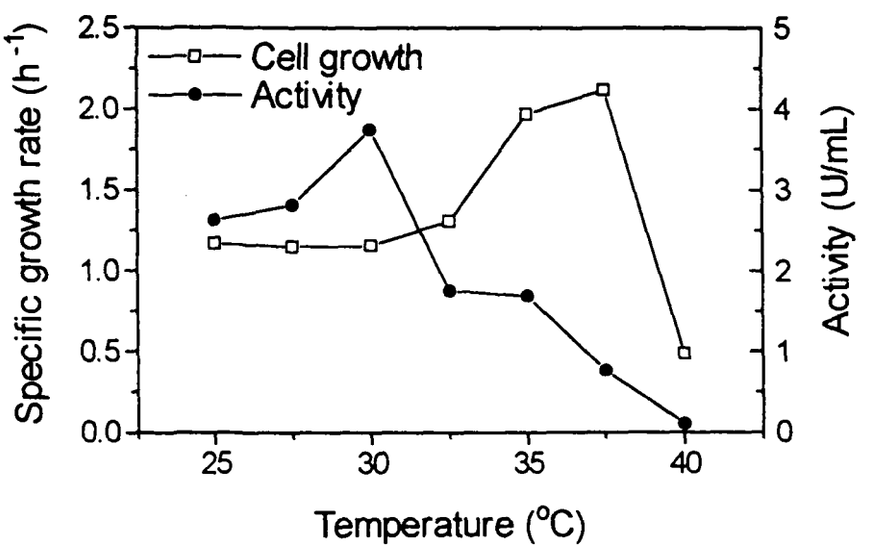


Figure 5.1 Effect of temperature on cell growth and enzyme production in basal medium I in a 1 L bioreactor

The optimum temperature for CGTase production varies greatly for different microorganisms. For mesophillic organisms the optimum lies between 30-40 °C, whereas for thermophillic organisms it is between 50-60 °C. *Bacillus cereus* RJ-30 grows at a maximum specific growth rate of 0.88 h^{-1} in a glucose-based medium (Jamuna et al., 1993). Makela et al. (1990) have studied CGTase production from *Bacillus circulans* (ATCC 21783) at temperatures 30-42 °C and observed maximum CGTase production at 37 °C. Yan and Lin (1993) studied CGTase production for alkalophillic *Bacillus* sp. No. 562 at 20 to 65 °C and found out that both cell growth and enzyme production were maximum at 37 °C. *Klebsiella pneumoniae* M 5 al is cultivated at 28 °C while *Klebsiella oxytoca* 19-1 is grown at 37 °C. However, optimum temperatures for maximum enzyme production for these organisms have not been reported.

Effect of pH

Cell growth and CGTase production were studied in basal medium (I) at different controlled pH values from 5.5 to 7.0 (Figure 5.2). Specific growth rate was almost the same as that observed when AS-22 was grown at a pH of 6.0-7.0, whereas CGTase production was optimum at pH of 6.0. Only 15% activity was observed at pH of 5.5 as compared to that at pH of 6.0. When AS-22 was cultivated in basal medium I at controlled and uncontrolled initial pH of 7.0, cell growth and enzyme production were higher with uncontrolled pH 7.0 (Figure 5.3).

To evaluate the effect of pH control, in media containing different substrate concentrations, fermentations were carried out at controlled and uncontrolled initial pH 7.0 with basal medium II containing 10 or 20 g/L tapioca starch (Figures 5.4-5.7). At higher starch concentration, controlled pH of 7.0 resulted in better enzyme production than uncontrolled initial pH 7.0. The reason for this type of behaviour is that high substrate concentration results in higher acid production indicated by lower pH. When 10 g/L starch is used, pH goes down to 5.4 but increases rapidly (Figure 5.5). However, at 20 g/L starch concentration (Figure 5.7), pH goes down to 4.2 and remains there for nearly 2 h and then increases slowly. This indicates that fermentation at high starch concentration produces more acid, thus decreasing the growth rate and enzyme production. Further studies were performed at a controlled pH of 7.0.

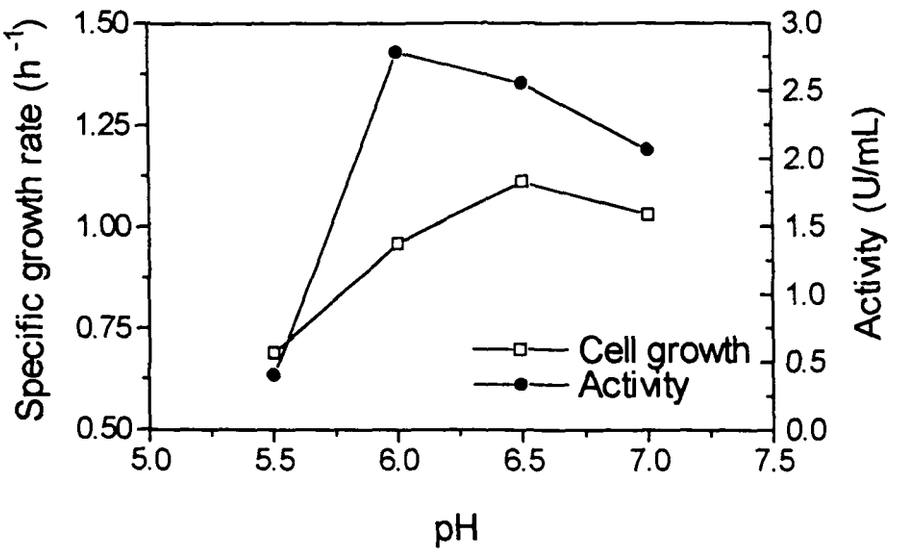


Figure 5.2 Cell growth and enzyme production at different controlled pH's in basal medium I at 30 °C in 1 L (or 14 L) bioreactor

For alkalophillic bacteria, the pH is generally kept between 9-10 by adding Na_2CO_3 in the medium (Nakamura and Horikoshi 1976a; Makela et al., 1990; Yan and Lin 1993). The specific growth rate of alkalophillic *Bacillus firmus* was nearly constant between pH 7.5-9.0, whereas CGTase production was maximum at pH 8.5 (Goel 1995). Moreover, pH control did not significantly affect enzyme production; experiments with medium containing 10 g/L Na_2CO_3 resulted in same enzyme activity as that observed in experiments at controlled pH at 9.0. Makela et al. (1990) have also studied growth of *Bacillus circulans* in the presence of different concentrations of Na_2CO_3 and observed no effect on enzyme production in the range of 10-15 g/L Na_2CO_3.

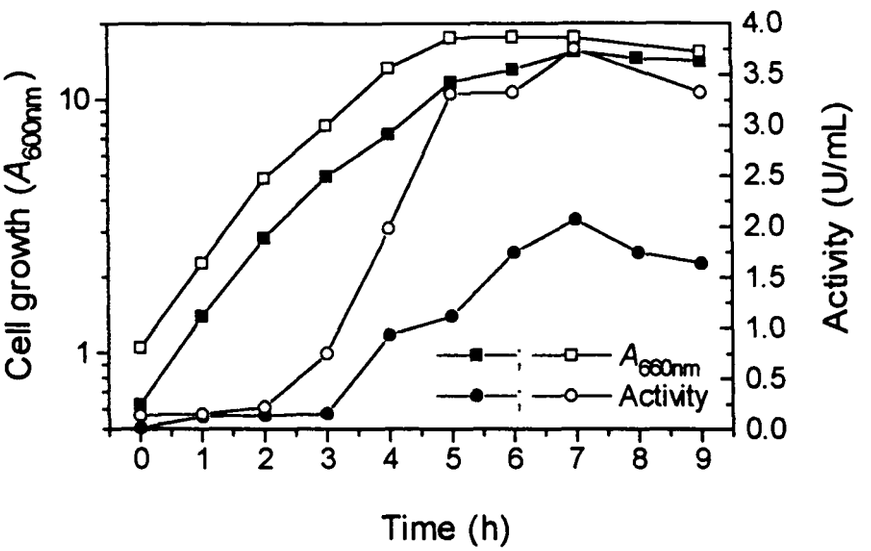


Figure 5.3 Cell growth and enzyme production at controlled and uncontrolled pH of 7.0 in basal medium I in a 1 L bioreactor. The open symbols represent fermentation carried out at uncontrolled pH whereas closed symbols represent fermentation carried out at a controlled pH.

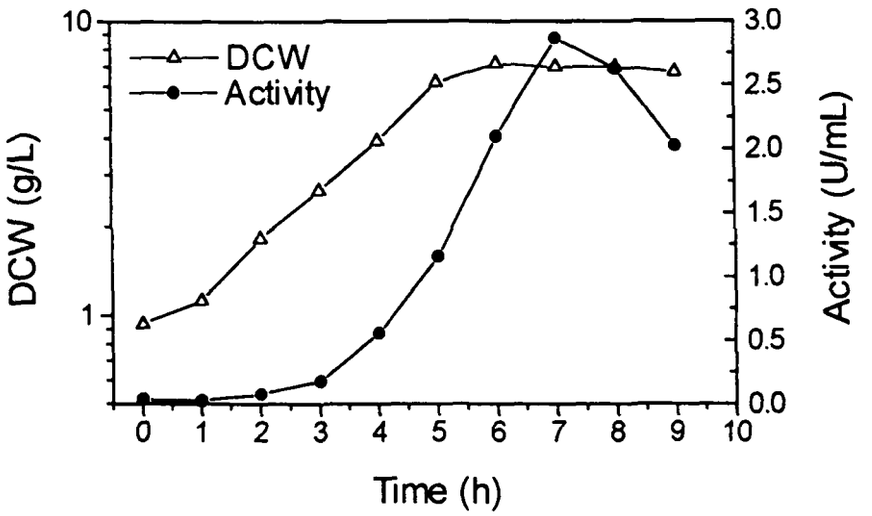


Figure 5.4 Cell growth and enzyme production in basal medium II at controlled initial pH of 7.0 in a 14 L bioreactor

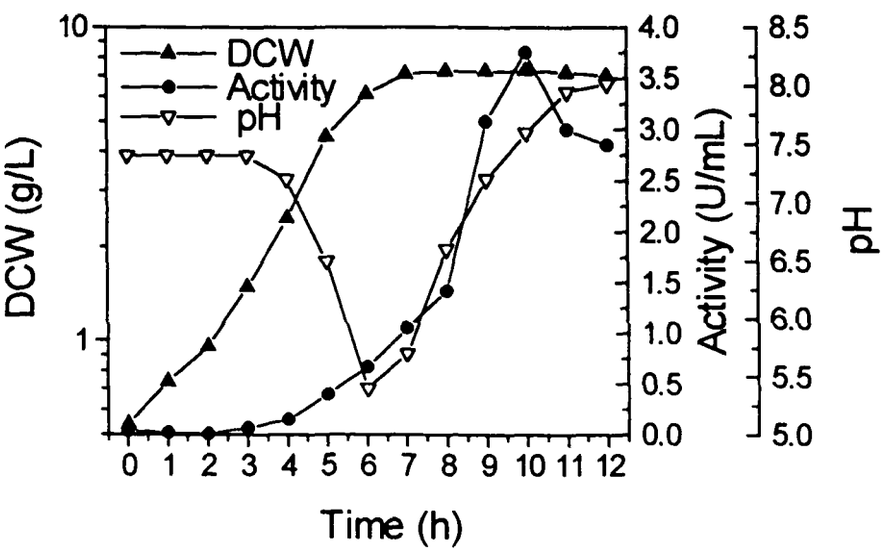


Figure 5.5 Cell growth and enzyme production in basal medium II at uncontrolled initial pH of 7.0 in a 14 L bioreactor

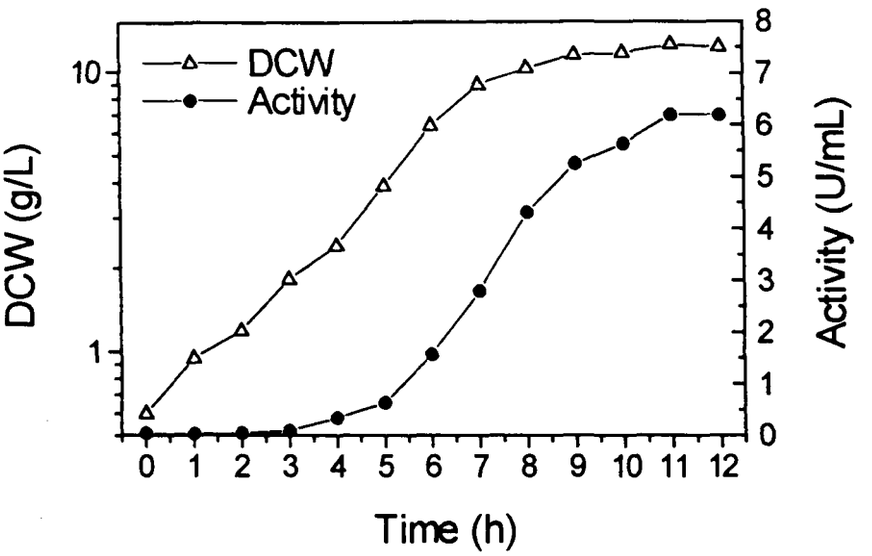


Figure 5.6 Cell growth and enzyme production with 20 g/L tapioca starch in basal medium II at controlled initial pH of 7.0 in a 14 L bioreactor

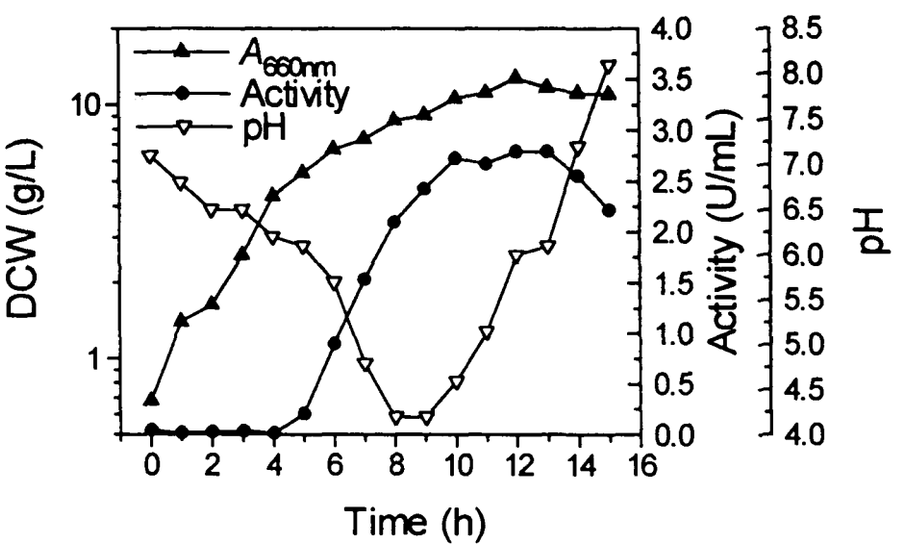


Figure 5.7 Cell growth and enzyme production with 20 g/L tapioca starch in basal medium II at uncontrolled initial pH of 7.0 in a 14 L bioreactor

Effect of agitation speed

AS-22 was cultivated in basal medium II at different agitation speeds from 400-600 rpm in 14 L fermenters (Figures 5.8-5.10). At 400 rpm, dissolved oxygen (DO) concentration decreased below 20% of saturation at 4 h, remained at zero for two hours and started rising after 8 h incubation period. Thus, at low agitation rate, there is a severe oxygen limitation. The maximum specific growth rate was 0.59 h^{-1} at 400 rpm as compared to 0.71 h^{-1} at 500 rpm (Table 5.1). There was a marginal further increase in specific growth rate at 600 rpm and no improvement in enzyme activity. The DO concentration profiles at 500 and 600 rpm were almost similar, reaching a minimum of 26% at 6 h in the 500 rpm run and that of 44% at 6 h in the 600 rpm run. The enzyme production in the 400 rpm was 82% of that at 500 and 600 rpm runs. At 400 rpm, low cell yield was obtained as compared to that at 500 and 600 rpm runs (Table 5.1). The oxygen limiting conditions may have resulted in incomplete utilization of the substrate or formation of side products. The agitation speed of 500 rpm was used in further experiments.

DO concentration is an important fermentation parameter, which can affect cellular metabolism and growth (Harrison 1972). Goel (1995) has studied CGTase production in *Bacillus firmus* fermentations at different agitation speeds. He observed severe DO limitation at 400 rpm resulting in less cell growth and enzyme production as compared to the values at 500 and 600 rpm. Jamuna et al. (1993) have also reported CGTase production from *Bacillus cereus* RJ-30 at different agitation speeds ranging from 300 to 700 rpm with starch as the carbon source. They reported increase in specific growth rate from 0.29 to 0.36 h^{-1} with increase in agitation speed. However, maximum enzyme production was observed at 500 rpm.

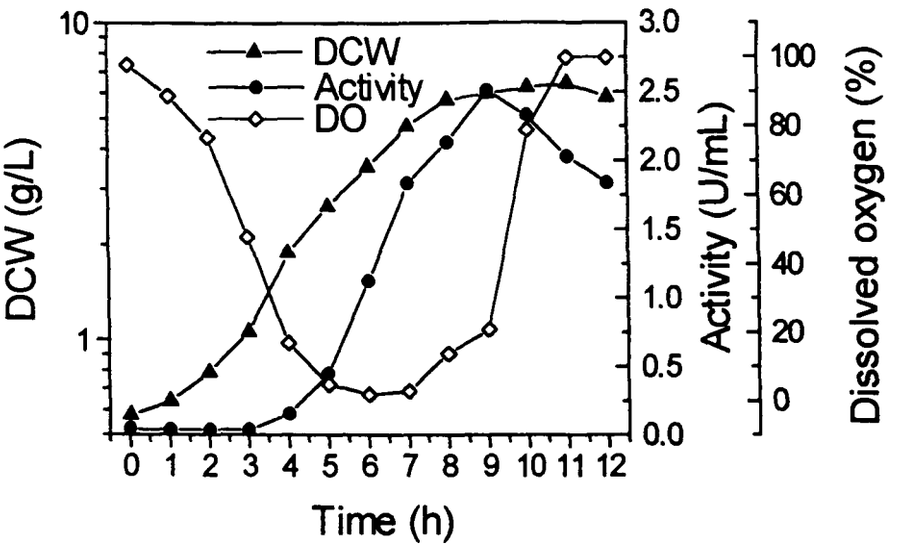


Figure 5.8 Cell growth and enzyme production at agitation speed of 400 rpm

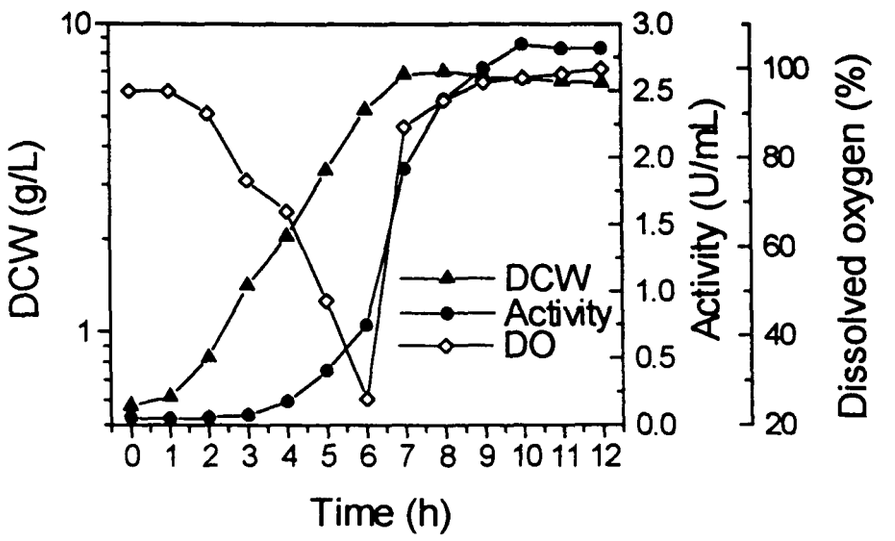


Figure 5.9 Cell growth and enzyme production at agitation speed of 500 rpm

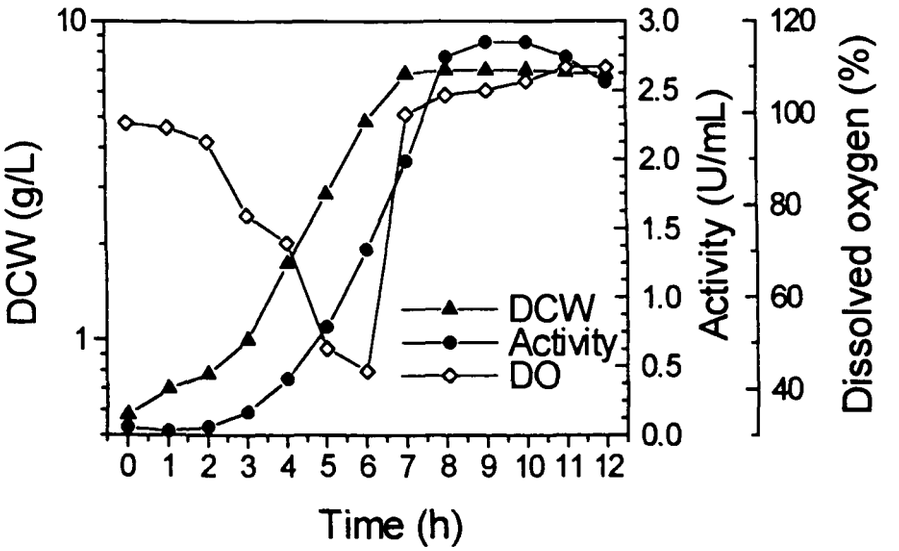


Figure 5.10 Cell growth and CGTase production at agitation speed of 600 rpm

RPM	Cell mass X_{max} (g/L)	Activity P_{max} (U/mL)	Specific growth rate μ_{max} (h^{-1})	Cell yield X_{max} / Total substrate (g/g)	Specific enzyme productivity P_{max} / X_{max} (U/g)
400	6.44	2.33	0.59	0.64	361.8
500	7.03	2.85	0.71	0.70	405.4
600	7.04	2.85	0.74	0.70	404.8

Table 5.1 Cell mass and CGTase production yields in various rpm runs in batch culture

Effect of substrate concentration

Inducer concentration plays a very important role in enzyme production. The effect of concentrations of tapioca starch and dextrin on cell growth and enzyme production was studied in 1 L and 14 L fermenters in basal medium II. The tapioca starch concentration was varied from 10 to 30 g/L, whereas dextrin concentration was varied from 10 to 50 g/L (Table 5.2). In 1 L fermenter, the specific growth rate decreased with an increase in tapioca starch concentration. However, in 14 L fermenter, the specific growth rate was the same at 10 and 20 g/L tapioca starch concentrations and decreased at 30 g/L concentration. The enzyme production increased two-fold when tapioca starch concentration was increased from 10 to 20 g/L in both the fermenters. However, at a higher starch concentration of 30 g/L, less enzyme was produced. Cell mass increased with increase in tapioca starch concentration, although maximum enzyme activity was obtained with 20 g/L tapioca starch.

When dextrin was used as the carbon source, specific growth rate decreased with increase in dextrin concentration. Total cell mass increased with increase in dextrin concentration up to 40 g/L and remained almost constant at higher dextrin concentrations. With increase in dextrin concentration, the enzyme production initially increased, reached a maximum and subsequently decreased. The maximum enzyme activity was obtained with 40 g/L dextrin.

These results indicate that above certain concentration, if other nutrients are kept limiting, substrate inhibition occurred. Effect of substrate concentration on enzyme production has not been studied well in literature. Chen et al. (1994) have also observed substrate inhibition at higher than 20-30 g/L soluble starch concentration for alkalophilllic *Bacillus* sp. (ATCC 31007). We have optimized concentrations of different media components in shake flask experiments using statistical experimental design approach. This optimum medium composition was then used in fermenters, leading to similar increase in CGTase production compared to that in basal medium. The components in the optimized medium were as follows: carbon source – dextrin (E. Merck, India), nitrogen source – peptone, yeast extract as additional nutrient source, phosphorus source – $NH_4H_2PO_4$ and mineral source – $MgSO_4$. The carbon source from

this medium was replaced with dextrin from another manufacturer (Anil Starch, India) because of unavailability. The nitrogen source (peptone) was also replaced by urea, a cheap chemical, because AS-22 can use urea as a nitrogen source and produces high CGTase levels (Table 3.3). When AS-22 was grown in this substituted optimum medium, the enzyme production was 32.8 U/mL as compared to 32.5 U/mL with earlier medium (Figure 5.11). The CGTase production was observed in two phases. In the first phase, the enzyme was produced in along with cell growth. As the cell growth slowed down at 8 h, CGTase production stopped. However, when cell growth stopped completely at 10 h, CGTase production resumed. More than half the enzyme was produced in the stationary phase. As this medium resulted in good cell growth and enzyme activity, it was further used in fed-batch and continuous culture studies to achieve high cell and enzyme productivity.

Substrate (g/L)		Cell mass X_{max} (g/L)	Activity P_{max} (U/mL)	Specific growth rate μ_{max} (h^{-1})	Cell yield X_{max} / Total substrate (g/g)	Specific enzyme productivity P_{max} / X_{max} (U/g)
Tapioca starch in 1 L fermenter	10	7.17	2.67	0.57	0.72	372.38
	20	15.76	6.77	0.45	0.78	429.56
	30	19.22	2.93	0.34	0.64	152.44
Tapioca starch in 14 L fermenter	10	7.03	2.87	0.61	0.70	408.25
	20	12.39	6.19	0.63	0.62	499.59
	30	18.20	2.78	0.54	0.60	152.74
Dextrin in 1 L fermenter	10	5.20	4.59	0.57	0.52	882.69
	20	9.10	8.65	0.41	0.45	1067.90
	30	13.49	13.72	0.36	0.45	1017.04
	40	19.41	16.86	0.31	0.48	868.62
	50	19.26	15.02	0.31	0.38	779.85

Table 5.2 Effect of carbon source concentration on cell mass and enzyme production in 1 L and 14 L fermenters

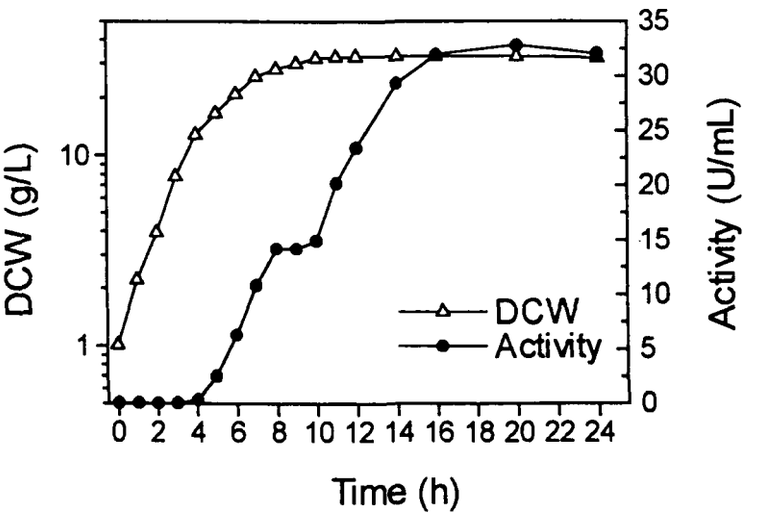


Figure 5.11 CGTase production with a substituted medium in 1 L bioreactor

Effect of carbon sources on CGTase induction

Production of most CGTases requires the presence of starch (or a component of starch) in the medium. However, the exact identity of this inducer is unknown. To investigate CGTase induction in more detail, different malto-oligosaccharides (G_1 to G_{10}), CDs, dextrin and starch were tested for their effectiveness in inducing CGTase production (Figures 5.12-5.18, 5.24). CGTase production was also studied in presence of glucose/ maltose with starch/α-CD (Figures 5.19-5.23). AS-22 was cultivated in basal medium II by replacing tapioca starch with these carbon sources (10 g/L). Samples were analyzed for residual substrate and conversion products. The specific growth rate and maximum enzyme activity obtained in all the carbon sources tested is shown in Table 5.3.

Fermentation using glucose-based medium

AS-22 efficiently uses glucose as a carbon source, resulting in the highest specific growth rate of 0.55 h^{-1} (Figure 5.12). Further, no other sugars were detected in the culture broth at any time. The enzyme production was totally repressed in the presence of glucose.

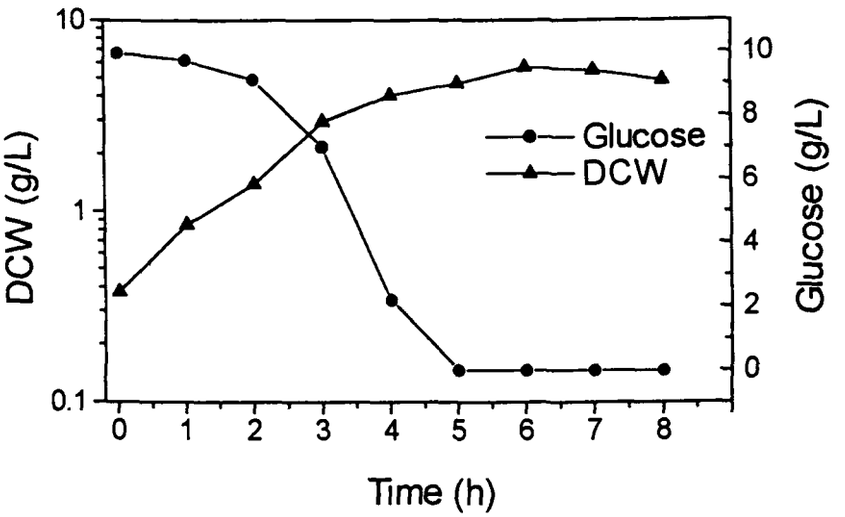


Figure 5.12 Cell growth and CGTase production in 1 L fermenter in basal medium II with the carbon source replaced by glucose (10 g/L). CGTase activity was not detected in any sample.

Fermentation using maltose-based medium

The utilization of maltose was slower than that of glucose (Figure 5.13). Moreover, a diauxic growth pattern was observed. When broth samples were analysed by HPLC, it was observed that G_4 (2-3 g/L) was present which decreased along with decrease in maltose (results not shown). This indicates that G_2 was probably converted to G_4 by coupling of two G_2 molecules by basal CGTase activity. Only 0.17 U/mL activity was detected.

Fermentation using maltotriose-based medium

With maltotriose as the carbon source, the specific growth rate was only 0.33 h^{-1}, indicating that it is utilized even slower than maltose (Table 5.3). However, CGTase activity was two-fold higher than observed with maltose-based medium (Figure 5.14). When culture broth was analysed for presence of sugars, G_2 and G_4 were detected. This again indicates that these are probably produced by disproportionation reaction of CGTase.

Substrate (g/L)	Maximum specific growth rate (μ_{max})	Maximum activity (P_{max})
Glucose (10)	0.55	ND
Maltose (10)	0.39	0.17
Maltotriose (10)	0.33	0.34
Malto-oligosaccharides (10)	0.33	1.99
Dextrin (10)	0.51	4.59
Starch (10)	0.38	2.87
α-CD (10)	0.48	2.95
β-CD (10)	0.36	2.62
γ-CD (10)	0.39	1.50
Glucose (10) and starch (10)	0.34	1.99
Maltose (10) and starch (10)	0.45	1.39
Glucose (10) and α-CD (10)	0.51	0.13
Maltose (10) and α-CD (10)	0.47	3.46
Starch (20)	0.39	6.19

ND = Not detected

Table 5.3 Effect of various carbon sources on cell growth and enzyme production

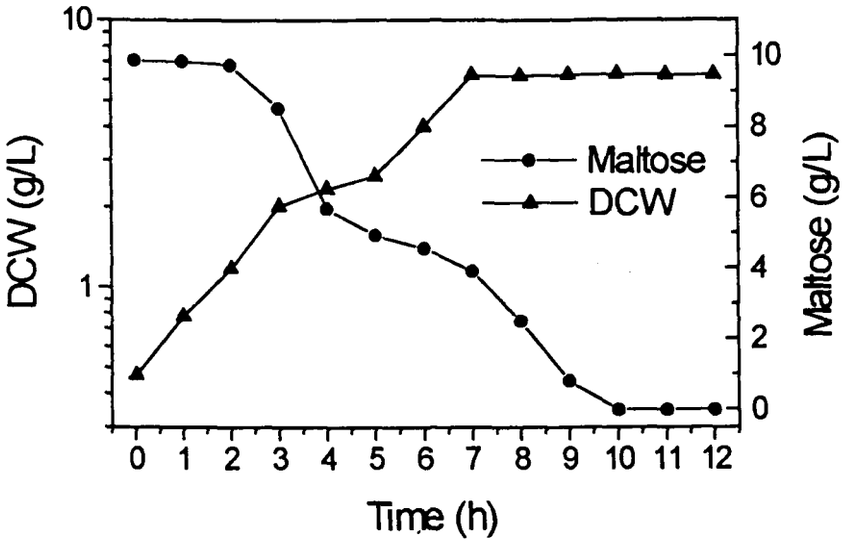


Figure 5.13 Cell growth and CGTase production in 1 L fermenter in basal medium II with the carbon source replaced by maltose (10 g/L).

Fermentation using medium with malto-oligosaccharide mixture (G_4-G_{10})

AS-22 was cultivated in basal medium II containing malto-oligosaccharide mixture (G_4 to G_{10}) as the carbon source (Figure 5.14). In the sample immediately after inoculation, 0.71 g/L α-CD was detected. It was probably formed by cyclization reaction of CGTase carried over from the inoculum. The concentration of α-CD reached a maximum of 0.82 g/L in 4 h and then decreased abruptly (results not shown). The maximum CGTase activity was 65% of that obtained with starch-based medium.

Fermentation using dextrin-based medium

AS-22 grows very well on dextrin with a specific growth rate of 0.51 h^{-1} (Figures 5.14 - 5.15). The enzyme production was also the highest (4.59 U/mL) among all the carbon sources tested. When culture broth was analysed by HPLC, 2.49 g/L α-CD concentration was detected at the sample immediately after inoculation. The concentration of α-CD

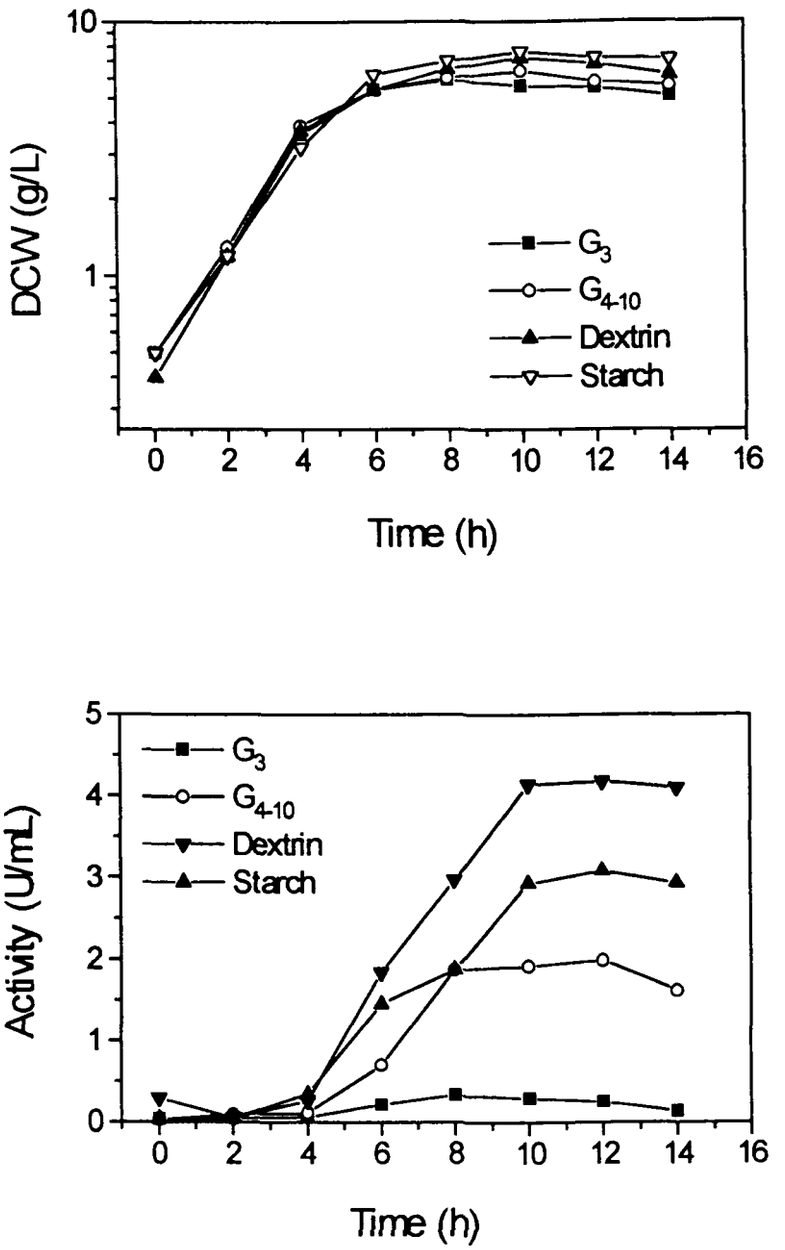


Figure 5.14 Cell growth and CGTase production in shake flasks in basal medium II with the carbon source replaced by G_3 or G_{4-10} or starch (10 g/L).

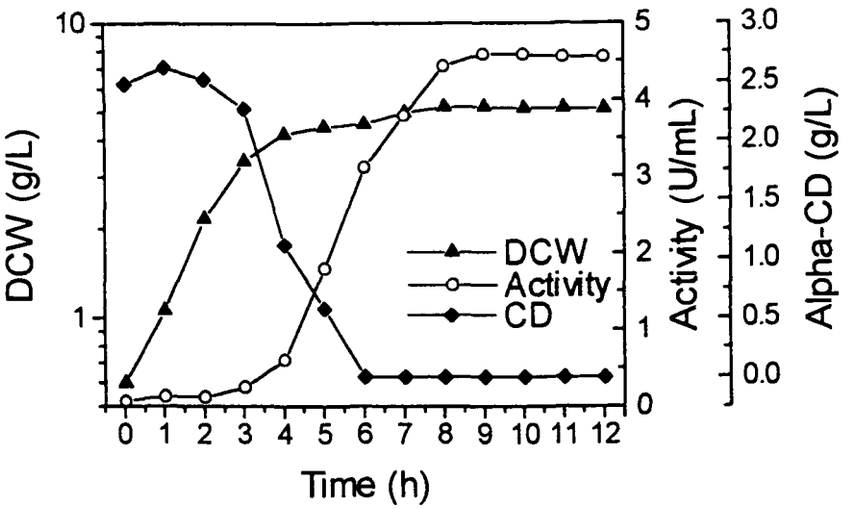


Figure 5.15 Cell growth and CGTase production in 1 L fermenter in basal medium II with the carbon source replaced by dextrin (10 g/L).

reached a maximum within 1 h and started decreasing afterwards. It appears that components of dextrin are consumed preferentially, followed by α-CD consumption/conversion. Among the malto-oligosaccharides, only G_4 was detected in all the samples (HPLC standards available were G_1-G_7 and the three CDs).

Fermentation using starch-based medium

The specific growth rate was 0.38 h^{-1} when tapioca starch was used as the carbon source (Figure 5.16, 5.24). The sample immediately after inoculation contained 3.3 g/L α-CD and 1.58 g/L G_6. The concentration of α-CD decreased with time and no α-CD was detected after 5 h incubation. However, concentration of G_6 reached a maximum (1.76 g/L) in 3 h and then decreased abruptly.

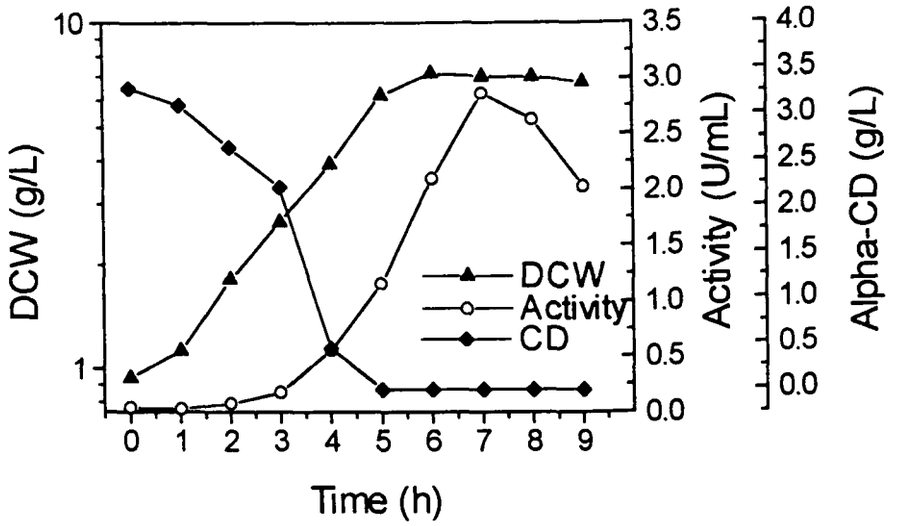


Figure 5.16 Cell growth and CGTase production in 1 L fermenter in basal medium II.

Fermentation using α-CD-based medium

As compared to starch-based medium, the specific growth rate as well as enzyme activity was higher in the α-CD based medium (Figure 5.17). Interestingly, when only α-CD was used as the carbon source, almost no malto-oligosaccharides were detected in the medium. However, α-CD concentration decreased with time. This clearly indicates that α-CD is transported inside the cell as such without breaking it into simpler sugars.

Fermentation using β- or γ-CD-based medium

The specific growth rates with β- and γ-CD-based media were lower than that with α-CD-containing medium (Figure 5.18, 5.19). The media with β-CD resulted in better enzyme production than that with γ-CD.

There are interesting differences in the pattern of uptake of the three CDs. In the experiment with α-CD, the CD was consumed right from the beginning at a rapid rate. CGTase production was negligible till α-CD was present in the medium. When it was almost completely consumed, rapid CGTase production started. In this phase the cell

growth was probably supported by carbon from other components from the medium such as urea and yeast extract. With β-CD as the carbon source, there was very slow degradation/uptake of this CD till 6 h, after which the CD was rapidly degraded/transported. Thus, in this case, the other carbon sources were preferred to β-CD. Suprisingly, the pattern of CGTase production was almost the same. In the fermentation with γ-CD as the carbon source, the degradation/uptake of this CD was quite slow throughout the fermentation. The cell growth was also appreciable slower.

Beta-CD is also probably taken as such inside the cell. Small amounts of α-CD as well as G_6 and G_7 were detected in the fermentation with γ-CD-based medium indicating that γ-CD is (at least partially) disproportionated by CGTase and then used.

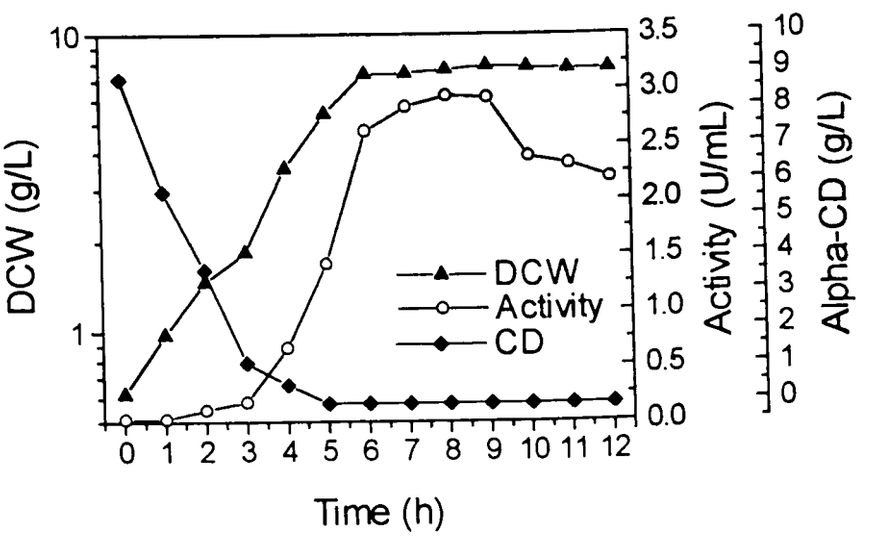


Figure 5.17 Cell growth and CGTase production in 1 L fermenter in basal medium II with the carbon source replaced by α-CD (10 g/L).

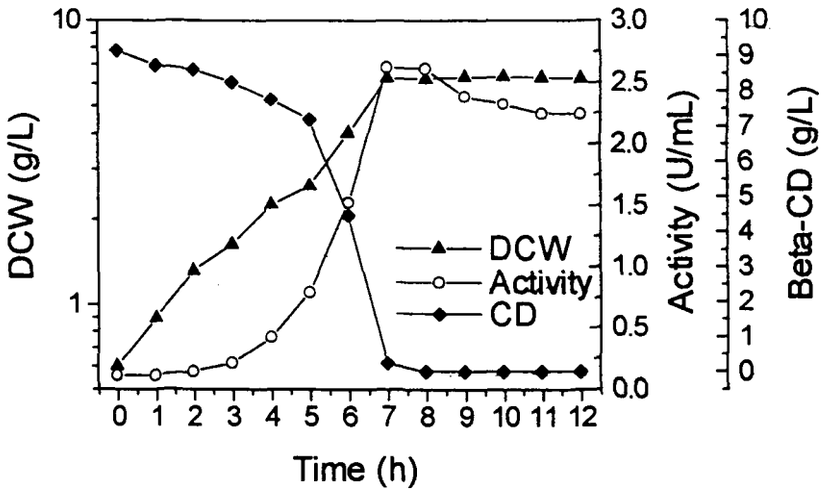


Figure 5.18 Cell growth and CGTase production in 1 L fermenter in basal medium II with the carbon source replaced by β-CD (10 g/L).

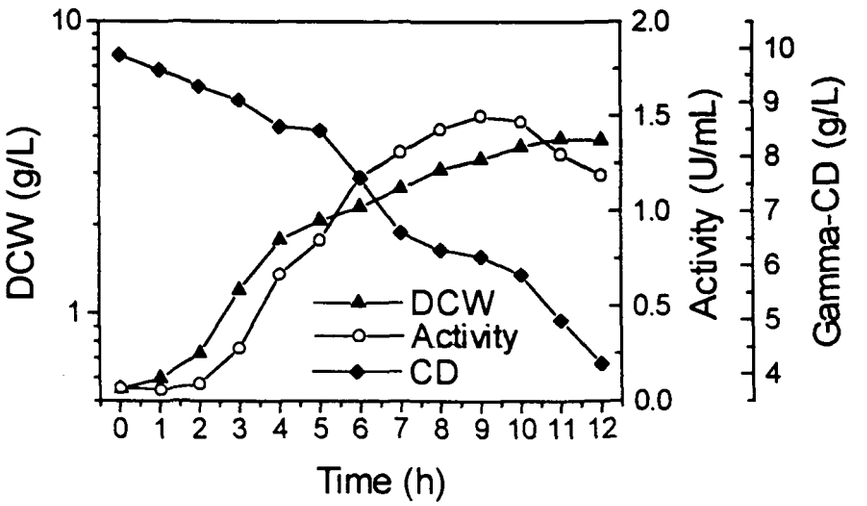


Figure 5.19 Cell growth and CGTase production in 1 L fermenter in basal medium II with the carbon source replaced by γ-CD (10 g/L).

Fermentation using glucose and tapioca starch containing medium

When glucose was added in presence of starch, only small amount of α-CD (0.79 g/L) was synthesized (Figure 5.20). Alpha-CD concentration increased only after glucose was exhausted from the medium. A typical diauxic growth was obtained, with glucose being the preferred carbon source. Moreover, enzyme was induced only in the second phase of growth. Detailed time-profiles of individual oligosaccharides are shown in Table 5.4. Enzyme activity was started appearing only after 8 h incubation. These results indicate that α-CD is the final inducer of CGTase production in this organism.

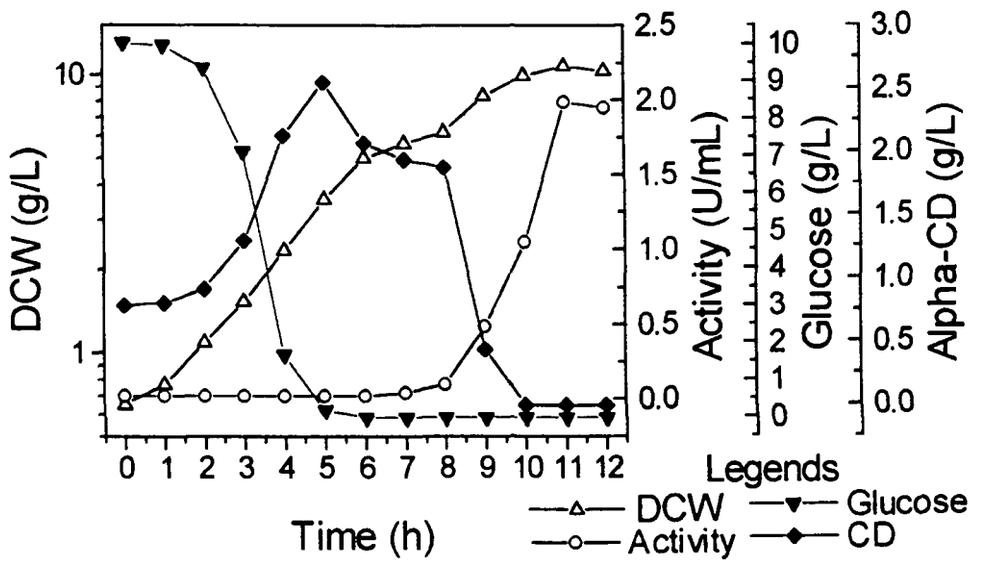


Figure 5.20 Cell growth and CGTase production in 1 L fermenter in basal medium II with the additional glucose (10 g/L) carbon source.

Fermentation using glucose and α-CD containing medium

Surprising results were obtained when AS-22 was cultivated in presence of glucose and α-CD (Table 5.4). When glucose is added in presence of α-CD as carbon source, almost all α-CD was converted into malto-oligosaccharides right at the beginning of the fermentation. Glucose probably serves as an acceptor molecule and α-CD as the donor in coupling reaction catalysed by CGTase. As we know that specific coupling and

Time (h)	G_1	G_2	G_3	G_5	G_6	G_7	α-CD
0	10.00	0.59	0.34	ND	ND	ND	0.79
1	9.97	0.72	0.35	0.4	0.53	1.48	0.81
2	9.36	0.81	0.37	0.39	0.44	1.49	0.92
3	7.14	1.04	0.27	0.29	0.34	1.23	1.30
4	1.68	2.91	0.02	0.02	0.07	0.38	2.13
5	0.2	2.72	ND	ND	ND	ND	2.55
6	ND	1.4	ND	ND	ND	ND	2.07
7	ND	1.2	ND	ND	ND	ND	1.94
8	ND	0.45	ND	ND	ND	ND	1.88
9	ND	ND	ND	ND	ND	ND	0.44
10	ND	ND	ND	ND	ND	ND	ND
11	ND	ND	ND	ND	ND	ND	ND
12	ND	ND	ND	ND	ND	ND	ND

ND = Not detected

Table 5.4 Formation of major malto-oligosaccharides and CDs by AS-22 when grown on glucose (10 g/L) and tapioca starch (10 g/L). All values are given in g/L.

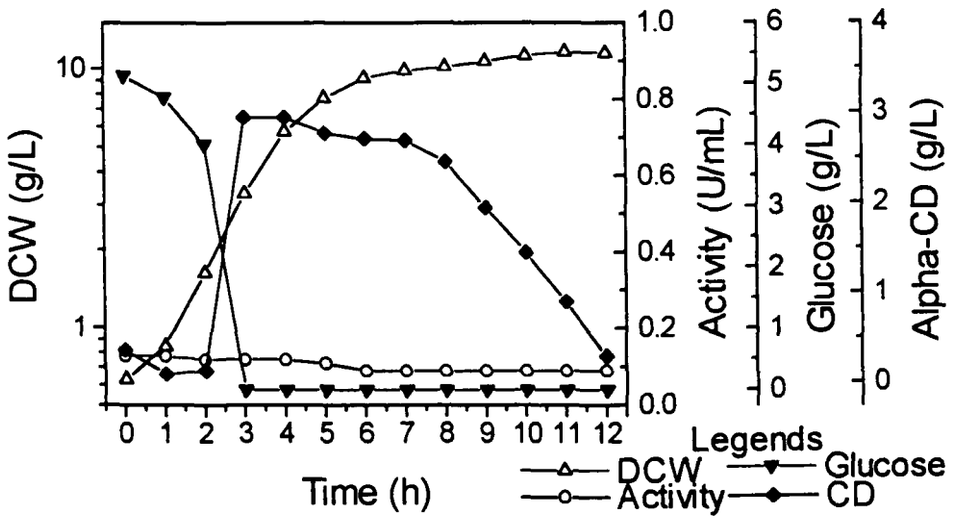


Figure 5.21 Cell growth and CGTase production in 1 L fermenter in basal medium II with the carbon source replaced by glucose and α-CD, 10 g/L each.

disproportionation activities are very high for this enzyme (Table 2.2). No activity was observed when AS-22 was grown in presence of glucose and α-CD (Figure 5.21). Although, initially all of the α-CD is used in coupling or disproportionation reaction, as glucose is consumed, the malto-oligosaccharides are again converted back to α-CD. However, catabolic repression by glucose is so strong that, even in presence of α-CD, CGTase could not be induced.

Fermentation using maltose and tapioca starch containing medium

In the experiment with maltose and tapioca starch as the carbon sources, the starch was rapidly converted to α-CD immediately after inoculation; 2.24 g/L α-CD was detected in the 0 h sample (Table 5.6; Figure 5.22). The malto-oligosaccharides, G_3 to G_6, were found in the culture broth; G_4 was the predominant one. Even though α-CD concentration was high initially, the enzyme production was lower than that in the medium containing glucose and starch. The pattern of CGTase production indicates that maltose also represses CGTase production, but it is not a strong repressor like glucose.

Time (h)	G_1	G_2	G_3	G_4	G_5	G_6	G_7	α-CD	β-CD
0	5.15	2.85	2.67	1.27	1.95	1.44	0.95	0.37	0.02
1	4.79	2.95	2.65	1.25	1.95	1.11	0.86	0.10	0.03
2	4.03	2.71	2.46	1.25	1.89	1.07	0.75	0.14	0.02
3	ND	ND	ND	ND	ND	ND	ND	2.96	ND
4	ND	ND	ND	ND	ND	ND	ND	2.96	0.08
5	ND	0.17	0.19	ND	ND	ND	ND	2.78	0.24
6	ND	0.08	0.05	ND	ND	ND	ND	2.72	0.15
7	ND	ND	ND	ND	ND	ND	ND	2.70	0.08
8	ND	ND	ND	ND	ND	ND	ND	2.46	0.09
9	ND	ND	ND	ND	ND	ND	ND	1.95	0.07
10	ND	ND	ND	ND	ND	ND	ND	1.45	0.06
11	ND	ND	ND	ND	ND	ND	ND	0.90	ND
12	ND	ND	ND	ND	ND	ND	ND	0.30	ND

ND = Not detected

Table 5.5 Formation of major malto-oligosaccharides and CDs by AS-22 when grown on glucose (10 g/L) and α-CD (10 g/L). All values are given in g/L.

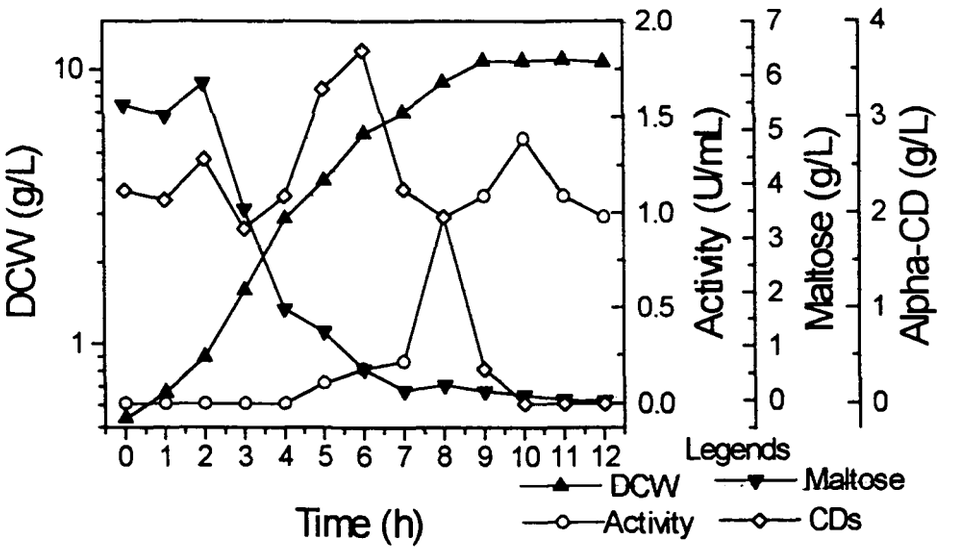


Figure 5.22 Cell growth and CGTase production in 1 L fermenter in basal medium II with the additional maltose (10 g/L) as carbon source.

Fermentation using maltose and α-CD containing medium

Use of α-CD with maltose resulted in higher CGTase activity than in media with tapioca starch and maltose (Figure 5.23). In 0 h sample, 4.52 g/L of maltose and 2.85 g/L of α-CD were detected (Table 5.7). In addition, high concentration of G_6 was observed at 0 h, indicating coupling and disproportionation action of CGTase from the inoculum.

The inoculum contains a lot of CGTase, equivalent to about 10 U/g of substrate. This enzyme immediately acts on the carbon source in the medium and catalyses cyclization, coupling and/or disproportionation reactions depending upon the nature of the substrate. This is probably the reason for the surprising results, such as presence of large amount of α-CD in experiments with dextrin and starch, as well as the rapid disappearance of α-CD when used as the carbon source.

Time (h)	G_1	G_2	G_3	G_4	G_5	G_6	α-CD	β-CD
0	0.12	5.46	1.33	3.48	0.64	1.36	2.24	ND
1	0.14	5.27	1.47	3.80	0.73	1.31	2.14	ND
2	0.27	5.87	1.46	2.84	0.64	1.30	2.57	ND
3	0.23	3.56	1.54	3.33	1.02	1.34	1.84	ND
4	0.52	1.69	1.57	3.40	0.78	1.01	2.18	ND
5	0.56	1.27	1.06	3.20	0.63	1.0	2.62	0.48
6	0.19	0.58	0.58	2.40	ND	ND	2.80	0.90
7	0.08	0.18	0.21	2.0	ND	ND	1.60	0.65
8	0.20	0.28	0.26	1.60	ND	ND	0.90	1.06
9	0.17	0.17	0.09	0.83	ND	ND	0.29	0.07
10	0.08	0.1	ND	0.84	ND	ND	ND	ND
11	ND	0.01	ND	0.84	ND	ND	ND	ND
12	ND	ND	ND	0.80	ND	ND	ND	ND

ND = Not detected

Table 5.6 Formation of major malto-oligosaccharides and CDs by AS-22 when grown on maltose (10 g/L) and tapioca starch (10 g/L). All values are given in g/L.

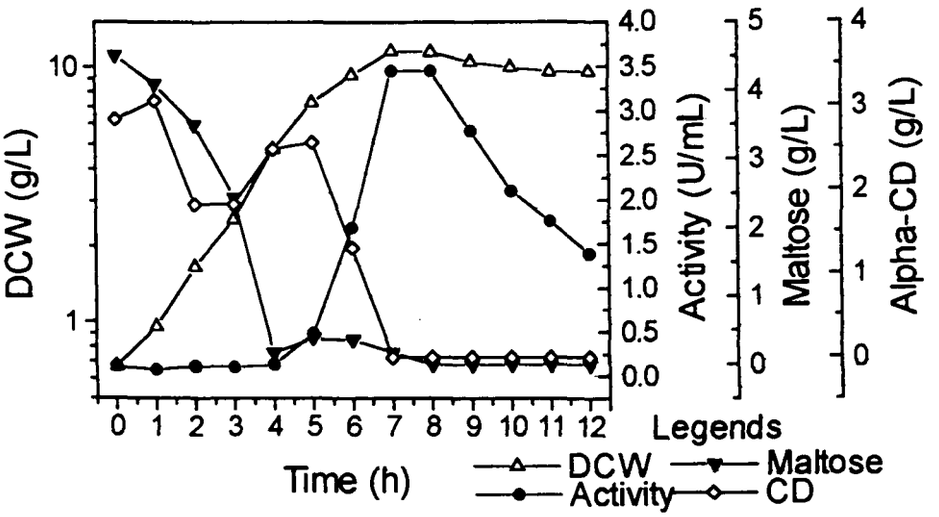


Figure 5.23 Cell growth and CGTase production in 1 L fermenter in basal medium II with the carbon source replaced by maltose and α-CD, 10 g/L each.

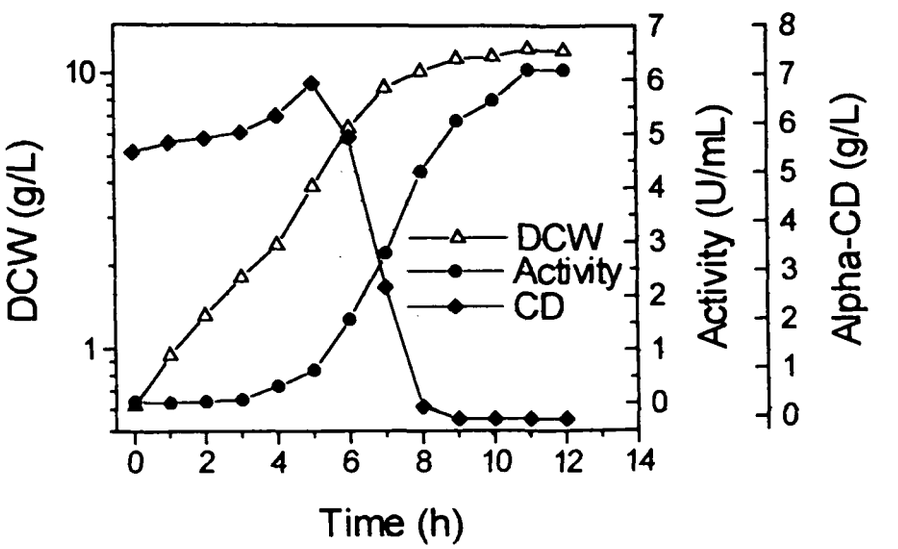


Figure 5.24 Cell growth and CGTase production in 1 L fermenter in basal medium II with 20 g/L tapioca starch

Time (h)	G_1	G_2	G_3	G_4	G_5	G_6	G_7	α-CD	β-CD
0	0.31	4.52	0.76	0.21	0.44	4.95	0.43	2.85	ND
1	0.15	4.10	0.80	0.38	0.64	2.04	0.36	3.07	0.23
2	0.12	3.52	0.86	0.64	0.87	3.12	0.34	1.84	0.07
3	0.09	2.46	0.78	0.69	1.00	3.67	0.30	1.84	ND
4	ND	0.18	0.42	0.07	0.32	4.02	0.15	2.50	ND
5	ND	0.39	0.19	ND	0.16	2.21	0.09	2.58	ND
6	ND	0.36	0.22	ND	ND	1.86	ND	1.31	1.08
7	ND	0.18	0.08	ND	ND	0.5	ND	ND	ND
8	ND	ND	ND	ND	ND	0.1	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	ND	ND	ND	ND	ND	ND	ND	ND	ND
12	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND= Not detected

Table 5.7 Formation of major malto-oligosaccharides and CDs by AS-22 when grown on maltose (10 g/L) and α-CD (10 g/L). All values are given in g/L.

Based on the above results, we propose the following hypothesis for induction of CGTase in *Klebsiella pneumoniae* AS-22. The organism, when grown in the presence of starch or dextrin, converts the substrate to CDs and malto-oligosaccharides using the basal CGTase from the inoculum. It grows initially on malto-oligosaccharides in the medium and keeps aside CDs as reserve carbon source to be used whenever required. Once all linear malto-oligosaccharides are consumed, the organism utilizes CDs as carbon source. Now the large amounts of CDs available can not be broken into simple sugars easily with the basal enzyme level, therefore, it has to either take up CDs as such or degrade them into simple sugars and utilize them as carbon source. If CDs are transported as such inside the cell, the cell needs to produce intracellular CDase to degrade it to simple sugars. CGTase can also degrade the CDs in the medium through coupling and disproportionation reactions. The relative extent of these two mechanisms could not be estimated based on our results. Measurement of intracellular CDase production, in presence of various carbon sources, needs to be carried out (along with CGTase) to determine whether these enzymes are induced simultaneously. Our results indicate that α- and β-CD are directly taken inside the cell, whereas γ-CD is degraded to α- and β-CD or malto-oligosaccharides. It also appears that CGTases are probably induced by CDs and not by any other chain length malto-oligosaccharide.

Fiedler et al. (1996) have described a novel starch utilization pathway in *Klebsiella oxytoca*. They have cloned a DNA fragment from *Klebsiella oxytoca* in *Escherichia coli*. The transformed *E. coli* can grow on α- or β-CD as carbon sources. On the *Klebsiella oxytoca* chromosome, the DNA fragment is located immediately upstream of the *cgt* gene, which is known to code for CGTase in *Klebsiella oxytoca* (Binder et al., 1986). According to these workers, α- and β-CDs are directly transported inside the cell by a specific uptake system, linearised by cytoplasmic CDase and malto-oligosaccharides thus produced are channeled into the maltodextrin-degradation route, involving the activity of maltodextrin phosphorylase and amylomaltase. In this case, if CDs are directly taken up inside the cell and can be utilized as a carbon and energy source, then extracellular CGTase need not be induced for growth in CD-based media. Induction of CDase is sufficient for this purpose. It is known that a cluster of ten *cym* genes, which contains four genes for maltose and linear maltodextrin uptake system and one for a putative

cytoplasmic CDase, is located immediately upstream of the *cgt* gene. It is possible that this cluster of genes would be regulated in a coordinated fashion.

Nishida et al. (1997) have studied regulation of CGTase production from *Bacillus ohbensis* in the presence of glucose and soluble starch at transcriptional level. They observed that in the presence of starch, a large number of copies of *cgt* mRNA were found only at the end of the exponential growth phase. These mRNAs rapidly declined in the stationary phase. When glucose was added to the medium along with soluble starch, *cgt* mRNAs were not detected, indicating that glucose strongly represses expression of the *cgt* gene. However, these researchers also hinted at the possibility of regulation of CGTase production at the post-transcriptional level, besides the negative transcriptional regulation by glucose.

Nogrady et al. (1995) detected intracellular CGTase activity when *Bacillus macerans* was grown in the presence of starch. The intracellular activity could not be detected with glucose, probably because the maximum activity obtained with glucose was 13-times lower than that with soluble starch, indicating that CGTase production is inducible with starch. Presence of glucose strongly represses CGTase synthesis, but some basal extra- and intracellular activity is detected. The only known constitutively produced CGTase has been reported by Jamuna et al. (1993). They found that *Bacillus cereus* RJ-30 produces CGTase to a very high level with glucose as a carbon source, even in the absence of starch. They have reported maximum enzyme production with xylose as the carbon source.

Pocsi et al. (1998b) also studied CGTase production using G_1-G_3, G_7, CDs and soluble starch as carbon sources. They simply monitored extracellular CGTase activity during fermentations with *Bacillus macerans* and observed that no enzyme was induced when G_1-G_3 is used as carbon source. In the presence of G_7, CGTase production was about 65% of that observed with soluble starch. Use of CDs, on the other hand, resulted in CGTase production at only one-third the level obtained with soluble starch. In fact, with G_1-G_3, they have reported 16-20% activity as compared to that with soluble starch, which might be the basal activity present for this organism. These results differ from ours, in that we have reported higher enzyme production with α-CD than with starch.

Fed-batch studies

Fed-batch fermentation is commonly used to achieve high cell density and high product yields for many microorganisms (Kim et al., 1996; Lee et al., 1994; Mao et al., 1992; Meesters et al., 1996; Patkar and Seo, 1992; Yoo et al., 1988). It is very difficult, if not impossible, to obtain high cell densities in a simple batch operation. Use of high concentrations of media components invariably leads to substrate inhibition, shifting of metabolism to produce undesirable side products, which can be inhibitory to cell growth and product formation. Moreover, in the batch mode cell growth rate cannot be controlled easily, leading to dissolved oxygen limitations at higher cell densities. Such limitations also result in side-product formation and growth inhibition. The advantages of fed-batch fermentation include: (a) good control of growth limiting substrate concentration in the fermenter, (b) efficient control of cell growth rate by controlled feeding thus avoiding dissolved oxygen limitation and side-product formation, (c) possibility of changing medium composition during product formation phase. Different substrate feeding strategies have been reported in literature, for example, constant feeding, exponential feeding, DO-stat, pH-stat and feeding glucose by monitoring glucose level (Kwon et al., 1996; Lee 1996; Sakamoto et al., 1994; Yee and Blank 1992). Fed-batch fermentation has been successfully used for α-amylase production in *Bacillus* (Kim et al., 1997b; Kole and Gerson 1989; Yoo et al., 1988). There are only two reports of fed-batch fermentation for CGTase production (Chen et al., 1994; Park et al., 1997).

As can be seen from the results in the previous section, high carbon source concentrations cause substrate inhibition leading to lower cell yields and enzyme levels. Moreover, our results show that AS-22 grows efficiently utilizing glucose as a carbon source. It can also produce CGTase if glucose is exhausted and starch is present in the medium. With these results in mind, we attempted to achieve high cell densities and enzyme productivity in fed-batch cultivation with DO-based addition of feeds containing glucose or dextrin.

When batch run was started with 10 g/L glucose in basal medium II (4 L volume), fed with 200 g/L glucose (4 L) and then fed with medium containing high concentration of starch (75 g/L, 2 L). No enzyme activity was detected in the broth at any time (results

not shown). Glucose is known to strongly repress CGTase production. It appears that the repression of CGTase formation was so strong that even after subsequent addition of starch-based medium CGTase was not produced.

In another attempt, we started the batch run with starch containing medium, fed medium containing glucose to achieve high cell density (Figure 5.25). Once high cell density was obtained, starch-based medium was fed to induce CGTase production. It was thought that during the initial batch growth on starch, the protein synthesis machinery inside the cells would be geared towards CGTase production. Even though a glucose-based feed was added during the cell growth phase, glucose-repression would be overcome quickly after addition of starch-based medium. However, it was observed that initial enzyme activity in the batch decreased rapidly when glucose addition was started and could not be recovered again even after addition of starch. There might be three reasons for this kind of behaviour. First, concentration of starch may be insufficient to induce CGTase production at such a high cell density. Thus, an inducer that can be fed at very high concentration may be required. Second, the catabolic repression of CGTase by

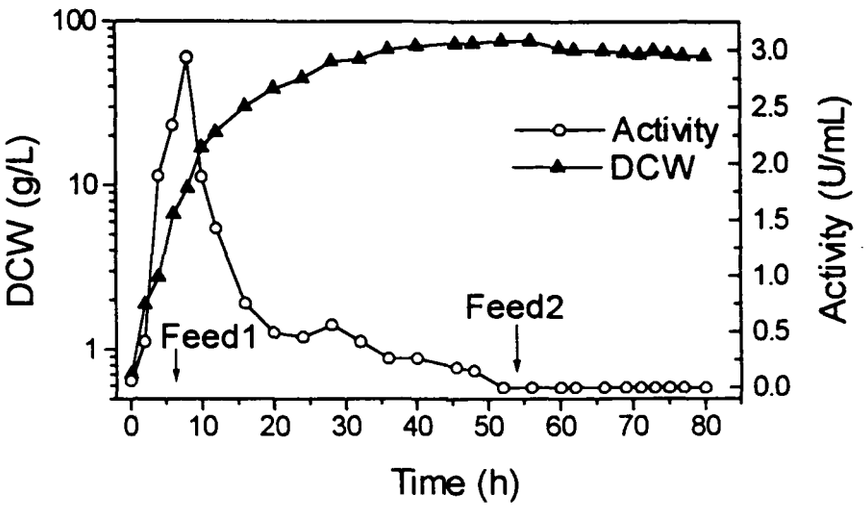


Figure 5.25 High cell density cultivation of AS-22 using glucose-based medium. The carbon source in batch medium, feed1 and feed2 were 20 g/L tapioca starch, 400 g/L glucose and 75 g/L tapioca starch, respectively.

glucose, which seems to be not correct as can be seen from results obtained with growth on carbon source glucose and starch together (Figure 5.20). Third, starch addition was started when growth was virtually stopped. CGTase production being growth associated, if addition of inducer could have been started when cells were actively growing, results could have been probably differently.

Fed-batch fermentations have rarely been used for CGTase production. One possible reason may be the difficulty in handling high concentrations of starch: 100 g/L solution becomes practically immovable. Therefore, we tried controlled feeding of another substrate, i.e. dextrin, which can be fed at high concentration and results in very good CGTase induction. As specific cell growth rate with dextrin was almost the same as that with glucose (Table 5.3), we thought that high cell density could also be achieved with direct feeding of dextrin-based medium. Further, if high cell density is obtained with glucose-based medium, CGTase induction is likely to be problematic. The batch run was

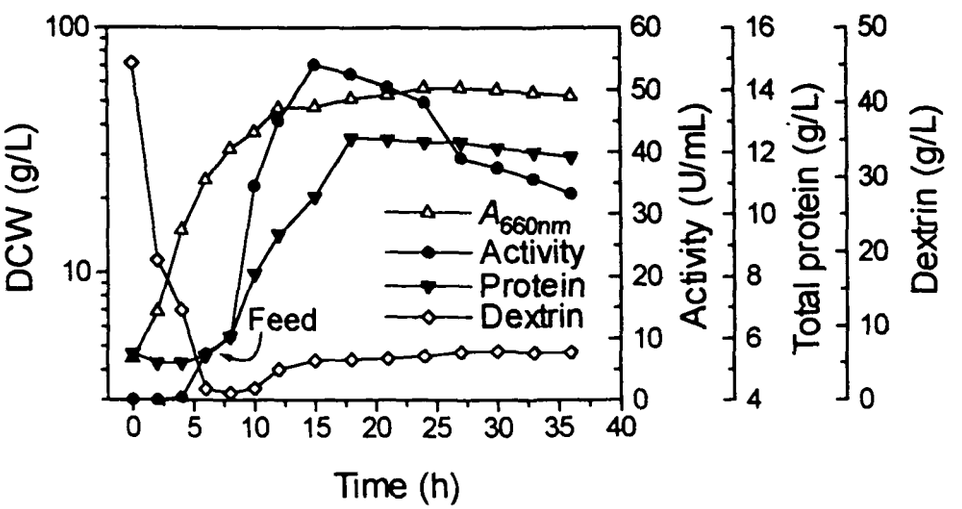


Figure 5.26 High cell density cultivation of AS-22 using dextrin-based medium. The carbon source in batch medium and feed was 49.3 and 172.5 g/L dextrin, respectively.

started with optimized medium and the fermenter was fed with 2X optimum medium (with respect to carbon and nitrogen source) (Figure 5.26). CGTase activity increased 1.7-fold in 15 h as compared to that in the batch operation. However, at longer fermentation times the activity continuously decreased. Maximum cell growth was obtained at 24 h, which also decreased later on. HPLC analysis of fermentation broth samples showed that concentration of α-CD decreased continuously after reaching a peak of 4.13 g/L at 2 h, and never increased to more than 0.15 g/L after 10 h of fermentation. With higher cell mass in the fed-batch fermentations, the enzyme activity should have been higher than observed. The exact nature of CGTase induction is not known. Although starch and dextrin are known to be good inducers, these sources are rather heterogenous consisting of malto-oligosaccharides of varying lengths. It is possible that only certain malto-oligosaccharides are the direct inducers of CGTase production and the concentration of these inducers needs to be controlled at optimum levels. CGTase is produced in a growth-associated fashion in batch fermentations. Thus, the specific growth rate may significantly influence CGTase production rate. In fact, our results in continuos culture fermentations show this dependence. Further studies need to be done to optimize the growth rate during the fed-batch phase, composition of feeds and timing of induction.

There are only two reports on fed-batch fermentation for CGTase production. Park et al. (1997) have reported fed-batch fermentation using recombinant *Escherichia coli* harboring CGTase gene of *Bacillus macerans*. They obtained very high yields of cell mass (29-fold increase) and enzyme production (31-fold increase) as compared to that in batch fermentation. Chen et al. (1994) obtained 50% more CGTase production as compared to that in batch operation using alkalophillic *Bacillus* sp (ATCC 31007).

Continuous culture studies

The continuous culture experiments were performed with 20 g/L tapioca starch in basal medium II or optimized dextrin medium at dilution rates (D) of 0.1 to 0.6 h^{-1}. The steady-state values of cell mass, enzyme activity, total or residual sugars and total proteins in the broth at different dilution rates are shown in Figures 5.28 and 5.30, whereas cell mass and enzyme productivities are shown in Figures 5.29 and 5.31. In

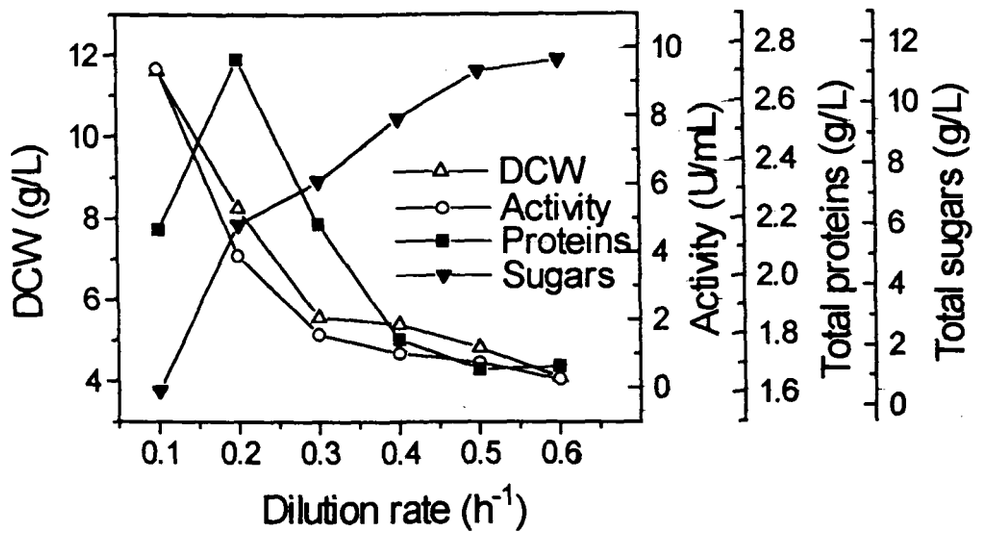


Figure 5.28 Cell growth and CGTase production in continuous culture with feed containing 20 g/L tapioca starch in basal medium II in 1 L fermenter

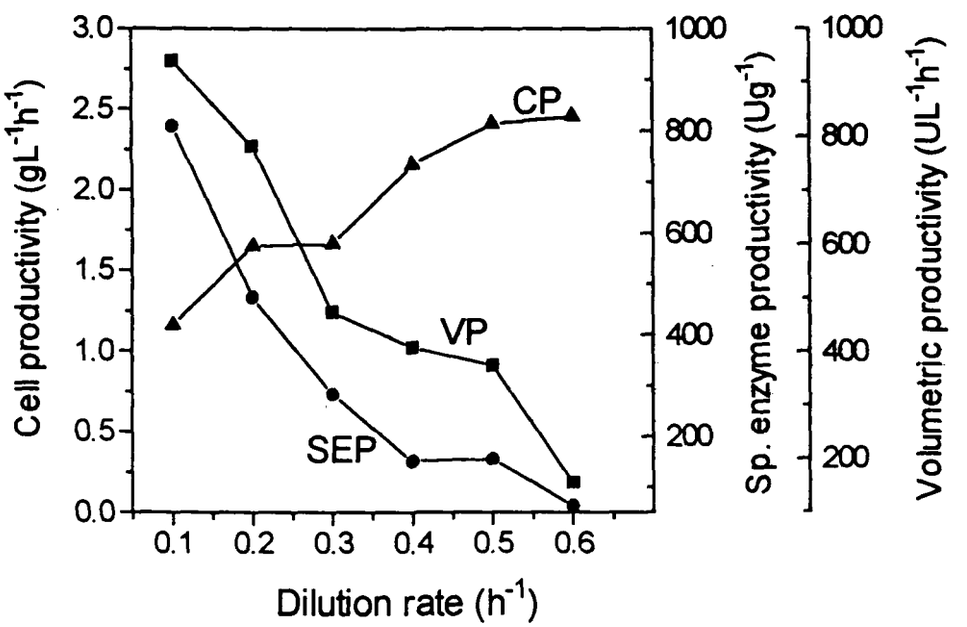


Figure 5.29 Cell and CGTase productivities in continuous culture with 20 g/L tapioca starch in 1 L fermenter. CP represents cell productivity, SEP represents specific enzyme productivity and VP represents volumetric productivity

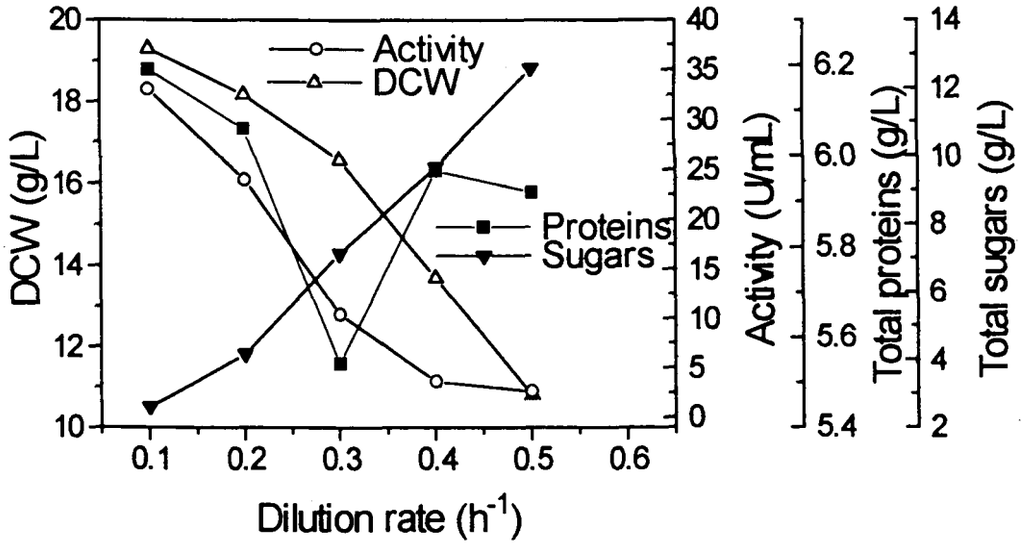


Figure 5.30 Cell growth and CGTase production in continuous culture with feed containing optimized dextrin medium in 1 L fermenter

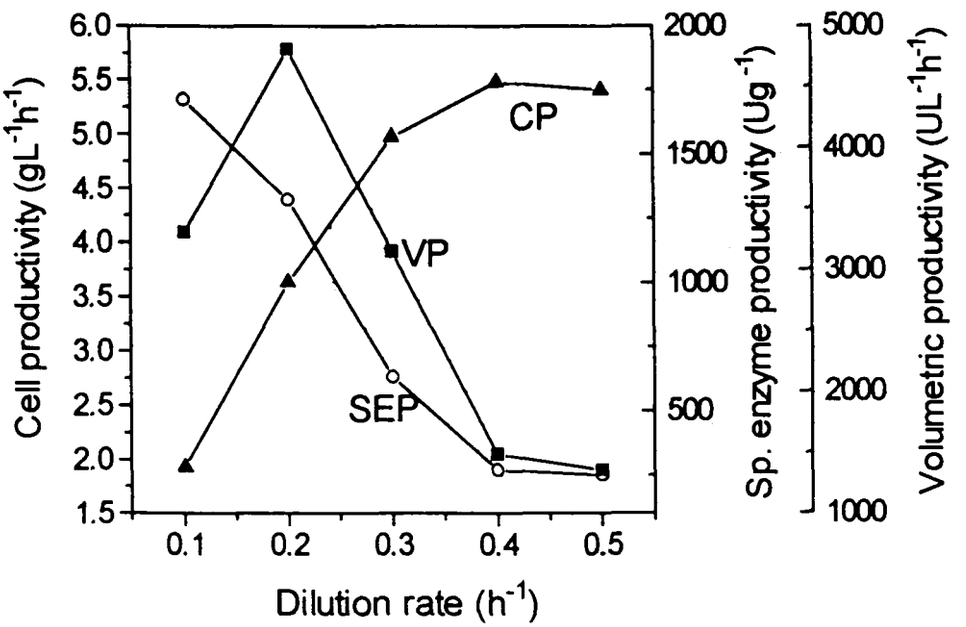


Figure 5.31 Cell and CGTase productivities in continuous culture with feed containing optimized dextrin medium in 1 L fermenter. CP represents cell productivity, SEP represents specific enzyme productivity and VP represents volumetric productivity

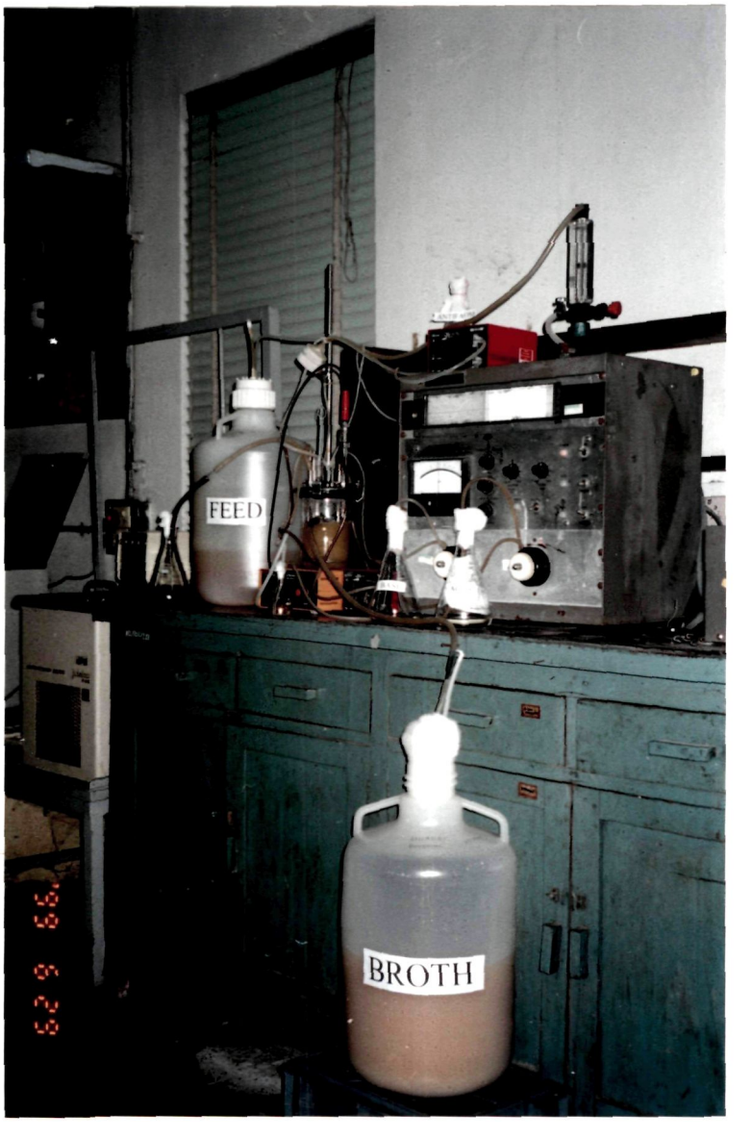


Figure 5.32 Continuous culture bioreactor used in this study

Mode	Substrate	Time or Residence time (h)	Cell mass (g/L)	Activity (U/mL)	Volumetric productivity (U/Lh^{-1})
Batch	Tapioca starch (20 g/L)	12	15.76	6.77	564
	Dextrin (Optimized medium)	24	32.89	32.82	1367
Fed-batch	Dextrin (2X optimum medium)	15	56.97	54.07	3605
Continuous	Tapioca starch (20 g/L)	10	11.62	9.4	940
	Dextrin (Optimized medium)	5	18.18	24.06	4812

Table 5.7 Comparison of results obtained in batch, fed-batch and continuous culture studies

experiments with starch-based feed CGTase activity increased to a maximum of 15.2 U/mL at the dilution rate of 0.1 h^{-1}. This activity is 2.3 times higher than that observed in batch culture. The activity decreased after 24 h at same dilution rate and remained steady at 9.4 U/mL. Similar results were obtained with dextrin containing medium. Maximum activity of 36.5 U/mL was obtained at 36 h at dilution rate of 0.1 h^{-1}. The activity subsequently decreased and remained steady at 24.1 U/mL. Cell mass and enzyme activity decreased with increase in dilution rate in both the experiments. The maximum volumetric productivity was obtained at the dilution rate of 0.1 h^{-1} in starch-based medium and 0.2 h^{-1} in dextrin-based medium. The specific enzyme productivity was highest at the lowest dilution rate, whereas cell mass productivity increased with increase in dilution rate and remained almost constant in both the experiments. Amount of total sugar or residual dextrin increased at higher dilution rates, indicating that substrate is not utilized completely, which probably resulted in substrate inhibition giving low enzyme activity. Table 5.7 shows comparison of results obtained with starch as well as dextrin containing media in batch, fed-batch and continuous culture studies. The

volumetric enzyme productivity was 1.66-fold higher in continuous culture as compared to batch fermentations in tapioca starch-based medium. In optimized dextrin-based medium, the corresponding increase in volumetric enzyme productivity was 2.64-fold in fed-batch culture and 3.52-fold in continuous culture as compared to batch run.

Lane and Pirt (1973) have studied production of *Bacillus macerans* CGTase in a starch containing minimal medium in continuous culture. They obtained 2.75-fold increase in enzyme productivity in continuous culture as compared to that in batch. The maximum enzyme activity was obtained with the lowest dilution rate of 0.05 h^{-1}. Jamuna et al. (1993) have observed 3.1-fold increase in CGTase productivity in continuous culture of *Bacillus cereus* RJ-30 using a glucose-containing medium. Bender (1981, 1986) has also reported synthesis of CGTase from *Klebsiella pneuminiae* M 5 al in continuous culture, details of which are not available.

Conclusions

A detailed study on CGTase production from *Klebsiella pneumoniae pneumoniae* AS-22 in batch, fed-batch and continuous mode of operations in automated bioreactors is presented. CGTase production was improved by optimization of fermentation parameters using tapioca starch as the carbon source. The maximum CGTase activity obtained with tapioca starch was 6.77 U/mL, whereas that with dextrin was 32.82 U/mL in batch experiments. Induction of enzyme production was investigated with different carbon sources in shake flasks and fermenters. Glucose was found to strongly repress CGTase production while dextrin and starch was found to induce CGTase production. Alpha-CD was found to be the probable final inducer of CGTase production. High cell density of 76 g/L dry cell weight was achieved with controlled feeding of glucose-based medium. Fed-batch cultivation with dextrin-based medium resulted in 1.7-fold higher enzyme activity and 2.64-fold higher volumetric productivity. Continuous culture experiments resulted in 1.66 and 3.52-fold higher volumetric enzyme productivity with tapioca starch and dextrin-based medium, respectively, as compared to that in batch fermentations.

References

References are listed in Chapter 7

CHAPTER 6

General discussion and concluding remarks

Cyclodextrins (CDs) were discovered by Villiers in 1891. However, it was only during 1980's that their properties and uses in various fields were extensively explored for the first time. The price of CDs fell down in mid-eighties due to discovery of new CGTase producing bacteria and higher scale of production. Although potential market for CDs is continuously expanding, as indicated by increasing number of CD related publications in all fields, the market for CDs is still limited because of relatively high cost of production (Schmid 1989). There are three main factors responsible for this situation. First, general use of CDs is limited because of their high cost. Second, availability of α- and γ-CD is limited. Third, use of CDs in food and pharmaceuticals is not approved in some countries. Estimated selling prices in the world market for α-, β- and γ-CD are $30, $5-20 and $60-200 per Kg respectively. The worldwide consumption of β-CD alone was about 2,500 tonnes per year in 1995, increasing yearly by 10-15% (The figures about prices and consumption were provided by Dr. Szejtli at Cyclolabs, Hungary). The prices of CDs need to go down further to about $5/kg for β-CD and $10-20/kg for α- and γ-CD to increase their use. This is only possible if highly economical CD production processes are developed and used for commercial production. In India, all the cyclodextrin demand is met through imports. The local price is about Rs. 500/kg for β-CD, which is the most commonly used CD. Few companies have shown significant interest in the purchase of cyclodextrin manufacturing technology, if available.

This thesis presents the results obtained in an extensive study of cyclodextrin glycosyltransferase (CGTase) enzyme from *Klebsiella pneumoniae pneumoniae* AS-22. The thesis covers many topics including strain isolation, identification, enzyme purification, biochemical characterisation, media optimization, fermentation optimization and improvement of enzymatic conversion of starch to cyclodextrins. The thesis conclusions are summarised below.

Isolation of strain

A large number of starch degrading organisms were isolated and screened for CGTase secretion by a direct specific HPLC-based method. This method also allowed us to characterise the enzymes in terms of their selectivity towards production of different CDs. We undertook study on isolation of new CGTase producing culture, mainly for two

reasons. First, very few α- and γ-CGTases have been reported. Second, costs of α- and γ-CDs are much higher than that of β-CD. We were successful in isolating an organism which produced a CGTase specific for α-CD production. However, even after isolating hundreds of starch degrading cultures, we were not able to discover a CGTase with good specificity towards γ-CD production. The α-CGTase possessed several novel characteristics and was therefore chosen for further study. The novel properties of this enzyme are predominant production of α-CD from starch and capability of converting raw starch directly without gelatinization. The culture growth rate was very fast, which was beneficial not only for rapid fermentation studies but also for prospective commercial utilization. This new organism (AS-22) was identified taxonomically as *Klebsiella pneumoniae pneumoniae*.

Purification and properties

The enzyme was purified to homogeneity; the pure preparation gives a single band on SDS-PAGE using the highly sensitive silver-staining method (Chapter 2). One interesting characteristic of the pure enzyme was the absence of any net hydrolysis activity. This indicates that CGTase produced by AS-22 is a true CGTase, without any amylolytic activity. The enzyme had good activity over a broad pH range, between pH 5.5 to 9.0, and was also stable in the pH range of 6-9. Although, temperature stability of this CGTase is low above 30 °C, the addition of $CaCl_2$ significantly increased its temperature stability. Moreover, storage stability at 30 °C can also be improved by addition of 30% (v/v) glycerol. Absolutely no loss of activity was observed when pure enzyme preparation was stored at -20 °C in 1 mM $CaCl_2$ for a period of two years.

The effectiveness of this CGTase in CD production was investigated using different gelatinized and raw starches (Chapter 2). Very few CGTases degrade and produce CDs from raw starch. This enzyme produces α-CD from raw starch with the same yield as that from gelatinized starch. For production of α-CD, the optimum substrate concentrations were 100 g/L gelatinized soluble starch or 125 g/L raw wheat starch. About 21% (w/w) conversion of soluble starch to total CDs was obtained in 4 h and the ratio of α : β : γ-CD was 81 : 12 : 7. With raw wheat starch, 20% (w/w) conversion of to

total CDs was obtained in 4 h and the ratio of α : β : γ-CD was 89 : 9 : 2. The conversions obtained here, at high starch concentrations, in such short reaction times, and without using any complexing agent for CD production, compare favourably with values reported in literature.

CGTase production

Media optimization was the first logical step for increasing enzyme production. We optimized concentrations of different media components (Chapter 3) using systematic study based on statistical experimental designs at shake flask level. The optimized medium was used to check the enzyme production level in fermenters, because ultimately enzyme has to be produced in large scale fermenters. We obtained about 9-fold increase in enzyme activity (32.5 U/mL) as compared to that in basal medium (2.4 U/mL) in shake flask. Similar increase was also obtained in fermenters. We have successfully shown that studies carried out at shake flask level can be extrapolated to fermenters, as carrying out such media optimization directly in fermenters is time-consuming.

α-CD production

The cyclodextrin yields obtained with enzymatic conversion of starch are comparatively low. The conversion to CDs usually decreases at higher substrate concentrations (Horikoshi 1979; Jamuna et al., 1993). For economical production of CDs, the property of CD to form inclusion complex with specific complexing agent is exploited for enhancement of conversion to a particular CD. The specific complexing agent, when added to the reaction mixture, drives the equilibrium of the reaction towards formation of a particular CD (Schmid 1996b). We screened many complexing agents (Chapter 4) with the objective of predominant production of α-CD with raw wheat starch. Production of CDs with raw starch in the presence of complexing agents has not been studied before. We obtained 42.5% (w/w) conversion of raw wheat starch to α-CD in the presence of 2% (v/v) n-butanol. Addition of this complexing agent resulted in another advantage of very low production of β-CD (3%) and negligible amounts of γ-CD and malto-oligosacchrides. Such good selectivity is particularly useful and a purification of α-CD from the reaction mixture will be greatly simplified.

Gelatinized starch is very difficult to use at high concentrations; it becomes highly viscous and difficult to handle. Therefore, concentrated gelatinized starch is usually thinned using α-amylase or CGTase before CD production. This thinning needs to be controlled very accurately because 'over-thinning' significantly reduces conversion to CDs in the subsequent step (Schmid 1996b). The present CGTase directly attacks raw wheat starch, thus eliminating the energy-intensive gelatinization and difficult to control thinning step. There is also another option of using dextrin, which can be prepared in very high concentrations (up to 600 g/L). It is known that the ratios of enzyme to substrate and substrate to complexing agent have a significant influence on the the extent of conversion of substrate to CDs. We optimized production of α-CD using a factorial design approach with dextrin, enzyme and complexing agent concentrations as three independent variables. The optimum concentrations for α-CD production from dextrin were 500 g/L dextrin, 3% (v/v) n-hexanol and 15 U of the enzyme per g of starch. About 12.1% (w/w) conversion of dextrin to α-CD was obtained in 6 h and the ratio of α : β : γ-CD was 91 : 3 : 6.

It is difficult to increase the concentration of α-CD in the reaction mixture above 50 g/L. This could be as a result of inhibition of CGTase activity by α-CD at high concentrations. We found that this CGTase is strongly inhibited by α-CD; 50% inhibition was observed with 4 g/L initial concentration of α-CD. If the CD can be removed from the reaction mixture as it is formed, higher conversions should be possible. We tested this possibility by using an ultrafiltration membrane bioreactor, with continuous removal of CD in the permeate. However, α-CD yield was not enhanced as compared to without ultrafiltration membrane.

CGTase induction

The mechanism of induction of CGTase is not properly understood yet. We tried to investigate how CGTase synthesis is induced in *Klebsiella pneumoniae pneumoniae* AS-22 by growing this organism in presence of various carbon sources and monitoring their utilization in the fermentation broth (Chapter 5). Alpha-CD was found to be the strong inducer of CGTase production in this organism. When the organism was grown in

the presence of dextrin or starch as carbon source, about 50% of the carbon source was immediately converted into α-CD by the enzyme present in the inoculum as soon as the inoculum was added. Alpha-CD was utilized within 3-4 h of growth and as α-CD was consumed CGTase started appearing in the culture broth. When only α-CD was used as the carbon source, the amount of CGTase produced was even higher than that with starch as the carbon source. The use of β- or γ-CDs as carbon sources also resulted in CGTase production, although at a relatively low level. Presence of glucose strongly repressed CGTase production. Low levels of CGTase were detected with maltotriose (G_3) as the carbon source. The malto-oligosaccharide mixture containing G_4-G_{10} resulted in 65% enzyme activity as compared to starch. The question of the exact nature of the inducer still remains unsolved.

Fiedler et al. (1996) have reported that CDs (α and β) are transported inside the cell by a specific uptake system, linearised by cytoplasmic CDase (a CD degrading enzyme) and the malto-oligosaccharides produced are channeled into the maltodextrin-degradation route. Feederle *et al.* (1996) also support this observation and have purified and characterised cytoplasmic CDase from *Klebsiella oxytoca*. Our experimental data agrees with these observations, although a more direct validation would require radio-actively labeled substrates.

Fermentation studies

Although production of CGTase is the first step in CD production, there are very few reports on fermentation studies on CGTase producing organisms. The optimization of fermentation parameters is a direct approach for achieving high enzyme yields from a new organism. We have extensively studied and optimized various fermentation parameters in 1 and 12 L fermenters for maximum enzyme production (Chapter 5). Experiments at different substrate concentrations demonstrated that an optimum substrate concentration is required for maximum enzyme production. Fed-batch fermentations are usually used for achieving high cell density and enzyme productivity. We have also performed fed-batch fermentations using a feeding strategy based on dissolved oxygen concentration and obtained 76 and 57 g/L dry cell mass concentrations with glucose and dextrin feeding, respectively. In the experiment with glucose-based feed, CGTase

production was not induced even after switching over to a starch-based feed. Controlled feeding of dextrin in another fed-batch experiment resulted in 1.7-fold more enzyme production as compared to that observed in batch experiments. Continuous culture experiments with feeds containing tapioca starch or dextrin as carbon sources resulted in 1.66-fold and 3.52-fold increased CGTase volumetric productivity, respectively, as compared to that in batch culture.

Further work

This thesis was part of an on-going effort in the Biochemical Engineering group towards development of a process for cyclodextrin production. Currently, we have two promising enzymes, the α-CD specific CGTase reported in this study and another raw-starch degrading CGTase (which produces primarily β- and γ-CDs) studied earlier in our group (Goel, 1995; Goel and Nene 1995a; Gawande et al., 1999). Both these enzymes have been extensively characterised in terms of their biochemical properties and utility towards CD production. The means of fermentative production, concentration and stable formulation have also been standardised. Still a lot of work needs to be done in establishing a viable process for CD production. At a minimum this would require (a) cost-effective means of separation of individual CDs from the reaction mixture, (b) scale-up of enzyme production and purification, (c) scale-up of enzymatic conversion and CD separation.

References

References are listed in Chapter 7

CHAPTER 7

References

Abdel-Fattah A. F., Ismail A. S. and Sobieh U. I. (1994) Purification and properties of cyclodextrin glucosyltransferase from *Bacillus macerans* 314. *Al-Azhar J. Microbiol.*, **26**: 16-25

Abe S., Nagamine Y., Omichi K. and Ikenaka T. (1991) Investigation of the active site of *Bacillus macerans* cyclodextrin glycosyltransferase by use of modified maltooligosaccharides. *J. Biochem.*, **110**: 756-761

Abelian V. A., Adamian M. O., Abelian L. A., Balayan A. M. and Afrikian E. G. (1995a) A novel cyclomaltodextrin glucanotransferase from a halophilic bacterium. *Biochemistry (Moscow)*, **60**: 665-670

Abelian V. A., Afyan K. B., Avakian Z. G., Melkumyan A. G. and Afrikian E. G. (1995b) Cyclomaltodextrin glucanotransferases from thermophilic actinomycetes. *Biochemistry (Moscow)*, **60**: 1223-1229

Abelian V. A., Gasparien A. V., Avakian Z. G. and Afrikian E. G. (1991) Extracellular cyclodextrin glycosyltransferase from the thermophilic bacteria *Bacillus* sp.. *Biokhimiya*, **56**: 1578-1582

Abelian V. H., Shamtsian M. M., Chu D. -C., Kim M. and Yamamoto T. (1993) Production of cyclodextrins along with rice protein concentrate and sugar syrup from rice bran. *Chem. Express*, **8**: 365-368

Achary A., Hariharan K. A., Bandhyopadhyaya S., Ramachandran R. and Jayaraman K. (1997) Application of numerical modelling for the development of optimized complex medium for D-hydantoinase production from *Agrobacterium radiobacter* NRRL B 11291. *Biotechnol. Bioeng.* **55:** 148-154

Ahn J. -H., Hwang J. -B. and Kim S. -H. (1990) Cyclodextrin glucanotransferase from *Bacillus stearothermophilus*: purification by affinity chromatography and its properties. *Kor. J. Appl. Microbiol. Biotechnol.*, **18**: 585-590

Ahn J. -H., Hwang J. -B., Kim K. -E. and Kim S. -H. (1991) Purification of cyclodextrin glucanotransferase by affinity chromatography. *Kor. J. Appl. Microbiol. Biotechnol.*, **19**: 113-114

Akimaru K., Yagi T. and Yamamoto S. (1991a) Cyclomaltodextrin glucanotransferase-producing moderate thermophile, *Bacillus coagulans*. *J. Ferment. Bioeng.*, **71**: 63-65

Akimaru K., Yagi T. and Yamamoto S. (1991b) Purification and properties of *Bacillus coagulans* cyclomaltodextrin glucanotransferase. *J. Ferment. Bioeng.*, **71**: 322 – 328

Alcalde M., Plou F. J., Andersen C., Martin M. T., Pedersen S. and Ballesteros A. (1999) Chemical modification of lysine side chains of cyclodextrin glycosyltransferase from *Thermoanaerobacter* causes a shift from cyclodextrin glycosyltransferase to α-amylase specificity. *FEBS Lett.*, **445**: 333-337

Alcalde M., Plou F. J., Pastor E. and Ballesteros A. (1998) Effect of chemical modification of cyclodextrin glycosyltransferase (CGTase) from *Thermoanaerobacter* sp. on its activity and product selectivity. In: Enzyme

engineering XIV. Annals of the New York Academy of Sciences, Vol. 864. Laskin A. I., Li G. -X. and Yu Y. -T. (Eds) pg. 183-187, New York

Allenza P., Clifft C. G. and Morrell M. J. (1991) Novel *Bacillus* and *Pseudomonas* for manufacturing cyclodextrin glycosyltransferase. US 500195 (CA 115: 25927)

Aoki H., Yao D. and Misawa M. (1987) Production and characterization of a thermostable bacterial cyclodextrin glycosyltransferase. *Prog. Biotechnol.*, **3**: 81 -93

Armbruster F. C. (1970) Isolating pure α-cyclodextrin. US 3541077 (CA 74: 32887)

Armbruster F. C. (1988) Use of cyclohexane in the production of pure aplha- and beta-cyclodextrins. In: *Proceedings of the Fourth International Symposium on Cyclodextrins*. Huber O. and Szejtli J. (Eds), pg. 33-39, Kluwer, Dordrecht, Netherlands

Armbruster F. C. and Jacaway W. A. Jr. (1972) Enzymic production of α-cyclodextrin. US 3 640 847 (CA 76: 125427)

Armbruster F. C. and Kooi E. R. (1969) Cyclodextrin. US 3 425 910 (CA 70: 79360)

Bar R. (1996) Applications of cyclodextrins in biotechnology. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 423-440, Pergamon, U. K. (review)

Beadle J. B. (1969) Analysis of cyclodextrin mixtures by gas chromatography of their dimethylsilyl ethers. *J. Chromatogr.*, **42**: 201-206

Bender H. (1977a) Cyclodextrin glucanotransferase from *Klebsiella pneumoniae*. I Formation, purification and properties of the enzyme from *Klebsiella pneumoniae* M 5 al. *Arch. Microbiol.*, **111**: 271-282

Bender H. (1977b) Cyclodextrin glucanotransfearse from *Klebsiella pneumoniae* 2. Significance of the enzyme for the metabolism of cyclodextrin by *Klebsiella pneumoniae* M 5 al. *Arch. Microbiol.*, **113**: 49-56

Bender H. (1981a) A photometric assay for the initial rate of the cyclization reaction catalyzed by the cyclodextrin glycosyltransferase. *Anal. Biochem.*, **114**: 158-162

Bender H. (1981b) Enzymology of cyclodextrins. In: *Proceedings of the First International Symposium on Cyclodextrins*, Budapest, 1981, Szejtli J. (Ed), pg. 77-87, D. Reidel Publishing Company, Dordrecht

Bender H. (1983a) An improved method for the preparation of cyclooctaamylose, using starches and the cyclodextrin glycosyltransferase of *Klebsiella pneumoniae* M 5 al. *Carbohydr. Res.*, **124**: 225-233

Bender H. (1983b) Cyclodextrin glycosyltransferases from *Klebsiella pneumoniae* M 5 al and *Bacillus macerans*: Quantitative analysis by HPLC. *Carbohydr. Res.*, **117**: 1-11

Bender H. (1985) Studies of the inhibition by malto-oligosaccharides of the cyclization reaction catalyzed by the cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* M 5 al with glycogen. *Carbohydr. Res.*, **135**: 291-302

Bender H. (1986) Production, characterization and application of cyclodextrins. *Adv. Biotechnol. Processes* **6**: 31-71

Bender H. (1990) Studies of the mechanism of the cyclization reaction catalyzed by the wild type and a truncated α-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* M 5 al, and the β-cyclodextrin glycosyltransferase from *Bacillus circulans* strain 8. *Carbohydr. Res.*, **206**: 257-267

Bender H. (1991) On the role of histidine residues in cyclodextrin glycosyltransferase: chemical modification with diethyl pyrocarbonate. *Carbohydr. Res.*, **209**: 145-153

Bender M. L. and Komiyama M. (1978) Cyclodextrins chemistry. Springer-Verlag, New York

Bergsma J., Bruinenberg P. M., Hokse H. and Meiberg J. B. M. (1988) Cyclodextrins from potato starch. Recent developments. In: *Proceedings of International Symposium on Cyclodextrins*, 4th, Huber O. and Szejtli J. (Eds). pg. 41-46., Kluwer, Dordrecht, Netherlands

Berna P., Moraes F. F., Barbotin J. N., Thomas D. and Vijaylakshmi M. A. (1994) One step affinity purification of a recombinant cyclodextrin glycosyltransferase by [Cu (II), Zn (II) tendem column] immomobilized metal ion affinity chromatography. In: *Advances in Molecular Biology*, JAI Press Inc.

Binder F., Huber O. and Böck A. (1986) Cyclodextrin-glycosyltransferase from *Klebsiella pneumoniae* M 5 al: cloning, nucleotide sequence and expression. *Gene*, **47**: 269-277

Blum H., Beier H. and Gross H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, **8**: 93-99

Bovetto L. J., Backer D. P., Villette J. R., Sacard P. J. and Bouquelet S. J. L (1992a) Cyclomaltodextrin glucanotransferase from *Bacillus circulans* E 192 I. Purification and characterization of the enzyme. *Biotechnol. Appl. Biochem.*, **15**: 48-58

Bovetto L. J., Villette J. R., Fontaine I. F., Sicard P. J. and Bouquelet S. J. L. (1992b) Cyclomaltodextrin glucanotransferase from *Bacillus circulans* E 192 II. Action patterns. *Biotechnol. Appl. Biochem.*, **15**: 59-68

Box G. P., Hunter W. G. and Hunter J. S. (1978) Statistics for experiments: An introduction to design, data analysis and model building. John Wiley and Sons Inc, New York

Brunet C., Lamare S. and Legoy M. -D. (1998) Studies of specific cyclodextrin production starting from pure maltooligosaccharides using *Thermoanaerobacter* sp. cyclodextrin glycosyltransferase. *Biocatalysis Biotransformation*, **16**: 317-327

Brunt K. (1982) Rapid separation of linear and cyclic oligo-saccharides on a cation exchange resin using a calcium ethylele diamine tetraacetate solute as eluent. *J. Chromatogr.*, **246**: 145-151

Cami P. and Majou D. (1992) Manufacture of cyclodextrins fron starch using *Bacillus ohbensis* cyclodextrin glycosyltransferase. EP Appl. 48 19 03 (CA 117: 129975)

Chan K., Shin H. -D. and Lee Y. -H. (1995) Selection of the constitutive mutant of *Bacillus firmus* var. alkalophilus and its characteristics of cyclodextrin glucanotransferase production. *J. Microbiol. Biotechnol.*, **5:** 61-67

Chen W. C., Lin J. M. and Liu K. J. (1994) Production of cyclodextrin glucanotransferase by fed-batch fermentation with sugar supply. *Zhongguo Nongye Huaxue Huizhi*, **32**: 565-73 (Chinese)

Chiu C. P. (1987) Cyclodextrins: 4. Effect of sodium lauryl sulphate (SDS) on the yield of cyclodextrins from starches. *Shih Pin K'o Hsueh (Taipei)*, **14**: 327-334 (Ch)

Chung H. -J., Yoon S. -H., Lee M. -J., Kim M. -J, Kweon K. -S., Lee I. -W., Kim J. -W., Oh B. -H., Lee H. -S., Spiridonova V. A. and Park K. -H. (1998) Characterization of a thermostable cyclodextrin glucanotransferase isolated from *Bacillus stearothermophilus* ET1. *J. Agric. Food Chem.*, **46**: 952-959

Cramer F. and Steinle D. (1955) Action of amylase from *Bacillus macerans*. *Justus Liebigs Anal. Der Chemie.*, **595**: 81-100

Creminon C., Pilard F., Grassi J., Perly B. and Pradelles P. (1994) A sensitive and specific enzyme immunoassay for cyclomaltoheptaose and some derivatives. *Carbohydr. Res.*, **258**: 179-186

Dalmia B. K., Schutte K. and Nikolov Z. L. (1995) Domain E of *Bacillus macerans* cyclodextrin glucanotransferase: An independent starch-binding domain. *Biotechnol. Bioeng.*, **47**: 575-584

Davies L. (1993) Efficiency in research, development, and production: The statistical design and analysis of chemical experiments. Royal Society of Chemistry, Cambridge

Delbourg M. F., Drouet Ph., De Moraes F., Thomas D. and Barbotin J. N. (1993) Effect of PEG and other additives on cyclodextrin production by *Bacillus macerans* cyclomaltodextrin-glycosyl-transferase. *Biotechnol. Lett.*, **15**: 157-162

DePinto J. A. and Campbell L. L. (1968) Purification and properties of the amylase of *Bacillus macerans*. *Biochemistry*, 7: 114-120

Desai L. and Pai J. S. (1996) Fermentation, purification and properties of cycloamylose (cyclodextrin) glucano transferase (CGTase) from *Bacillus macerans*. Indian J. Microbiol., **36**: 29-32

Do E. -J., Park J. -B. and Lee Y. -H. (1993a) Screening of a new alkalophilic strain for overproduction of cyclodextrin glucanotransferase. *Kor. J. Appl. Microbiol. Biotechnol.*, **21**: 119-124

Do E. -J., Shin H. -D., Kim C. and Lee Y. -H. (1993b) Selection and characterization of catabolite repression resistant mutant of *Bacillus firmus* var. alkalophilus producing cyclodextrin glucanotransferase. *J. Microbiol. Biotechnol.*, **3**: 78-85

Endo T., Nagase H., Ueda H., Kobayashi S. and Nagai T. (1997a) Isolation, purification, and characterization of cyclomaltodecaose (ε-cyclodxetrin), cyclomaltoundecaose (ζ-cyclodextrin) and cyclomaltotridecaose (θ-cyclodextrin). *Chem Pharm. Bull.* **45**: 532-536

Endo T., Nagase H., Ueda H., Shigihara A., Kobayashi S. and Nagai T. (1997b) Isolation, purification and characterization of cyclomaltotetradecaose (ι-cyclodxetrin), cyclomaltopentadecaose (κ-cyclodextrin), cyclomaltohexadecaose (λ-cyclodextrin), and cyclomaltoheptadecaose (μ-cyclodextrin). *Chem Pharm. Bull.* **45**: 1856-1859

Endo T., Nagase H., Ueda H., Shigihara A., Kobayashi S. and Nagai T. (1998) Isolation, purification and characterization of cyclomaltooctadecaose (ν-cyclodxetrin), cyclomaltononadecaose (ξ-cyclodextrin), cyclomaltoeicosaose (o-cyclodextrin), and cyclomaltoheneicosaose (π-cyclodextrin). *Chem Pharm. Bull.* **46**: 1840-1843

Endo T., Ueda H., Kobayashi S. and Nagai T. (1995) Isolation, purification, and characterization of cyclomaltododecaose (η-cyclodextrin). *Carbohydr. Res.* **269**: 369-373

Englbrecht A., Harrer G., Lebert M. and Schmid G. (1990) Biochemical and genetic characterization of a CGTase from an alkalophilic bacterium forming primarily γ-cyclodextrin. In: *Minutes of 5th International Symposium on Cyclodextrins*, 1990, Paris. Dunchene D. (Ed) pg. 25-31, de Sante, Paris

Feederle E., Pajatsch M., Kremmer E. and Böck A. (1996) Metabolism of cyclodextrins by *Klebsiella oxytoca* M 5 al: purification and characterisation of a cytoplasmically located cyclodextrinase. *Arch. Microbiol.*, **165**: 206-212

Ferrarotti S. A., Rosso A. M., Marechal M. A., Krymkiewicz N. and Marechal L. R. (1996) Isolation of two strains (S-R type) of *Bacillus circulans* and purification of a cyclomaltodextrin-glucanotransferase. *Cellular Mol. Biol.*, **42**: 653-657

Fiedler G., Pajatsch M. and Böck A. (1996) Genetics of a novel starch utilization pathway present in Klebisella oxytoca. *J. Mol. Biol.*, **256**: 279-291

Flaschel E., Andriamboavonjy E. R. and Renken A. (1996) Transfer and disproportionation reaction of the cyclodextrin glucanotransferase from *Klebsiella pneumoniae*. In: *Proceedings of the Eight International Symposium on Cyclodextrins*, 1996, Budapest, Szejtli J. and Szente L. (Eds) pg. 45-50, Kluwer, Dordrecht, Netherlands

Flaschel E., Landert J. P., Spiesser D. and Renken A. (1984) The production of α-cyclodextrin by enzymatic degradation of starch. *Ann. N. Y. Acad. Sci.*, **434**: 70-77

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

French D. and Rundle R. E. (1942) The molecular weights of the Schardinger α- and β-dextrins. *J. Am. Chem. Soc.* **64**: 1651-1653

French D., Knapp D. W. and Pazur J. H. (1950) Studies on Schardinger dextrins. VI. Molecular size and structure of γ-dextrin. *J. Am. Chem. Soc.* **72**: 5150-5152

French D., Pulley A. O., Efenburger J. A., Rougvie M. A. and Abdullar M. (1965) Studies on the Scahrdinger dextrins. XII. The molecular size and structure of the δ-, ε-, ζ-, η-dextrins. *Arch. Biochem. Biophys.* **111**: 153-160

Freudenberg K and Jacobi R. (1935) Schardinger's dextrins from starch. *Ann.* **518**: 102-108 (CA 29: 6213)

Freudenberg K. and Cramer F. (1948) Constitution of the Schardinger dextrins α, β and γ. *Naturforsch* **3b**: 464 (CA 44:1036)

Freudenberg K. and Meyer-Delius M. (1938) The Schardinger dextrins from starch. *Ber.* **71B**: 1596-1600 (CA 32: 8373)

Freudenberg K. and Rapp W. (1936) Starch and Schardinger dextrins. *Ber.* **69B**: 2041-2045 (CA 30: 8172)

Freudenberg K., Blomqvist G., Ewald L. and Soff K. (1936) Hydrolysis and acetolysis of starch and of the Schardinger dextrins. *Ber.* **69B**: 1258-1266 (CA 30: 5567)

Freudenberg K., Boppel H. and Meyer-Delius M. (1938) Starch. *Naturwissenschaften* **26**: 123-124 (CA 32: 5655)

Freudenberg K., Schaaf E., Dumpert G. and Ploetz T. (1939) New views on starch. *Naturwissenschaften* **27**: 850-853 (CA 34: 2632)

Frijlink H. W. Visser J. and Drenth B. F. H. (1987) Determination of cyclodextrins in biological fluids by high performance liquid chromatography with negative colorimetric detection using post-column complexation with phenolphthalein. *J. Chromatogr.*, **415**: 325-333

Fujita K., Ishigami H., Hara K. (1989) Separation and purification of cyclodextrin on ion exchange chromatography. In: *Proceedings of the Research Society of Japan Sugar Refineries Technologists* **37**: 93-103

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

Fujiwara S., Kakihara H., Woo K. B., Lejeune A., Kanemoto M., Sakaguchi K. and Imanaka T. (1992) Cyclization chracteristics of cyclodextrin glucanotransferase are conferred by the NH_2-terminal region of the enzyme. *Appl. Environ. Microbiol.*, **58**: 4016-4025

Fuwa H. (1954) A new method for microdetermination of amylase activity by the use of amylose as the substrate. *J. Biochem (Tokyo)*, **41**: 583-603

Gawande B. N. and Patkar A. Y. (1998) An improved process for the preparation of α-cyclodextrin glycosyltransferase enzyme. Indian Patent Application No. 1104/DEL/98

Gawande B. N. and Patkar A. Y. (1999) Application of factorial designs for optimization of cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae pneumoniae* AS-22. *Biotechnol. Bioeng.*, **64**: 168-173

Gawande B. N., Goel A., Patkar A. Y. and Nene S. N. (1999) Purification and properties of a novel raw starch degrading cyclomaltodextrin glucanotransferase from *Bacillus firmus*. *Appl. Microbiol. Biotechnol.*, **51**: 504-509

Gawande B. N., Singh R. K., Chauhan A. K., Goel A. and Patkar A. Y. (1998). Optimization of cyclomaltodextrin glucanotransferase production from *Bacillus firmus*. *Enzyme Microb. Technol.* **22:** 288-291

Georganta G., Kaneko T., Kudo T. and Horikoshi K. (1991) Expression of the CGTase gene of alkalophilic *Bacillus* no. 38-2 in various hosts. *Starch*, **43**: 361-363

Georganta G., Kaneko T., Nakamura N., Kudo T. and Horikoshi K. (1993) Isolation and partial properties of cyclomaltodextrin glucanotransferase-producing alkalophilic *Bacillus* sp. from a deep-sea mud sample. *Starch*, **45**: 95-99

Glickstein D. and Bar R. (1996) Isolation and characterization of a *Bacillus circulans* strain and its CGTase. In: *Proceedings of the Eight International Symposium on Cyclodextrins*, 1996, Budapest, Szejtli J. and Szente L. (Eds) pg. 63-66, Kluwer, Dordrecht, Netherlands

Goel A. (1995) Studies on the enzyme cyclodextrin glycosyltransferase from *Bacillus* sp. Ph. D. Thesis, University of Pune

Goel A. and Nene S. (1995a) A novel cyclomaltodextrin glucanotransferase from *Bacillus firmus* that degrades raw starch. *Biotechnol. Lett.*, **17**: 411-416

Goel A. and Nene S. (1995b) Modifications in the phenolphthalein method for spectrophotometric estimation of beta cyclodextrin. *Starch* **47**: 399-400

Griffiths D. W. and Bender M. L. (1973) Cycloamyloses as catalysts. *Adv. Cat.*, **23**: 209-261

Hale W. S. and Rawlins L. C. (1951) Amylase of *Bacillus macerans*. *Cereal Chem.*, **28**: 49-58

Hamaker K. and Tao B. Y. (1993) Screening of gamma cyclodextrin-producing recombinant *E. coli* using congo red dye on solid complex media. *Starch*, **45**: 181-182

Han I. -K. and Lee Y. -H. (1991) Production of cyclodextrin from raw starch in the agitated bead reaction system and its reaction mechanism. *Kor. J. Appl. Microbiol. Biotechnol.*, **19**: 163-170 (CA 119: 26773)

Han N. S. and Tao B. Y. (1999a) Detection of native and recombinant *Bacillus macerans* cyclodextrin glycosyltransferase using microtiter ELISA techniques. *Enzyme Microbial Technol.*, **24**: 35-40

Han N. S. and Tao B. Y. (1999b) A simple method to express soluble, highly active cyclodextrin glycosyltransferase in recombinant *E. coli*. *Biotechnol. Tech.*, **13**: 631-635

Hanus J., Kucera J., Kodet J., Masilkova A. (1974) Starch gel for gel filtration. *J. Chromatogr.*, **97**: 270-272

Harata K. (1996) Crystallographic studies. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 279-304, Pergamon, U. K. (review)

Harata K., Haga K., Nakamura A., Aoyagi M. and Yamane K. (1996) X-ray structure of cyclodextrin glucanotransferase from alkalophlic *Bacillus* sp. 1011. Comparision of two independent molecules at 1.8 Å resolution. *Acta Cryst.* **D52**: 1136-1145

Harrison D. E. F. (1972) Physiological effects of dissolved oxygen tension and redox potential on growing population of microorganisms. *J. Appl. Chem. Biotechnol.*, **22**: 417-440

Hashimoto H. (1996) Cyclodextrins in foods, cosmetics and toiletries. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 483-502, Pergamon, U. K. (review)

Hedges A. R. (1992) Cyclodextrin: Production, properties and applications, pp. 319-333. In: F. Schenk and R. E. Hebeda (Eds.), Starch hydrolysis products. VCH publishers, New York

Hellman J., Paavilainen S. and Mantsala P. (1992) Expression in *E. coli* and purification of intra-cellular proteins by fusion to cyclomaltodextrin glucanotransferase. *J. Biotechnol.*, **26**: 275-288

Hofmann B. E., Bender H. and Schulz G. E. (1989) Three-dimentional structure of cyclodextrin glycosyltransferase from *Bacillus circulans* at 3.4 Å resolution. *J. Mol. Biol.*, **209**: 793-800

Hokse H. (1980) Analysis of cyclodextrin by high performance liquid chromatography. *J. Chromatogr.*, **189**: 98-100

Hokse H. (1983) Colorimetric analysis of cyclomaltooctaose (γ-cyclodextrin). *Carbohydr. Res.*, **114**: 303-305

Horikoshi K. (1979) Production and industrial applications of β-cyclodextrin. *Proc. Biochem.*, **14**: 26-30

Horikoshi K. and Akiba T. (1982) Cyclodextrin glycosyltransferase. In: Alkalophilic microorganisms - a new microbial world. pg. 104-110, Japan Scientific Society Press, Tokyo

Horikoshi K., Nakamura N., Matsuzawa N. and Yamamoto M. (1981) Industrial production of cyclodextrins. In: *Proceedings of the First International Symposium on Cyclodextrins*, Budapest, 1981, Szejtli J. (Ed), pg. 25-39, D. Reidel Publishing Company, Dordrecht

Hounsa C. G., Aubry J. M., Dubourguier H. C. and Hornez J. P. (1996) Application of factorial and Doehlert designs for optimization of pectate lyase production by a recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, **45:** 764-770

Hwang J. -B., Kim S. -H., Lee T. -K. and Yang H. -C. (1990) Production of cyclomaltodextrin from *Bacillus stearothermophilus*. *Kor. J. Appl. Microbiol. Biotech.* **18:** 578-584

ICUMSA method (1998) Determination of starch content in sugar and sugar house products. International Commission for Uniform Methods of Sugar Analysis (ICUMSA). Edition 1998, ICUMSA Publication Department, Norwich, England

Ismail A. S., Sobieh U. I. and Abdel-Fattah A. F. (1996) Biosynthesis of cyclodextrin glucosyltransferase and β-cyclodexrtin by *Bacillus macerans* 314 and properties of the crude enzyme. *Chem. Eng. J.*, **61**: 247-253

Ito Y., Hasegawa N. and Suzuki K. (1990) Enzymic manufacture of cyclodextrin from extruded starch. JP 01168295 (CA 112: 199974)

Jamuna R., Saswathi N., Sheela R. and Ramakrishna S. V. (1993) Synthesis of cyclodextrin glucosyltransferase by *Bacillus cereus* for the production of cyclodextrins. *Appl. Biochem. Biotechnol.*, **43**: 163-176

Japan Maize Products Co., Ltd., Japan (1980) α-cyclodextrin. JP 55102396 (CA 93: 219321)

Jeang C. -L. and Lin Y. -W. (1994) Evidence for the presence of essential histidines on cyclodextrin glucanotransferase from *Bacillus macerans*. *Biotechnol. Appl. Biochem.*, **19**: 85-92

Jeang C. -L., Chiang C. -M., Fang T. -Y. and Sung H. -Y. (1992) Purification and partial characterization of cyclodextrin glucanotransferase from *Bacillus macerans*. *J. Chi. Biochem. Soc.*, **21**: 68-77

Jermyn M. A. (1971) Increasing the sensitivity of the anthrone method for carbohydrate. *Anal. Biochem.*, **68**: 332-335

Jogdand V. V. and Karanth N. G. (1983) A suitable sampling device for highly viscous fermentation broth. *Biotechnol. Bioeng.*, **25**: 1677-1678

Jorgensen S. T., Tangney M., Jorgensen P. L., Diderichsen B. (1998) Integration and amplification of a cyclodextrin glycosyltransferase gene from *Thermoanaerobacter* sp. ATCC 53627 on the *Bacillus subtilis* chromosome. *Biotechnol. Tech.*, **12**: 15-19

Jorgensen S. T., Tangney M., Starnes R. L., Amemiya K. and Jorgensen P. L. (1997) Cloning and nucleotide sequence of a thermostable cyclodextrin glycosyltransferase gene from *Thermoanaerobacter* sp. ATCC 53627 and its expression in *Escherichia coli*. *Biotechnol Lett.*, **19**: 1027-1031

Kaneko T., Hamamoto T. and 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

Kaneko T., Kato T., Nakamura N. and Horikoshi K. (1987) Spectrophotometric determination of cyclization activity of β-cyclodextrin forming cyclomaltodextrin glucanotransferase. *J. Jpn. Soc. Starch Sci.*, **34**: 45-48

Kaneko T., Song K. -B., Hamamoto T., Kudo T. and Horikoshi K. (1989) Construction of a chimeric series of *Bacillus* cyclodextrin glucanotransferase and analysis of the thermal stabilities and pH optima of the enzymes. *J. Gen. Microbiol.*, **135**: 3447-3457

Kaneko T., Yoshida M., Yamamoto M., Nakamura N. and Horikoshi K. (1990) Production of cyclodextrins by simultaneous action of two CGTases from three strains of *Bacillus*. *Starch*, **42**: 277-281

Kato T. and Horikoshi K. (1984) Colorimetric determination of gamma cyclodextrin. *Anal. Chem.*, **56**: 1738-1740

Kato T. and Horikoshi K. (1986a) γ-Cyclodextrin: A new γ-cyclodextrin forming enzyme produced by *Bacillus subtilis* no. 313. *J. Jpn. Soc. Starch Sci.*, **33**: 137-143

Kato T. and Horikoshi K. (1986b) Cloning and expression of the *Bacillus subtilis* No. 313 γ-cyclodextrin forming CGTase gene in *Escherichia coli*. *Agric. Biol. Chem.* **50**: 2161-2162

Kim T. -J., Kim B. -C. and Lee S. -H. (1997a) Production of cyclodextrins using raw corn starch without a pretreatment. *Enzyme Microbiol. Technol.*, **20**: 506-509

Kim D. -O., Park K., Song J. -W. and Seo J. -H. (1997b) Enhanced production of maltotetraose-producing amylase by recombinant *Bacillus subtilis* LKS88 in Fed-batch cultivation. *J. Microbiol. Biotechnol.*, 7: 417-422

Kim G. J., Lee I. Y., Choi D. K., Yoon S. C. and Park Y. H. (1996) High cell-density cultivation of *Pseudomonas putida* BM01 using glucose. *J. Microbiol. Biotechnol.*, **6**: 221-224

Kim M. H., Sohn C. B. and Oh T. K. (1998) Cloning and sequencing of a cyclodextrin glycosyltransferase gene from *Brevibacillus brevis* CD 162 and its expression in *Escherichia coli*. *FEMS Microbiol. Lett.*, **164**: 411-418

Kim T. -J., Kim B. -C. and Lee S. -H. (1995) Production of cyclodextrins using moderately heat-treated cornstarch. *Enzyme Microbiol. Technol.*, **17**: 1057-1061

Kim T. -J., Lee Y. -D. and Kim H. -S. (1992) Production of cyclodextrins from unliquefied corn starch using CGTase in a membrane bioreactor. *Ann. N. Y. Acad. Sci.*, **672**: 552-557

Kim T. -J., Lee Y. -D. and Kim H. -S. (1993) Enzymatic production of cyclodextrins from milled corn starch in an ultrafiltration membrane bioreactor. *Biotechnol. Bioeng.*, **41**: 88-94

Kimura K., Ishii Y., Kataoka S., Takano T. and Yamane K. (1990) Expression of the β-cyclodextrin glucanotransferase gene of an alkalophilic *Bacillus* sp.# 1011 in *Escerichia coli* cells and characterization of the synthesized enzyme. *Agric. Biol. Chem.*, **54**: 641-648

Kimura K., Takano T. and Yamane K. (1987) Molecular cloning of the β-cyclodextrin synthetase gene from an alkalophilic *Bacillus* and its expression in *Escerichia coli* and *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.*, **26**: 149-153

Kitahata S. and Okada S. (1974) Action of cyclodextrin glycosyltransferase from *Bacillus megaterium* strain no. 5 on starch. *Agric. Biol. Chem.*, **38**: 2413-2417

Kitahata S. and Okada S. (1982a) Purification and some properties of Cyclodextrin glucanotransferase from *Bacillus stearothermophilus* TC-60. *J. Jpn. Soc. Starch Sci.*, **29**: 7-12

Kitahata S. and Okada S. (1982b) Comparision of action of cyclodextrin glucanotransferase from *Bacilluc megaterium*, *Bacillus circulans*, *Bacillus stearothermophilus* and *Bacillus macerans*. *J. Jpn. Soc. Starch Sci.*, **29**: 13-18

Kitahata S., Tsuyama N. and Okada S. (1974) Purification and some properties of cyclodextrin glycosyltransferase from a strain of *Bacillus* species. *Agri. Biol. Chem.*, **38**: 387-393

Kitahata S., Yoshikawa S. and Okada S. (1978) Determination of α-, β- and γ-cyclodextrins by high performance liquid chromatography. *J. Jpn. Soc. Starch Sci.*, **25**: 19-23

Kitamoto N., Kimura T., Kito Y. and Ohmiya K. (1992) Cloning and sequencing of the gene encoding cyclodextrin glucanotransferase from *Bacillus* sp. KC201. *J. Ferment. Bioeng.*, **74**: 345-351

Klein C. and Schulz G. E. (1991) Structure of cyclodextrin glycosyltransferase refined at 2.0 Å resolution. *J. Mol. Biol.*, **217**: 737-750

Klein C., Hollender J., Bender H. and Schulz G. E. (1992) Catalytic center of cyclodextrin glycosyltransferase derived from X-ray structure analysis combined with site directed mutagenesis. *Biochemistry* **31**: 8740-8746

Knegtel R. M. A., Strokopytov B., Penninga D., Faber O. G., Rozeboom H. J., Kalk K. H., Dijkhuizen L. and Dijkstra B. W. (1995) Crystallographic studies of the interaction of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 with natural substrates and products. *J. Biol. Chem.*, **270**: 29256-29264

Knegtel R. M. A., Wind R. D., Rozeboom H. J., Kalk K. H., Buitelaar R.M., Dijkhuizen L., Dijkstra B. W. (1996) Crystal structure at 2.3 Å resolution and revised nucleotide sequence of the thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM 1. *J. Mol. Biol.*, **256**: 611-622

Kobayashi S., Kainuma K. and Suzuki S. (1977) Cyclodextrin production. JP 52079039 (CA 87: 150201)

Kobayashi S., Kainuma K. and Suzuki S. (1978) Purification and some properties of *Bacillus macerans* cycloamylose (cyclodextrin) glucanotransferase. *Carbohydr. Res.*, **61**: 229-238

Kobayashi S., Kainuma K. and Suzuki S. (1983) Effect of surfactants on cyclization of *Bacillus macerans* CGT. *J. Jpn. Soc. Starch Sci.*, **30**: 62-68 (CA 99: 49495)

Kole M. M. and Gerson D. F. (1989) Ammonium controlled fed-batch fermentation for amylase production. *J. Ferment. Bioeng.*, **68**: 423-427

Kometani T., Terada Y. and Nishimura T., Takii H. and Okada S. (1994) Purification and characterization of cyclodextrin glucanotransferase from an alkalophilic *Bacillus* sp. and transglycosylation at alkaline pHs. *Biosci. Biotechnol. Biochem.*, **58**: 517-520

Komiyama M. (1996) Cyclodextrins as enzyme models. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 401-422, Pergamon, U. K. (review)

Kondo H., Nakatani H. and Hiromi K. (1976a) Analysis of mixtures of α- and β-cyclodextrins using fluorescent dyes. *Carbohydr. Res.*, **52**: 1-10

Kondo H., Nakatani H. and Hiromi K. (1976b) Interaction of cyclodextrins with fluorescent probes and its applications to kinetic studies of amylase. *J. Biochem.*, **79**: 393-405

Kubota M., Matsuura Y., Sakai S. and Katsube Y. (1991) Molecular structure of *B. stearothermophilus* cyclodextrin glucanotransferase and analysis of substrate binding site. *J. Jpn. Soc. Starch Sci.*, **38**: 141-146

Kwon S., Kim S. and Kim E. (1996) Effects of glycerol on β-lactamase production during high cell density cultivation of recombinant *Escherichia coli*. *Biotechnol. Prog.*, **12**: 205-208

Laakso S., Leivo P., Makela M. and Korpela T. (1984) A polarographic cyclodextrin assay based on linoleate-cyclodextrin complex formation in a lipoxygenase reaction. *Starch*, **36**: 432-435

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685

Landert J. -P., Flaschel E. and Renken A. (1981) A photometric test for the cyclisation activity of cyclodextrin-glycosyltransferases. In: *Proceedings of the First International Symposium on Cyclodextrins*, Budapest, 1981, Szejtli J. (Ed), pg. 89-94, D. Reidel Publishing Company, Dordrecht

Lane A. G. and Pirt S. J. (1973) Production of cyclodextrin glycosyltransferase by batch and chemostat culture of *Bacillus macerans* in chemically determined medium. *J. Appl. Chem. Biotechnol.*, **23**: 309-321

Larsen K. L., Duedahl-Olesen L., Christensen H. J. S., Mathiesen F., Pedersen L. H. and Zimmermann W. (1998a) Purification and charcterisation of cyclodextrin glycosyltransferase from *Paenibacillus* sp. F8. *Carbohydr. Res.*, **310**: 211-219

Larsen K. L., Christensen H. J. S., Mathiesen F., Pedersen L. H. and Zimmermann W. (1998b) Production of cyclomaltononaose (δ-cyclodextrin) by cyclodextrin glycosyltransferases from *Bacillus* spp. and bacterial isolates. *Appl. Microbiol. Biotechnol.*, **50**: 314-317

Lawson C. L., Bergsma J., Bruinenberg P. M., de Vries G. E., Dijkhuizen L. and Dijkstra B. W. (1990) Maltodextrin-dependent crystallization of cyclomaltodextrin glucanotransferase from *Bacillus circulans*. *J. Mol. Biol.*, **214**: 807-809

Lawson C. L., van Montfort R., Strokopytov B., Rozeboom H. J., Kalk K. H., de Vries G. E., Penninga D., Dijkhuizen L. and Dijkstra B. W. (1994) Nucleotide sequence and x-ray structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form. *J. Mol. Biol.*, **236**: 590-600

Lee I. Y., Choi E. S., Kim G. J., Nam S. W., Shin Y. C., Chang H. N. and Park Y. H. (1994) Optimization of fed-batch fermentation for production of poly-β-hydroxybutyrate in Alcaligenes eutrophus. *J. Microbiol. Biotechnol.*, **4**: 146-150

Lee J. -H., Choi K. -H., Choi J. -Y., Lee Y. -S., Kwon I. -B. and Yu J. -H. (1992) Enzymatic production of α-cyclodextrin with the cyclodextrin glucanotransferase of *Klebsiella oxytoca* 19-1. *Enzyme Microb. Technol.*, **14**: 1017-1020

Lee K. C. P. and Tao B. Y. (1994) High-level expression of cyclodextrin glycosyltransferase in *E. coli* using a T7 promoter expression system. *Starch* **46**: 67-74

Lee S. -L and Chen W. -C. (1997) Optimization of medium composition for the production of glucosyltransferase by *Aspergillus niger* with response surface methodology. *Enzyme Microb. Technol.*, **21:** 436-440

Lee S. Y. (1996) High cell-density culture of *Escherichia coli*. *TIBTECH*, **14**: 98

Lee Y. -D. and Kim H. -S. (1991a) Enzymatic production of cyclodextrin from unliquefied corn starch in an atrition bioreactor. *Biotechnol. Bioeng.*, **37**: 795-801

Lee Y. -D. and Kim H. -S. (1991b) Enhancement of enzymic production of cyclodextrins by organic solvents. *Enzyme Microb. Technol.*, **13**: 499-503

Lee Y. -D. and Kim H. -S. (1992) Effect of organic solvents on enzymatic production of cyclodextrins from unliquefied corn starch in an attrition bioreactor. *Biotechnol. Bioeng.*, **39**: 977-983

Lee Y. -H. and Park D. -C. (1991) Enzymic synthesis of cyclodextrin in an heterogeneous enzyme reaction system containing insoluble extruded starch. *Kor. J. Appl. Microbiol. Biotechnol.*, **19**: 514-520 (CA 119: 47514)

LeGendre N., Mansfield M., Weiss A. and Matsudaira P. (1993) In: A practical guide to protein and peptide purification for microsequencing. Matsudaira P. (Ed), pg. 71, Academic Press, London

Lejeune A., Sakaguchi K. and Imanaka T. (1989) A spectrophotometric assay for the cyclization activity of cyclomaltohexaose (α-cyclodextrin) glucanotransferase. *Anal. Biochem.*, **181:** 6-11

Li W., Zhou J., Zhang L., Corke H. and Yang L. (1996) Factors affecting the determination of β-cyclodextrin by phenolphthalein spectrophotometry. *Anal. Lett.*, **29**: 1201-1213

Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.*,**193**: 265-275

Makela M. J. and Korpela T. K. (1988) Determination of the catalytic activity of cyclomaltodextrin glucanotransferase by maltotriose-methyl orange assay. *J. Biochem. Biophys. Methods*, **15**: 307-318

Makela M. J., Korpela T. K. and Laakso S. V. (1987) Colorimetric determination of beta cyclodextrin: two assay modifications based on molecular complexation of phenolphthalein. *J. Biochem. Biophys. Methods*, **14**: 85-92

Makela M. J., Korpela T. K., Puisto J. and Laakso S. V. (1988a) Nonchromatographic cyclodextrin assays: Evaluation of sensitivity, specificity, and conversion mixture applications. *J. Agric. Food Chem.*, **36**: 83-88

Makela M. J., Mattsson P., Schinina M. E. and Korpela T. K. (1988b) Purification and properties of cyclomaltodextrin glucanotransferase from an alkalophilic *Bacillus*. *Biotechnol. Appl. Biochem.*, **10**: 414-427

Makela M. J., Mattsson P. and Korpela T. (1989) Specific adsorbents in isolation and purification of cyclodextrins. *Biotechnol. Appl. Biochem.*, **11**: 193-200

Makela M. J., Paavilainen S. K. and Korpela T. K. (1990) Growth dynamics of cyclomaltodextrin glucanotransferase producing *Bacillus circulans* var. *alkilophilus*. *Can. J. Microbiol.*, **36**: 176-182

Mao W., Pan R. and Freedman D. (1992) High production of alkaline protease by *Bacillus licheniformis* in a fed-batch fermentation using a synthetic medium. *J. Ind. Microbiol.*, **11**: 1-6

Marechal L. R., Rosso A. M., Marechal M. A., Krymkiewicz N. and Ferrarotti S. A. (1996) Some properties of a cyclomaltodextrin-glucanotransferase from *Bacillus circulans* DF 9 R type. *Cellular Mol. Biol.*, **42**: 659-664

Martin C. and Smith A. M. (1995) Starch biosynthesis. *Plant Cell* **7**: 971-985

Maruo S., Kyotani Y., Yamamoto H., Miyazaki K., Ogawa T., Kojima M. and Ezure Y. (1993) Effect of moranoline, 4-o-α-D-glucopyranosylmoranoline and their *N*-substituted derivatives on thermostability of cyclodextrin glycosyltransferase, glucoamylase, and β-amylase. *Biosci. Biotech. Biochem.*, **57**: 1294-1298

Matioli G., Zanin G. M., Guimarães M. F. and de Moraes F. F. (1996) Screening for gamma-CGTase. In: *Proceedings of International Symposium on Cyclodextrins, 8th*, Szejtli J. and Szente L. (Eds), pg. 55-58, Kluwer, Dordrecht, Netherlands

Matioli G., Zanin G. M., Guimarães M. F. and de Moraes F. F. (1998) Production and purification of CGTase of alkalophylic *Bacillus* isolated from Brazilian soil. *Appl. Biochem. Biotechnol.* **70-72**: 267-275

Mattsson P., Battchikova N., Sippola K. and Korpela T. (1995) The role of histidine residues in the catalytic act of cyclomaltodextrin glucanotransferase from *Bacillus circulans* var. *alkalophilus*. *Biochem. Biophys. Acta*, **1247**, 97-103

Mattsson P., Korpela T., Paavilainen S. and Makela M. (1991) Enhanced conversion of starch to cyclodextrins in ethanolic solutions by *Bacillus circulans* var.alkalophilic cyclomaltodextrin glucanotransferase. *Appl. Biochem. Biotechnol.*, **30**: 17-28

Mattsson P., Makela M. and Korpela T. (1988) Isolation and purification of gamma-cyclodextrin by affinity chromatography. In: *Proceedings of International Symposium on Cyclodextrins*, 4th, Huber O. and Szejtli J. (Eds). pg. 65-70., Kluwer, Dordrecht, Netherlands

Mattsson P., Pohjalainen T. and Korpela T. (1992) Chemical modification of cylomaltodextrin glucanotransferase from *Bacillus circulans* var. *alkalophilus*. *Biochem. Biophys. Acta.*, **1122**: 33-40

Matzuzawa M., Kawano M., Nakamura N. and Horikoshi K. (1975) An improved method for the preparation of Schardinger β-dextrin on a industrial scale by cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. (ATCC 21783). *Starch* **27**: 410-413

McClenahan W. S., Tilden E. B. and Hudson C. S. (1942) A study of the products obtained from starch by the action of the amylose of *Bacillus macerans*. *J. Am. Chem. Soc.* **64**: 2139-2144

Means G. E. and Feeny R. E. (1971) Chemical modification of proteins. Holden-Day, Inc., San Francisco

Meesters P. A. E. P.; Huijberts G. N. M. and Eggink, G. (1996) High cell density cultivation of the lipid accumulation yeast *Cryptococcus curvatus* using glycerol as a carbon source. *Appl. Microbiol. Biotechnol.*, **45**: 575-579

Miller G. L. (1959) Use of dinitro salicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, **31**: 426-428

Miyazawa I., Ueda H., Nagase H., Endo T., Kobayashi S. and Nagai T. (1995) Physicochemical properties and inclusion complex formation of δ-cyclodextrin. *Eur. J. Pharm. Sci.* **3**: 153-162 (CA 123: 65655)

Montgomery D. C. (1991) Design and analysis of experiments. 3rd. (Ed.), John Wiley & Sons, New York

Mori S., Goto M., Mase T., Matsuura A., Oya T. and Kitahata S. (1995) Reaction conditions for the production of γ-cyclodextrin by cyclodextrin glucanotransferase from *Brevibacterium* sp. No. 9605. *Biosci. Biotechnol. Biochem.*, **59**: 1012-1015

Mori S., Hirose S., Oya T. and Kitahata S. (1994) Purification and properties of cyclodextrin glucanotransferase from *Brevibacterium* sp. No. 9605. *Biosci. Biotechnol. Biochem.*, **58**: 1968-1972

Morita T., Yoshida N. and Karube I. (1996) A novel synthesis method for cyclodextrins from maltose in water-organic solvent systems. *Appl. Biochem. and Biotechnol.*, **56**: 311-324

Mosi R. Sham H., Uitdehaag J. C. M., Ruiterkamp R., Dijkstra B. W. and Withers S. G. (1998) Reassessment of acarbose as a transition state analogue inhibitor of cyclodextrin glycosyltransferase. *Biochemistry*, **37**: 17192-17198

Nagai T. and Ueda H. (1996) Aspects of drug formulations with cyclodextrins. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 441-450, Pergamon, U. K. (review)

Nakamura A., Haga K. and Yamane K. (1993) Three histidine residues in the active center of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011:

Effect of the replacement on pH dependence and transition-state stabilization. *Biochemistry*, **32**: 6624-6631

Nakamura A., Haga K. and Yamane K. (1994a) Four aromatic residues in the active center of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011. *Biochemistry*, **33**: 9929-9936

Nakamura A., Haga K. and Yamane K. (1994b) The transglycosylation reaction of cyclodextrin glucanotransferase is operated by a Ping-Pong mechanism. *FEBS Lett.*, **337**: 66-70

Nakamura A., Haga K., Ogawa S., Kuwano K., Kimura K. and Yamane K. (1992) Functional relationships between cyclodextrin glucanotransferase from an alkalophilic *Bacillus* and α-amylases. Site-directed mutagenesis of the conserved two Asp and one Glu residues. *FEBS Lett.*, **296**: 37-40

Nakamura N. and Horikoshi K. (1976a) Characterization and some cultural conditions of a cyclodextrin glycosyltransferase producing alkalophilic *Bacillus* sp. *Agri. Biol. Chem.*, **40**: 753-757

Nakamura N. and Horikoshi K. (1976b) Purification and properties of cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. *Agri. Biol. Chem.*, **40**: 935-941

Nakamura N. and Horikoshi K. (1976c) Characterization of acid cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. *Agri. Biol. Chem.*, **40**: 1647-1648

Nakamura N. and Horikoshi K. (1976d) Purification and properties of neutral cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. *Agri. Biol. Chem.*, **40**: 1785-1791

Nakanishi K., Tomita N., Fukushige T., Akano H., Kawamura K. and Nakano S. K. (1993) Manufacture of cyclodextrin with cyclodextrin synthetase. JP 93103683 (CA 119: 47620)

Nishida T., Nakamura A., Masaki H. and Uozumi T. (1997) Regulation of cyclodextrin glucanotransferase synthesis in *Bacillus ohbensis*. *FEMS Microbiol. Lett.*, **149**: 221-226

Nitschke L., Heeger K., Bender H. and Schulz G. E. (1990) Molecular cloning, nucleotide sequence and expression in *E. coli* of the β-cyclodextrin gene from *Bacillus circulans* strain no. 8. *Appl. Microbiol. Biotechnol.*, **33**: 542-546

Nogrady N., Pocsi I. and Szentirmai A. (1995) Cyclodextrin glycosyltransferase may be the only starch degrading enzyme in *Bacillus macerans*. *Biotechnol. Appl. Biochem.*, **21**: 233-243

Nogrady N., Pocsi I. and Szentirmai A. (1996a) Isolation and characterization of the intra- and extra-cellular cyclodextrin glycosyltransferases of *Bacillus macerans*. *Acta Microbiol. Immunol. Hung.*, **43**: 268

Nogrady N., Pocsi I., Katona E., Jeney V., Boross P., Tozser J., Fachet J. and Szentirmai A. (1996b) Soluble cell-bound and extracellular cyclodextrin glycosyltransferases

of *Bacillus macerans* show identical enzymological characteristics and antigenicity. *J. Basic Microbiol.*, **36**: 335-340

Nogrady N., Pocsi I., Paffard S. M., Katona E., Miles R. J., Balazs A., Sandor E., Fachet J., Price R. G. and Szentirmai A. (1998) Development of ELISA and enzyme-linked immunofiltration assay (ELIFA) methods for monitoring cyclodextrin glycosyltransferase (CGTase) production and bacterial growth in *Bacillus macerans* batch cultures. *J. Biotechnol.*, **60**: 15-22

Nomoto M., Chen C. C. and Sheu D. C. (1986) Purification and characterization of cyclodextrin glucanotransferase from an alkalophilic bacterium of Taiwan. *Agric. Biol. Chem.*, **50**: 2701-2707

Nomoto M., Sheu D. C., Chen S. J., Yen T. M., Yen C., Liao W. and Yang C. P. (1984) Cyclodextrin glucanotransferase from alkalophilic bacteria of Taiwan. *Agric. Biol. Chem.*, **48**: 1337-1338

Norberg E. and French D. (1950) Schardinger dextrins. III. Redistribution reactions of macerans amylase. *J. Am. Chem. Soc.* **72**: 1202-1205 (CA 44: 6394)

Norman B. E. and Jorgensen S. T. (1992) *Thermoanarobacter* sp. CGTase: its properties and application. *J. Jpn. Soc. Starch Sci.*, **39**: 101-108

Oakes J. V., Shevmaker C. K. and Stalker D. M. (1991) Production of cyclodextrins, a novel carbohydrate in the tubers of transgenic potato plants. *Bio/Technol.*, **9**: 982-986

Ohnishi M., Azuma T. and Kubota M. (1994) Cycloamylose glucanotransferase-catalysed cyclization of a substrate maltose: Modification with *N*-bromosuccimide on the tryptophan residues. *Starch*, **46**: 276-279

Ohnishi M., Ota U., Abe M., Tonomura B. and Kubota M. (1992) Effects of modification of the tryptophan residues of cyclodextrin glucanotransferase with *N*-bromosuccinimide on the enzyme catalyzed hydrolysis (cleavage) of soluble starch and cyclomaltohexaose. *Carbohydr. Res.*, **227**: 285-291

Okada S. and Tsujama M. (1973) Enzymic conversion of starch to β-cyclodextrin. JP 73 40 996 (CA 79: 113990)

Orskov I. (1984) Bergey's manual of Systematic Bacteriology, Volume 1. pp. 461-465. N. R. Krieg and J. G. Holt (Eds.), Williams and Wilkins, Baltimore

Paloheimo M., Haglund D., Aha S. and Korhola M. (1992) Production of cycomaltodextrin glucanotransferase of *Bacillus circulans* var. alkalophilus ATCC 21783 in *Bacillus subtilis*. *Appl. Mirobiol. Biotechnol.*, **36**: 584-591

Park C. S., Park K. H. and Kim S. H. (1989) A rapid screening method for alkaline beta CGTase using phenolphthalein - methyl orange containing solid medium. *Agric. Biol. Chem.*, **53**: 1167-1169

Park C. S., Woo E. J. Kuk S. U., Seo B. C., Park K. H. and Lim H. (1992) Purification and charcterization of cyclodextrin glucanotransferase from *Bacillus* sp. E1. *Kor. J. Microbiol. Biotechnol.*, **20**: 156-163

Park Y. -C, Kim C. S., Kim C. I., Choi K. H. and Seo J. -H. (1997) Fed-batch fermentations of recombinant *Escherichia coli* to produce *Bacillus macerans* CGTase. *J. Microbiol. Biotechnol.*, **7**: 323-328

Parsiegla G., Schmidt A. K. and Schulz G. E. (1998) Substrate binding to a cyclodextrin glycosyltransferase and mutations increasing the γ-cyclodextrin production. *Eur. J. Biochem.*, **255**: 710-717

Patkar A and Seo J. -H. (1992) Fermentation kinetics of recombinant yeast in batch and fed-batch cultures. *Biotechnol. Bioeng.*, **40**: 103-109

Penninga D., Strokopytov B., Rozeboom H. J., Lawson C. L., Dijkstra B. W., Bergsma J. and Dijkhiuzen L. (1995) Site-directed mutations in tyrosine 195 of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 affect activity and product specificity. *Biochemistry* **34**: 3368-3376

Penninga D., van der Veen B. A., Knegtel R. M. A., van Hijum S. A. F. T., Rozeboom H. J., Kalk K. H., Dijkstra B. W. and Dijkhuizen L. (1996) The raw starch binding domain of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251. *J. Biol. Chem.*, **271**: 32777-32784

Pho D. B., Roustan C., Tot A. N. T. and Pradel L. -A. (1977) Evidence for an essential glutamyl residue in yeast hexokinase. *Biochemistry*, **16**: 4533-4537

Pocsi I., Nogrady N. and Szentirmai A. (1998a) Assay for simultaneous determination of β-cyclodextrin glycosyltransferase (β-CGTase) and amylase. *Starch*, **50**: 36-38

Pocsi I., Nogrady N., Liptak A. and Szentirmai A. (1998b) Cyclodextrins are likely to induce cyclodextrin glycosyltransferase production in *Bacillus macerans*. *Folia Microbiol.*, **43**: 71-74

Pongsawasdi P. and Yagisawa M. (1987) Screening and identification of a cyclomaltodextrin glucanotrnasferase-producing bacteria. *J. Ferment. Technol.*, **65**: 463-467

Pongsawasdi P. and Yagisawa M. (1988) Purification and some properties of cyclomaltodextrin glucanotransferase from *Bacillus circulans*. *Agri. Biol. Chem.*, **52**: 1099-1103

Prema P., Sreedharan V. P., Raja K. C. M. and Ramakrishna S. V. (1990) Studies on cyclodextrin glycosyltransferase production and characterization. In: *Minutes of 5th International Symposium on Cyclodextrins*, 1990, Paris. Dunchene D. (Ed) pg. 46-49, de Sante, Paris

Pulley A. O. and French D. (1961) Studies of the Schardinger dextrins. XI. The isolation of new Schardinger dextrins. *Biochem. Biophys. Res. Commun.* **5**: 11-15

Raja K. C. M. and Ramakrishna S. V. (1994) Improved reaction conditions for preparation of β-cyclodextrin (β-CD) from cassava (*Manihot esculenta* crantz) starch. *Starch*, **46**: 402-403

Raja K. C. M., Sreedharan V. P., Prema P. and Ramakrishna S. V. (1990) Cyclodextrin from cassava (*Manihot esculenta* Crantz) starch: isolation and characterization as bromobenzene and chloroform clathrates. *Starch*, **42**: 196-198

Ramakrishna S. V., Saswathi, N., Sheela R. and Jamuna R. (1994) Evaluation of solid, slurry and submerged fermentations for the production of cyclodextrin glycosyltransferase by *Bacillus cereus*. *Enzyme Microb. Technol.*, **16**: 441-444

Rendleman J. A., Jr. (1992) Enhanced production of cyclomaltooctaose (γ-cyclodextrin) through selective complexation with C_{12} cyclic compounds. *Carbohydr. Res.*, **230**: 343-359

Rendleman J. A., Jr. (1996a) Influence of saccharides as inhibitors of cyclodextrin production. *Biotechnol. Appl. Biochem.*, **24**: 121-127

Rendleman J. A., Jr. (1996b) Enzymic conversion of malto-oligosaccharides and maltodextrin into cyclodextrin at low temperature. *Biotechnol. Appl. Biochem.*, 24: 129-137

Rendleman J. A., Jr. (1997) Enhancement of cyclodextrin production through use of debranching enzymes. *Biotechnol. Appl. Biochem.*, **26**: 51-61

Robertson E. F., Dannelly H. K., Malloy P. J. and Reeves H. C. (1987) Rapid isoelectric focusing in a vertical polyacrylamide mini gel system. *Anal. Biochem.*, **167**: 290-294

Sabioni J. G. and Park Y. K. (1992a) Cyclodextrin glycosyltransferase production by alkalophilic *Bacillus lentus*. *Rev. Microbiol.*, São Paulo, **23**: 128-132

Sabioni J. G. and Park Y. K. (1992b) Production and characterization of cyclodextrin glycosyltransferase from *Bacillus lentus*. *Starch* **44**: 225-229

Saenger W. (1980) Cyclodextrin inclusion compounds in research and industry. *Angew. Chem.*, **19**: 344-362

Saenger W. (1984) Structural aspects of cyclodextrins and their inclusion complexes. In: *Inclusion compounds. Vol. 3. Physical properties and applications*. Atwood J. L., Davies J. E. D. and MacNicol D. D. (Eds), pg. 231-259, Academic Press, London

Saha B. C. and Zeikus J. G. (1992) Cyclodextrin degrading enzymes. *Starch* **44**: 312-315

Sakai S. and Horie M. (1987) Enzymic cyclodextrin production enhancement by sucrose fatty acid esters. JP 87100297 (CA 107: 132671)

Sakai S., Kubota M., Yamamoto K., Nakada T., Torigoe K., Ando O. and Sugimoto T. (1987) Cloning of cyclodextrin glucanotransferase genes from *B. stearothermophilus* and *B. macerans*. *J. Jpn. Soc. Starch Sci.*, **34**: 140-147

Sakamoto S., Iijima M., Matsuxawa H. and Ohita T. (1994) Production of thermophilic protease by glucose-controlled fed-batch culture of recombinant *Escherichia coli*. *J. Ferment. Bioeng.*, **78**: 304

Salva T. J. G., Rodrigues J. A. D. and Colombo C. A. (1997) Purification and determination of some properties of cyclodextrin glycosyltransferase from *Bacillus circulans* N° 76 using response surface analysis. *Revista de Microbiologia*, **28**: 13-21

Sato M. and Nakamura N. (1974) Production of cyclodextrin. JP 74 92 288 (CA 84: 119962)

Sato M. and Yagi Y. (1991) Properties of CGTases from three types of *Bacillus* and production of cyclodextrins by the enzymes. In: *Biotechnology of amylodextrin oligosaccharides,* ACS symposium series 458, Friedman R. B. (Ed) pg. 125-137, Am. Chem. Soc., Washington D. C.

Sato M., Yagi Y., Nagano H. and Ishikura T. (1985a) Determination of CGTase from *Bacillus ohbensis* and its optimum pH using HPLC. *Agric. Biol. Chem.*, **49**: 1189-1191

Sato M., Nagano H., Yagi Y. and Ishikura T. (1985b) Increasing γ-cyclodextrin yield. JP 60 227 693 (CA 104: 128250)

Sato M., Watanabe M., Nagano H. and Yagi Y. (1994) Selective degradation of β- and γ-cyclodextrin by co-digestion using a CGTase from *Bacillus ohbensis* and α-glucosidase for efficient α-cyclodextrin recovery. *Biotechnol. Lett.*, **16**: 703-708

Sawaguchi Y., Aikawa M., Kikuchi K. and Sasaki K. (1990) Effective production of γ-cyclodextrin and /or α-glucosylglycyrrhizin with cyclodextrin glucanotransferase. JP 022 55 095 (CA 114: 141647)

Schardinger F. (1903) *Z. Untersuch. Nahr. u. Genssm.* **6**: 865

Schardinger F. (1904) *Wien. Klim. Wochschr.* **17**: 207-209

Schardinger F. (1908) Formation of crystalline non-reducing polysaccharides from starch by microbic action. *Centr. Bakt. Parasitenk.*, Abt II **22**: 98-103 (CA 3: 910)

Schardinger F. (1911) The formation of crystalline polysaccharides (dextrins) from starch paste by micro organisms. *Centr. Bakt. Parasitenk.*, Abt II **29**: 188-197 (CA 5: 2575)

Schmid G and Eberle H. -J. (1988) Manufacture of cyclo-octa-amylose by enzymic digestion of starch in the presence of a complexing agent. EP 291 067 (CA 111: 55856)

Schmid G. (1989) Cyclodextrin glycosyltransferase production: yield enhancement by over expression of cloned genes. *Trends Biotechnol.*, 7: 244-248 (review)

Schmid G. (1996a) Enzymology of cyclodextrins. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins.* Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 615-626, Pergamon, U. K. (review)

Schmid G. (1996b) Preparation and industrial production of cyclodextrins. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins.* Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 41-56, Pergamon, U. K. (review)

Schmid G., Englbrecht A., Schmid D. (1988) Cloning and nucleotide sequence of a cyclodextrin glycosyltransferase gene from the alkalophilic *Bacillus* 1-1. In: *Proceedings of International Symposium on Cyclodextrins*, 4th, Huber O. and Szejtli J. (Eds). pg. 71-76., Kluwer, Dordrecht, Netherlands (CA 112: 230460)

Schmidt A. K., Cottaz S., Driguez H. and Schulz G. E. (1998) Structure of cyclodextrin glycosyltransferase complexed with a derivative of its main product β-cyclodextrin. *Biochemistry* **37**: 5909-5915

Schulz G. E., Parsiegla G., Candussio A., Wich G. (1997) Recombinant cyclodextrin glycosyltransferase deletion mutants for the production of gamma cyclodextrin. DE 196, 15, 336 (CA 127: 356537)

Schwimmer S. and Garibaldi J. A. (1952) Further studies on the production , purification and properties of the Schardinger dextrinogenase of *Bacillus macerans*. *Cereal Chem.*, **29**: 108-122

Seres G., Jarai M., Piukowich S., Szigetvarinee G. M. and Szejtli J. (1985) Highly pure γ- and α-cyclodextrin. *DE 3446080 (CA 103: 194961)*

Shieh W. and Hedges A. (1993) Cyclodextrin production enhancement with cyclodextrin glucosyltransferase (CGTase) of *Bacillus*. PCT Int. Appl. WO 93 07 285 (CA 118: 253426)

Shin H. -D., Kim C. and Lee Y. -H. (1999) The roles of thyptophan and histidine residues in the catalytic activities of β-cyclodextrin glucanotransferase from *Bacillus firmus* var. *alkalophilus*. *J. Microbiol. Biotechnol.*, **9**: 62-69

Shiraishi F., Kawakami K., Marushima H. and Kusurioki K. (1989a) Effect of ethanol on formation of cyclodextrin from soluble starch by *Bacillus macerans* cyclodextrin glucanotransferase. *Starch*, **41**: 151-155

Shiraishi F., Kawakami K., Marushima H. and Kusunoki K. (1989b) Effects of aliphatic alcohols on formation of cyclodextrins from soluble starch by *Bacillus macerans* cyclodextrin glucanotransferase. *Starch*, **41**: 417-420

Sicard P. J. and Saniez M. H. (1987) Biosynthesis of cycloglycosyltransferase and obtention of its enzymic reaction products. In: *Cyclodextrins and their industrial uses*, Duchene D. (Ed) pg. 75-103, de Sante, Paris (review)

Sin K. -A., Nakamura A., Kobayashi K., Maski H. and Uozumi T. (1991) Cloning and sequencing of a cyclodextrin glucanotransferase gene from *Bacillus ohbensis* and its expression in *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, **35**: 600-605

Sin K. -A., Nakamura A., Masaki H. and Uozumi T. (1993) Extracellular production of *Bacillis ohbensis* CGTase by *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.*, **57**: 346-347

Sin K. -A., Nakamura A., Masaki H., Matsuura Y. and Uozumi T. (1994) Replacement of an amino acid residue of cyclodextrin glucanotransferase of *Bacillus ohbensis* doubles the production of γ-cyclodextrin. *J. Biotechnol.*, **32**: 283-288

Siripornadulsil S., Mongkolkul P. and Rimphanitchayakit V. (1992) Molecular cloning of cyclodextrin glucanotransferase gene from *Bacillus* A11 in *Escherichia coli*. *Microb. Util. Renewable Resour.*, **8**: 247-253 (CA 120: 46897)

Sjoholm C., Prowe S. and Antranikian G. (1998) An enzyme with cyclomaltodextrin glucanotransferase activity from *Thermoalcalibacter bogoriae*. WO 9813479 (CA 128: 280229)

Slominska L. and Sobkowiak B. (1997) Studies on cyclodextrin synthesis by novel cyclodextrin glucosyl transferase. *Starch*, **49**: 301-305

Snopek J., Smolkova-Keulemansova E., Cserhati T., Gahm K. H. and Stalcup A. (1996) Cyclodextrins in analytical separation methods. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 515-571, Pergamon, U. K. (review)

Solichien B. W. (1995) Sago starch as a substrate for cyclodextrin production. *Acta Horticulturae*, **389**: 179-200

Sophianopoulos A. J. and Warner I. M. (1992) Purification of β-cyclodextrin. *Anal. Chem.*, **64**: 2652-2654h

Spande T. F. and Witkop B. (1967) Tryptophan involvement in the function of enzymes and protein hormones as determined by selective oxidation with *N*-bromosuccinimide. In: *Methods in Enzymology Vol. 11. Enzyme structure*. Hirs C. H. W. (Ed), pg. 507-522, Academic Press Inc., New York

Sreenivasan S., Samant S. K. and Pai J. S. (1991) Preparation of cyclodextrin glycosyltransferase from *Bacillus macerans*. *Indian J. Microbiol.*, **31**: 381-385

Starnes R. L. (1991) Conversion of liquified starch to cyclodextrins using *Clostridium* cyclodextrin glycosyltransferase. WO 9109962 (CA 115: 134232)

Starnes R. L., Flint V. M. and Katkocin D. M. (1990) Cyclodextrin production with a highly thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacter* sp. In: *Minutes of 5th International Symposium on Cyclodextrins*, 1990, Paris. Dunchene D. (Ed) pg. 55-61, de Sante, Paris

Starnes R. L., Trackman P. C. and Katkocin D. M. (1989) Manufacture and use of thermostable cyclodextrin glycosyltransferase. WO 8903421 (CA 112: 156726)

Stavn A. and Granum P. E. (1979) Purification and physicochemical properties of an extracellular cycloamylose (cyclodextrin) glucanotransferase from *Bacillus macerans*. *Carbohydr. Res.*, **75**: 243-250

Steighardt J. and Kleine R. (1993) Production and immobilization of a proteinase-reduced cyclodextrin glucanotransferase preparation. *Appl. Microbiol. Biotechnol.*, **39**: 63-68

Strokopytov B., Knegtel R. M. A., Penninga D., Rozeboom H., Kalk K., Dijkhuizen L. and Dijkstra B. W. (1996) Structure of cyclodextrin glycosyltransferase complexed with a maltononaose inhibitor at 2.6 Å resolution. Implications for product specificity. *Biochemistry*, **35**: 4241-4249

Strokopytov B., Penninga D., Rozeboom H. J., Dijkhuizen L. and Dijkstra B. W. (1995) X-ray structure of cyclodextrin glycosyltransferase complexed with acarbose. Implications for the catalytic mechanism of glycosidases. *Biochemistry*, **34**, 2234-2240

Sundararajan P. R. and Rao V. S. R. (1970) Conformational studies on cycloamyloses. *Carbohydr Res.*, **13**: 351-358

Svensson B., Jespersen H., Sierks M. R. and MacGregor E. A. (1989) Sequence homology between putative raw-starch binding domains from different starch-degrading enzymes. *Biochem. J.*, **264**: 309-311

Szejtli J. (1982) Cyclodextrins and their inclusion complexes. Akademiai Kiado, Budapest

Szejtli J. (1984) Industrial applications of cyclodextrins. In: *Inclusion compounds. Vol. 3. Physical properties and applications*. Atwood J. L., Davies J. E. D. and MacNicol D. D. (Eds), pg. 331-390, Academic Press, London

Szejtli J. (1996a) Historical background. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 1-3, Pergamon, U. K. (review)

Szejtli J. (1996b) Chemistry, physical and biological properties of cyclodextrins. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 5-40, Pergamon, U. K. (review)

Szejtli J. (1996c) Use of cyclodextrins in chemical products and processes. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 603-614, Pergamon, U. K. (review)

Szente L. and Szejtli J. (1996) Cyclodextrins in pesticides. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 503-514, Pergamon, U. K. (review)

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 the *Bacillus macerans* cyclodextrin glucanotransferase gene. *J. Bacteriol.*, **166**: 1118-1123

Takano T., Miyauchi A., Tagaki H., Kadowaki K., Yamane K. and Kobayashi S. (1992) Expression of the cyclodextrin glucanotransferase gene of *Bacillus macerans* in *Bacillus brevis*. *Biosci. Biotech. Biochem.*, **56**: 808-809

Takeo K., Kondo Y. and Kuge T. (1970) Thin-layer chromatography of cyclodextrins. *Agric. Biol. Chem.*, **34**: 954

Tang S., Feng Z., Huang R., Zhang C., Ren D. (1990) Cloning and expression of β-cyclodextrin gene from an alkalophilic *Bacillus* sp. N-227 in *Escherichia coli*. *Gongye Weishengwu* **20**: 1-5 (Ch) (CA 112: 230774)

Tao B. Y. (1991) Cyclodextrin glucanotransferases: Technology and biocatalyst design. In: Enzymes in biomass conversion, ACS symposium series 460, Leatham G. F and Himmel M. E. (Eds) pg. 372-383, Am. Chem. Soc., Washington D. C. (review)

Terada Y., Yanase M., Takata H., Takaha T. and Osada 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

Thoma J. A., Dygert S. and Hsue K. (1965) A simple and specific assay for cyclodextrin transglucosylase. *Anal. Biochem.*, **13**: 91-99 (CA 63: 15163)

Tilden E. B. and Hudson C. S. (1939) The conversion of starch to crystalline dextrins by the action of a new type of amylose separated from cultures of *Aerobacillus macerans*. *J. Am. Chem. Soc.* **61**: 2900-2902

Tilden E. B. and Hudson C. S. (1942) Preparation and properties of the amylases produced by *B. macerans* and *B. polymyxa*. *J. Bacteriol.* **43**: 527-544

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. *J. Ferment. Bioeng.*, **75**: 89-92

Tomita K., Tanaka T., Fujita Y. and Nakanishi K. (1990) Some factors affecting the formation of γ-cyclodextrin using cyclodextrin glycosyltransferase from *Bacillus* sp. AL 6. *J. Ferment. Bioeng.*, **70**: 190-192

Tonkova A. (1998) Bacterial cyclodextrin glucanotransferase. *Enzyme Microbiol. Technol.*, **22**: 678-686 (review)

Toyo Jozo Co., Ltd., Japan. (1980) Enhancement of cyclodextrin production. JP 55 156 595 (CA 94: 172894)

Tsuchiyama Y., Yamamoto K. -I., Asou T., Okabe M., Yagi Y. and Okamoto R. (1991a) A novel process of cyclodextrin production by the specific adsorbents: Part 1. Screening of specific adsorbents. *J. Ferment. Bioeng.*, **71**: 407-412

Tsuchiyama Y., Nomura H., Okabe M. and Okamoto R. (1991b) A novel process of cyclodextrin production by the use of specific adsorbents: Part 2. A new reactor system for selective production of α-CD with specific adsorbent. *J. Ferment. Bioeng.*, **71**: 413-417

Tsuji H. (1995) Production of cyclodextrin glucanotransferase from *Corynebacterium* and application of this enzyme. JP 07, 00, 183 (CA 122: 208680)

Uekama K and Irie T. (1996) Pharmaceutical use of cyclodextrins in various drug formulation. In: *Comprehensive Supramolecular Chemistry Vol. 3. Cyclodextrins*. Atwood J. L., Davies J. E. D., MacNicol D. D. and Vogtle F. (Eds), pg. 451-481, Pergamon, U. K. (review)

Vakaliu H., Seres G. Miskolci-Torok M., Szejtli J., Jarai M. and (1977) Production of β-cyclodextrin. HU 173825

Vandamme E. J., Declercq C. and Debonne I. (1984) Dynamics of *Bacillus circulans* var. *alkalophilus* cyclodextrin glycosyltransferase fermentation In: *Proceedings of the Third European Congress on Biotechnology*, pg. 327-332, Verlag Chemie, Weinheim, Federal Republic of Germany

Vikmon M. (1981) Rapid and simple spectrophotometric method for determination of micro-amounts of cyclodextrins. In: *Proceedings of the First International Symposium on Cyclodextrins*, Budapest, 1981, Szejtli J. (Ed), pg. 69-74, D. Reidel Publishing Company, Dordrecht

Villette J. R., Helbecque N., Albani J. R., Sicard P. J. and Bouquelet S. L. J. (1993) Cyclomaltodextrin glucanotransferase from *Bacillus circulans* E 192: nitration with tetranitromethane. *Biotechnol. Appl. Biochem.*, **17**: 205-216

Villiers A. (1891) Fermentationde fecule par le ferment butyrique. *Compt. Rend.* **112**: 536-538

Vrbaski L and Lepojevic Z. (1991) Rapid identification of microbial starch degradation products from a complex nutrient medium by a thin-layer chromatographic method. *J. Chromatogr.*, **558**: 328-332

Wind R. D., Liebel W., Buitelaar R. M., Penninga D., Spreinat A., Dijkhuizen L. and Bahl H. (1995) Cyclodextrin formation by the thermostable α-amylase of *Thermoanaerobacterium thermosulfurigenes* EM 1 and reclassification of the enzyme as a cyclodextrin glycosyltransferase. *Appl. Environ. Microbiol.*, **61**: 1257-1265

Yagi Y., Kuono K. and Inui T. (1980) Cyclodextrins by reacting starch with an enzyme from *Micrococcus* microorganism. EP 0017247 (CA 94: 45606)

Yagi Y., Sato M. and Ishikura T. (1986) Comparative studies of CGTases from *Bacillus ohbensis*, *Bacillus macerans* and *Bacillus circulans* and production of cyclodextrins using those CGTases. *J. Jpn. Soc. Starch Sci.*, **33**: 144-151

Yamane I., Kan M., Minamoto Y., and Amatsuji Y. (1981) α-Cyclodextrin, a novel substitute for bovine albumin in serum-free culture of mammalian cells. *Proc. Jpn. Acad., Ser. B.*, **57**: 385-389 (CA 97: 212006)

Yan T. -R. and Lin T. -T. (1993) Characterization and some fermentation properties of a cyclodextrin glucanotransferase-producing alkalophilic *Bacillus* sp. No. 562. *J. Chin. Agri. Chem. Soc.*, **31**: 518-530

Yan T. -R. and Lin T. -T. (1995) Purification and characterization of a β-cyclodextrin glucosyltransferase from an alkalophilic *Bacillus* sp. *Biotechnol. Tech.*, **9**: 215-220

Yee L. and Blank H. W. (1992) Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. *Bio/Technol.*, **10**: 1550-1556

Yim D. G., Sato H. H., Park Y. H. and Park Y. K. (1997) Production of cyclodextrin from starch by cyclodextrin glycosyltransferase from *Bacillus firmus* and characterization of purified enzyme. *J. Ind. Microbiol. Biotechnol.*, **18**: 402-405

Yong J., Choi J. N., Park S. S., Park C. S., Park K. H. and Choi Y. D. (1997) Molecular cloning and characterization of gene for cyclodextrin glycosyltransferase from *Bacillus* sp. E1. *Agric. Chem. Biotechnol.*, **40**: 495-500

Yoo Y. J., Cadman T. W., Hong J. and Hatch R. T. (1988) Fed-batch fermentation for the production of α-amylase by *Bacillus amyloliquefaciens*. *Biotechnol. Bioeng.*, **31**: 426-432

Yu E. K. C., Aoki H. and Misawa M. (1988) Specific alpha-cyclodextrin production by a novel thermostable cyclodextrin glycosyltransferase. *Appl. Microbiol. Biotechnol.*, **28**: 377-379

Zhu Y., Knol W., Smits J. P. and Bol J. (1996) Fractional factorial design used for the production of nuclease P1 by *Penicillium citrinum* in solid-state fermentation. *Enzyme Microb. Technol.*, **18:** 108-112

Zsadon B., Otta K. H., Tudos F. and Szejtli J. (1979) Separation of cyclodextrins by HPLC. *J. Chromatogr.*, **172**: 490-492