STUDIES ON THE ENZYME CYCLODEXTRIN GLYCOSYL TRANSFERASE FROM *BACILLUS* SP.

A

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CERTIFICATE

Certified that the work incorporated in the thesis '**Studies on the enzyme cyclodextrin glycosyl transferase from *Bacillus* sp.**' submitted by Shri Anuj Goel was carried out by the candidate under my supervision / guidance. Such material as has been obtained from other sources has been duly acknowledged in the thesis

Dr. R. A. Mashelkar
Research Guide

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ABSTRACT

Cyclodextrin glycosyl transferase (CGTase, E.C.2.4.1.19) catalyses the formation of nonreducing cyclic malto-oligosaccharides called cyclodextrins (CDs) from starch. It is a unique enzyme that carries out reversible intermolecular and intramolecular transglycosylation and performs cyclization, coupling and disproportionation of maltooligosaccharides. The rings of 6, 7 or 8 glucose residues are called α-, β- or γ-CDs, respectively. Due to the electron dense hydrophobic inner cavities and hydrophilic outer surface, CDs have the ability to form inclusion complexes with a variety of compounds. Cyclodextrins have great economic importance and find a wide range of applications in food, pharmaceutical, cosmetic and agricultural industry.

An alkalotolerant soil isolate, identified as *Bacillus firmus*, producing the enzyme CGTase, was selected for the present study. The CGTase of this bacillus was free of true amylase activity and it degraded raw starch to produce cyclodextrins.

A phenolphthalein method for determination of CGTase activity was developed which improved the linearity of assay and compensated for the instability of reagents.

B. firmus was found to be a mesophilic organism and it could grow well between pH 7-10.5. Cell growth and CGTase activity were similar in media buffered with Na_2CO_3 or that with pH controlled at 9.0. CGTase production was induced by starch and repressed by simple sugars like glucose.

Cell growth and CGTase production studies were done in shake flasks and fermenters. Due to inherent oxygen limitation in shake flasks, higher cell mass as well as CGTase activity were obtained in fermenter runs. Though CGTase production appeared to be growth-associated in shake flask experiments, in fermenter, the enzyme was produced in late log phase. This difference in enzyme production was attributed to lower specific growth rate in shake flasks. In the fermenter studies at different agitation rates, the cell

growth was limited by oxygen unavailability at low agitation rates resulting in low enzyme yields.

Both sugars and proteins in the growth medium decreased with time during the cell growth. A mechanism for utilization of starch by *B. firmus* was postulated. Stoichiometric analysis was used to formulate an equation representing the aerobic growth of *B. firmus* under the conditions of study.

In continuous culture studies, high cell mass and CGTase activity was obtained at low dilution rates. Inhibition of CGTase synthesis by unutilized starch in the medium was observed. Further experiments in shake flask with cell supplementation and studies in a membrane recycle bioreactor revealed that CGTase production was dependent on the cell density in the reactor. Though high volumetric CGTase productivity was obtained in a cell recycle bioreactor as compared to that in batch and continuous cultures, relatively low enzyme yields were observed. This was thought to be due to incomplete permeation of enzyme through the microfiltration membrane or due to the sub-optimal starch concentrations in the reactor.

CGTase was purified about 100 fold to homogeneity by affinity chromatography on a physically modified starch and DEAE anion-exchange chromatography. The purified enzyme had a specific activity of 35 U/mg protein. It had a molecular weight of 78,000 Da as determined by SDS-PAGE. The pure enzyme had pH optimum of 6.0-7.5 and was stable over pH range 7-10 (4 ^{o}C) and at pH 7 (60 ^{o}C). Maximum enzyme activity was observed at 65 ^{o}C. However, CGTase was thermolabile and rapidly lost its activity at temperatures above 30 ^{o}C.

The CGTase rapidly decreased the viscosity of gelatinized starch. The enzyme attacked raw starch to produce CDs. The CDs production was maximum with tapioca starch followed by potato and corn starch. About 49% of tapioca starch (at 10 and 50 g/L) was converted to CDs. The main reaction products were β and γ-CDs with 40% and 8%

(w/w) yields respectively. On prolonged incubation small amount of α-CD was also produced. The ratio of CDs produced was dependent on the initial substrate concentration as well as reaction time. Scanning electron microscopy revealed the mode of splitting open of starch granules on treatment with CGTase.

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1. INTRODUCTION

Cyclodextrin glycosyl transferase (CGTase, cyclomaltodextrin glucanotransferase, E.C.2.4.1.19, 1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano) transferase (cyclizing)) is a unique amylolytic enzyme that catalyses the formation of nonreducing cyclic malto-oligosaccharides called cyclodextrins (CDs, cyclomaltodextrins, cycloamylose, schardinger dextrins) from starch. It is the only hydrolytic enzyme that carries out reversible intermolecular and intramolecular transglycosylation and performs cyclization, coupling and disproportionation of maltooligosaccharides.

$$G_n \underset{\text{transglycosylation}}{\overset{\text{cyclization}}{\rightleftharpoons}} cG_x + G_{(n-x)}$$

$$G_n + G_m \overset{\text{disproportionation}}{\rightleftharpoons} G_{(n-x)} + G_{(m+x)}$$

where G_n and G_m are 1,4-α-D-glucopyranosyl chains with 'n' and 'm' D-glucopyranosyl residues, cG_x is CD with 'x' glucose residues. The CDs most commonly produced have rings of 6, 7 or 8 glucose residues linked to each other by α-1,4 bond and are called α-, β- or γ-CDs respectively (Figure 1.1). These torroidal molecules have unique structures with hydrophilic outer surfaces and hydrophobic inner cavities. The electron dense cavities of CDs have the ability to form inclusion complexes with a variety of compounds ranging from polar molecules such as acids, amines and small ions, to highly apolar aliphatic or aromatic hydrocarbons and even gases. Due to this phenomenon CDs have great economic importance and find a wide range of applications in food, pharmaceutical, cosmetic and agricultural industry. Moreover, CDs are known to facilitate some chemical reactions by covalent or noncovalent interactions. Modified CDs can also act as biomimetic models for enzymes.

1.1 Objective of the present study

Considering the vast potential applications of CDs, these molecules have evoked great interest from researchers and entrepreneurs alike. There are at least eight companies all

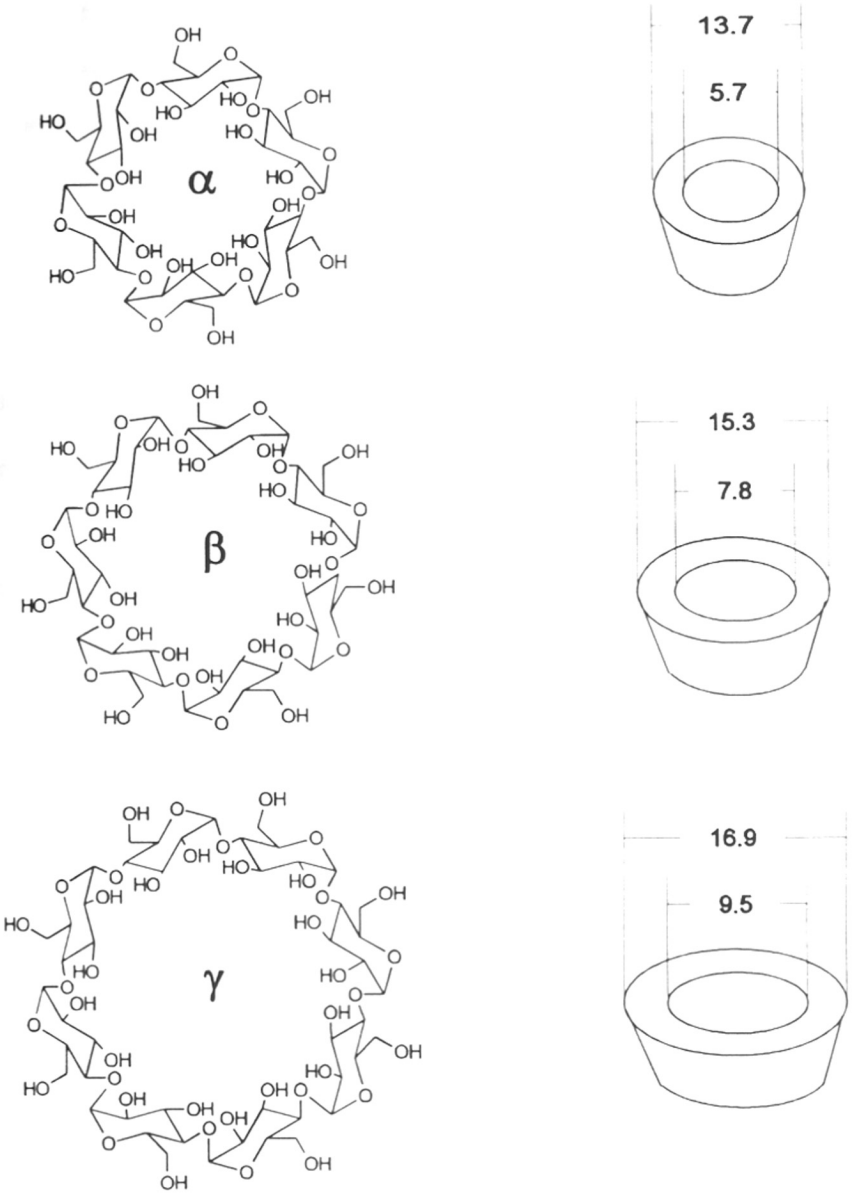

Figure 1.1: Structure of α, β, and γ-cyclodextrins. Dimensions in the figure are in angstroms.

over the world carrying out commercial production of CDs (4 in Europe, 3 in Japan and 1 in USA). However, the cost of CDs is one of the factors that hinders their wide acceptability. As the major cost centre in CD production is their separation from enzymic digests of starch, there is a constant search for CGTases that can produce specific CDs with minimum byproducts. Since CGTase is an inducible enzyme, its production is governed by the environmental conditions during growth of the producer strain. Limited information is available on the growth and CGTase production in various organisms. Therefore, this study was undertaken to :

— Isolate a fast growing microorganism that produces CGTase of desired characteristics.

— To study the growth of this organism under various conditions and elucidate their effect on CGTase production

— To purify the enzyme and characterize its properties

— To evaluate the action of CGTase on starch for CD production

1.2 Thesis presentation

The thesis has been divided mainly into three parts

— Review of the literature

This chapter contains a comprehensive survey of the work done on the enzyme CGTase, its production, purification, properties and uses of its product, cyclodextrins.

— Materials and methods

In this section the experimental protocols and methods have been described.

— Results and discussion

This part of the thesis describes the observations and the results obtained during the present study, their significance and comparison with previously reported information.

2. Literature Review

Cyclodextrins (CDs) were discovered more than a hundred years back by Villiers (1891). While working on the culture of '*Bacillus amylobacter*' he isolated crystalline dextrins which he called 'cellulosines' from the starch containing growth medium. Schardinger (1904) isolated the bacterium *Bacillus macerans* that produced CDs. Cyclodextrins were thought to be metabolic products of the bacteria until Tilden and Hudson (1939) isolated the enzyme capable of producing them *in vitro*. The enzyme was frequently named after the bacterium producing it, i.e., *Bacillus macerans* amylase (BMA) or enzyme (BME). The cyclic products were called 'Schardinger dextrins'. The names Schardinger enzyme or dextrinogenase and cyclodextrin transglucosidase were also used to describe the enzyme. However, later (Commission on Biochemical Nomenclature, 1973) the name cyclodextrin glycosyltransferase (CGTase) was adopted with an E. C. No. 2.4.1.19.

2.1 Microorganisms producing CGTase

Till now CGTases are known to be produced exclusively by bacteria. To date, only nine bacterial genera are documented to be producers of CGTase (Table 2.1). The enzyme is accumulated by almost all the species of the genus *Bacillus*. A few species of *Micrococcus*, *Brevibacterium*, *Corynebacterium*, *Thermoanaerobacter*, *Thermoanaerobacterium*, *Clostridium*, *Klebsiella* and *Pseudomonas* are the other CGTase producers. *Klebsiella* and *Pseudomonas* are the only gram-negative bacteria that are known to secrete CGTases. While the other bacteria grow aerobically, *Clostridium*, *Thermoanaerobacterium* and *Thermoanaerobacter* are obligate anaerobes. Virtually all of these bacteria have been isolated from soil or plant sources. The CGTase producing bacterium, *Bacillus macerans*, was first isolated from starch digest by Schardinger in 1904 (French, 1957). *B. macerans* was also isolated from rotting potatoes (Tsukuba university and ministry of agriculture, 1985) and dry onion powder (Stavn and Granum, 1979). While searching for CGTase with desired properties, the enzyme producing cultures have

Sr. No.	Organism	Conditions for growth and CGTase production		Assay method used	Activity *	Purification			Reference
		Temperature (°C)	Time (h)			Procedure	Sp. Activity (U/mg)	Yield %	
1	*Bacillus amyloliquifaciens* AL 35	37	12-16	Gluco-amylase	145 U/mL				Yu *et al.*, 1988
2	*Bacillus autolyticus* 11149	30	24	I_2	6.2 U/mL	UF→DEAE→ Sepharose CL6B(I)→ Sepharose CL6B(II) → Hydroxylappatite	332.5	31	Tomita *et al.*, 1993
3	*Bacillus circulans* ATCC 21783	37	late log and lag phase	MO-G3	30 μM/L/min				Makela and Korpela (1988)
4	*Bacillus circulans* ATCC 21783	37	50	Gluco-amylase	34.8 U/mL	Starch→BioGel P-100→DEAE cellulose →Sephadex G-100	81.1	35	Nakamura and Horikoshi, 1976b
5	*Bacillus circulans* ATCC 21783 neutral CGTase	37	50	Gluco-amylase	50.84 U/mL	Starch→ DEAE cellulose → Sephadex G-150→ DEAE cellulose	281	19	Nakamura and Horikoshi, 1976c
6	*Bacillus circulans* C31	37	4 days	I_2	30 U/mL	UF→Corn starch (βCD elution) →Biogel A500	6623.4	30	Pongsawasdi and Yagisawa, 1988

Table 2.1. Production and purification of CGTase in various bacteria (continued).

Sr. No.	Organism	Conditions for growth and CGTase production		Assay method used	Activity *	Purification			Reference
		Temperature (°C)	Time (h)			Procedure	Sp. Activity (U/mg)	Yield %	
7	*Bacillus circulans* E192	30	40	PHP	2.1 U/mg	$(NH_4)_2SO_4$ → DEAE→Q-Sepharose→βCD affinity	88	64	Bovetto *et al.*, 1992a
8	*Bacillus coagulans* A-147 FERM 9735	45	24	PHP	27.14				Kaneko *et al.*, 1990
9	*Bacillus lentus*	37	4 days	TCE-CD, I_2		$(NH_4)_2SO_4$ →DEAE cellulose → CM cellulose			Sabioni and Park, 1992a,b
10	*Bacillus licheniformis* IT 25	37		I_2, PHP, HPLC	1.63	$(NH_4)_2SO_4$+starch→ $(NH_4)_2SO_4$→ DEAE → crystalyzation	346	75	Aoki *et al.*, 1987
11	*Bacillus macerans*	37	5 days	I_2	0.84 U/mL	Starch→ $(NH_4)_2SO_4$ →DEAE cellulose → Sephadex G-100	127	17	Stavn and Granum, 1979
12	*Bacillus macerans* ATCC 8514	40	8-10 intracellular	I_2	31.6 U/mL	$MnCl_2$→$(NH_4)_2SO_4$ → DEAE → $(NH_4)_2SO_4$ → Prep electrophoresis	81.5	12	DePinto and Campbell, 1968
13	*Bacillus macerans* IAM 1243	40	48	Gluco-amylase	0.33 U/mL	Starch→ DEAE cellulose → DEAE cellulose→ crystalization	245	50	Kobayashi *et al.*, 1978; Kitahata and Okada, 1982b

Table 2.1. Production and purification of CGTase in various bacteria (continued).

Sr. No.	Organism	Conditions for growth and CGTase production		Assay method used	Activity *	Purification			Reference
		Temperature (°C)	Time (h)			Procedure	Sp. Activity (U/mg)	Yield %	
14	*Bacillus macerans* WKMW 506	37	3-4 days	PHP	0.2 CU/mL μM/mL/min	$(NH_4)_2SO_4$ partial			Steighardt and Klein, 1993
15	*Bacillus obhensis*					Acetone powder→starch→ DEAE cellulose →crystalization		36	Yagi *et al.*, 1986
16	*Bacillus* sp. no. 5	37	70	I_2	67 U/mL	$(NH_4)_2SO_4$ → Corn starch→$(NH_4)_2SO_4$ → DEAE → Sephadex G-75			Kitahata *et al.*, 1974
17	*Bacillus* sp. A2-5a	37	24-72	GLU, HPLC 1μmol/min	23.5 U/mL	Starch→$(NH_4)_2SO_4$ → Q-sepharose	590	51	Kometani *et al.*, 1994
18	*Bacillus* sp. AL-6	38	48	I_2, HPLC	3.75 U/mg protein	20% $(NH_4)_2SO_4$ → Starch→ DEAE → Sephadex A50→ DEAE A50	877.9	14.4	Fujita *et al.*, 1990
19	*Bacillus* sp. KC 201	37 pH 8-11		PHP					Kitamoto *et al.*, 1992
20	*Bacillus sp.* 290-3 *(B. firmus/B. lentus)*	37		HPLC	60 U/mg protein	γ-CD Sepharose			Englbrecht *et al.*, 1990

Table 2.1. Production and purification of CGTase in various bacteria (continued).

Sr. No.	Organism	Conditions for growth and CGTase production		Assay method used	Activity *	Purification			Reference
		Temperature (°C)	Time (h)			Procedure	Sp. Activity (U/mg)	Yield %	
21	*Bacillus* sp. HA3-3-2 ATCC 39612	37	4 days	I_2, CD-TCE	540 U/mL	$(NH_4)_2SO_4$ → Sephadex G-100→DEAE cellulose x 3	45,000	7	
22	*Bacillus* sp. No. 562			PHP		$(NH_4)_2SO_4$ → Sephadex A-50 →DEAE Sephacryl S-300	3108	32	Yan and Lin, 1993 & 1995
23	*Bacillus stearothermophilus*	55	48	I2	9.22 U/mL	$(NH_4)_2SO_4$→ β-CD-Sepharose affinity	3445	42	Joong-Hoon *et al.*, 1990
24	*Bacillus stearothermophilus* TC-60			I_2		$(NH_4)_2SO_4$+starch→ $(NH_4)_2SO_4$→ DEAE→ Biogel P-150	294	27	Kitahata and Okada, 1982a
25	*Bacillus subtilis* no. 313	37	3-5 days	BCG		$(NH_4)_2SO_4$ → DEAE→ Chromatofocussing PBC94→ Sephacryl S-200→ CM Toyoperl 650M	9.59	13.5	Kato and Horikoshi, 1986

Table 2.1. Production and purification of CGTase in various bacteria (continued).

Sr. No.	Organism	Conditions for growth and CGTase production		Assay method used	Activity *	Purification			Reference
		Temperature (°C)	Time (h)			Procedure	Sp. Activity (U/mg)	Yield %	
26	*Brevibacterium* sp. 9605	37	48	I_2	0.133 U/mL	UF→Butyl-Toyopearl 650M→ γ-CD Sepharose→ Toyopearl HW-55S	25.4	16	Mori *et al.*, 1994a
27	*Klebsiella oxytoca* 19-1	37, pH 7	9	MO	52 U/mL	Acetone ppt. 3.4 U/mg protein			Lee *et al.*, 1992
28	*Klebsiella pneumoniae* M 5 al (now called *K. oxytoca*)	28	12-24 log phase		0.7 U/mL	$(NH_4)_2SO_4$ → DEAE→polyamineP → $(NH_4)_2SO_4$	26.6	61.2	Bender, 1977a
29	*Thermoanaerobacterium thermosulphurigenes* EM1	Cloned in *E. coli*		PHP		α-CD Sepharose affinity	88	50	Wind *et al.*, 1995

* The CGTase activity reported in this column has been calculated from the available data if it is not reported directly.

Empty boxes indicate that data is not available.

Other CGTase producing bacteria belong to the genera *Clostridium* (Starnes, 1991), *Corynebacterium* (Amano-Pharma, 1979), *Micrococcus* (Yagi *et al.*, 1980), *Pseudomonas* (Allenza *et al.*, 1991), and *Thermoanaerobacter* (Novo, 1987). Detailed information for CGTases from these bacteria is not available.

Table 2.1. Production and purification of CGTase in various bacteria.

been screened from extreme environments like hot springs (*B. coagulans* A-147; Kaneko *et al.*, 1990), deep sea mud samples (*Bacillus* sp. no. 3-22; Georganta *et al.*, 1993) and highly alkaline environments (*Bacillus circulans,* ATCC 21783; Nakamura and Horikoshi, 1976a). A number of alkalophilic CGTase producing organisms have been isolated. These bacteria belong to genus *Bacillus* and can grow well between pH 7-10 (Nakamura and Horikoshi, 1976a; Nomoto *et al.*, 1984; Pongsawasdi and Yagisawa, 1987; Yan and Lin, 1993).

Most CGTase producing bacteria are mesophilic and grow at temperatures of 30-40 °C. *Thermoanaerobacter* sp. (ATCC 53627), a thermophile, produces CGTase when grown at 67 °C (Novo, 1987), whereas psycrophilic *Bacillus* sp. 3-22 can produce the enzyme even at 4 °C (Georganta *et al.*, 1993).

2.2 Classification of CGTases

CGTases are named after the predominant CD they produce during the initial phase of reaction. Thus α-, β-, or γ-CDs producing CGTase are called α-, β-, or γ-CGTases. The enzymes of *B. macerans* (Kobayashi *et al.*, 1978; Kitahata and Okada, 1982b) are α-CGTases. Other α-CGTases belong to *B. stearothermophilus* (Kitahata and Okada, 1982a), *B. amyloliquifaciens* (Yu *et al.*, 1988) and *Klebsiella oxytoca* (Bender, 1977a; Lee *et al.*, 1992). Beta-CGTases are from *B. circulans* (Nakamura and Horikoshi, 1976a; Bovetto *et al.*, 1992a), *B. autolyticus* (Tomita *et al.*, 1993); *B. megaterium* (Kitahata *et al.*, 1974), *Micrococcus* sp. (Yagi *et al.*, 1980) and *Thermoanaerobacter* sp. (Norman and Jorgensen, 1992). The enzymes from *B. subtilis* no. 313 (Kato and Horikoshi, 1988), *Bacillus* sp. AL-6 (Fujita *et al.*, 1990) and *Brevibacterium* sp. (Mori *et al.*, 1994a) belong to γ-CGTase subgroup. However, the classification is not species specific and the same species may produce two types of CGTases. *B. circulans* ATCC 9995 produces α-CGTase (Kitahata and Okada, 1982b). *B. coagulans* produces both α-CGTase

(Kaneko *et al.*, 1990) and β-CGTase (Akimaru *et al.*, 1991). *B. macerans* WKMW 506 produces β-CGTase (Steighardt and Kleine, 1993).

2.3 CGTase production

CGTase is an inducible enzyme and hence the wild strains producing it are grown in a medium containing starch or related compounds. CGTase production is strongly repressed in the presence of simple sugars. *B. cereus* RJ 30 (NCIMB 13123) is the only known organism that can produce CGTase in the presence of glucose or xylose as a carbon source (Jamuna *et al.*, 1993).

2.3.1 Media

Generally a complex nutrient medium consisting of a carbon source, a nitrogen source and inorganic salts is used for CGTase production. The carbohydrate source is a starchy material like potato or corn starch, soluble starch, maltodextrins, glycogen, potato gratings or wheat bran. Nitrogen is supplied predominantly in organic form although inorganic sources have also been used. These consist of peptone, yeast extract, soyabean hydrolysate, dry yeast, casein or meat extract and inorganic compounds like $(NH_4)_2SO_4$, NH_4Cl or urea. Lysamine, a commercial proteinaceous potato extract, has also been used to increase CGTase yields (Sicard and Saniez, 1987). Supplementation of nutrient medium with wheat bran extract also promotes CGTase production (*B. cereus* NCIMB 13123; Ramakrishna *et al.*, 1994).

Inorganic compounds, commonly incorporated into media, are sodium and potassium phosphates, $CaCl_2$, $MgCl_2$, $MgSO_4$ and Na_2CO_3. The medium pH is usually maintained around 7.0. However, alkalophilic bacteria are grown at pH 8-10, which is obtained by adding Na_2CO_3 as buffer.

2.3.2 *Enzyme production*

The CGTase producing bacteria are grown for a period of 1 to 4 days for enzyme production (Table 2.1). CGTase production appears to be growth associated and maximum enzyme is accumulated in the idiophase of the growth cycle. In some bacteria, CGTase production is biphasic. Some enzyme is released during the stationary/lysis phase (Makela *et al.*, 1990; Yan and Lin, 1993; Georganta *et al.*, 1993; Jamuna *et al.*, 1993). In *B. circulans* ATCC 21783 about 65% of total enzyme was obtained during the initial phase at the transition between the log and stationary phases (Makela *et al.*, 1990). The rest of the enzyme was produced by resting or spore-forming cells during the lysis phase. This two step production of CGTase is attributed partly to intracellular fraction of the enzyme that is released on lysis. In an earlier study involving the same bacterium, Vandamme *et al.* (1984) had actually observed upto 15% of total CGTase to be intracellular. Though the growth profiles of most of the CGTase producing bacteria have not been reported in literature, long incubation time (4-5 days, Table 2.1) indicate that cell lysis might be an important factor in the release of CGTase from these bacteria. *B. macerans* (ATCC 8514) CGTase is intracellular (De Pinto and Campbell, 1964). This enzyme is released from cells grown for 10-14 h in shake flask by disrupting the cells. CGTase from *K. pneumoniae* M5 al (Bender, 1977a), *K. oxytoca* (Lee *et al.*, 1992), *B. autolyticus* (Tomita *et al.*, 1993) and *B. amyloliquifaciens* (Yu *et al.*, 1988) are produced within 12–24 h.

Though most of the studies on CGTase production have been done in submerged culture, a few reports are available on enzyme production in slurry or solid state fermentation with media containing wheat bran or grated potatoes (Kitahata *et al.*, 1974; Steighardt and Klein, 1993; Ramakrishna *et al.*, 1994). Ramakrishna *et al.* (1994) cultivated CGTase producing *B. cereus* RJ30 in solid state and slurry fermentation with wheat bran. Compared to submerged culture in a defined nutrient medium (40 U/ml

CGTase activity), these workers obtained higher amount of CGTase in solid state and slurry fermentations (59 and 58 U/ml, respectively).

In a chemostat *K. pneumoniae* M5al was grown continuously for CGTase synthesis (Bender, 1986). At a dilution rate of 0.12 h^{-1}, with starch as limiting nutrient, 120 mg CGTase/L of culture filtrate was obtained after 3 days of cultivation in continuous mode. Jamuna *et al.* (1993) also reported CGTase production in continuous fermentation mode by *B. cereus* RJ30. The enzyme productivity increased linearly over the dilution rates studied (0.06 to 0.16 h^{-1}). A maximum productivity of 5.4 kU/L-h was obtained at 0.16 h^{-1} dilution rate at which the CGTase concentration was 27.5 U/mL. This was 3.1 times higher than that obtained in batch culture using the same nutrient medium. The same workers also reported continuous production of CGTase by *B. cereus* cells immobilized in calcium alginate beads. In a fluidized bed reactor containing 30 g beads with 16 g/L bacterial cells, a maximum activity of 60-68 U/mL CGTase was obtained at a productivity of 23 kU/L-h and 0.32 h^{-1} dilution rate over 240 h of operation.

2.4 Assays

A number of assays are currently in use depending on the convenience of researchers and the specific end use. A brief survey of these methods is presented in the following section.

The CGTase activity is estimated by measuring its dextrinizing, transglycosylation or cyclization activities. However, as all these activities of the enzyme are exhibited simultaneously, any CGTase assay based on any of the activities quantitates a combined enzyme activity.

2.4.1 Assays based on dextrinizing activity

CGTases that are essentially free of amylase activity can be assayed by measuring their dextrinizing activity. Most of the assays are based on loss of starch-iodine colour on

reacting with CGTase. The assay was first used by Hale and Rawlins (1951) when they defined one CGTase unit as the amount of enzyme that caused 50% change in transmission after 10 min of incubation. Various researchers have modified this assay to suit their specific work (Schwimmer and Garibaldi, 1952; Fuwa, 1954; Nakamura and Horikoshi, 1976b). Dextrinizing activity has also been determined by decrease in viscosity of starch solutions when treated with CGTase (Tilden and Hudson, 1939; McClenahan *et al.*, 1942).

2.4.2 Assays based on transglycosylation activity

The amount of donor, acceptor or linear dextrins produced in the transglycosylation (coupling) reaction can be measured and related to the enzyme activity. Nakamura and Horikoshi (1976b) devised an assay based on estimation of linear dextrins produced by coupling of α-CD to the acceptor sucrose. The dextrins produced by transglycosylation were hydrolyzed by glucoamylase and the reducing sugars released were related to CGTase activity. Kometani *et al.* (1994) estimated the amount of salicin coupled to starch by HPLC to measure transglycosylation activity. To study the transglycosylation reaction specifically, Nakamura *et al.* (1994) used a protected aglycone 3-ketobutylidene-2-chloro-4-nitromaltopentaoside. This molecule could not act as an acceptor in the CGTase reaction as it is modified at the C-4 hydroxyl of its non-reducing end.

2.4.3 Assays based on cyclization activity

Quantification of the initial cyclization activity of CGTase is the most specific and direct approach to assay its activity. The cyclodextrins produced can be detected either by their ability to form clathrates with a number of organic molecules or by chromatography. A number of non-chromatographic assays for CDs have been reported in literature (Makela *et al.*, 1988). Tilden and Hudson (1942) developed a method based on microscopic examination of CD-iodine clatharates. This assay is qualitative and is suitable only for

bacterial strains which produce α-CDs as the main product of CGTase reaction. Similarly, α-CD forming CGTase can be quantified by monitoring α-CD-trichloroethylene (TCE) complex that precipitates readily in aqueous phase. Either the threshold value of serially diluted enzyme that forms CD-TCE precipitate is determined (Nomoto *et al.*, 1984) or the CDs can be quantified from the residual starch in the supernatant (Kitahata and Okada, 1975). The absorbance of iodine solution in the ultraviolet spectrum is enhanced by the presence of α-CD. Based on this observation Bender (1981a) described a photometric assay for α-CD forming CGTase. This assay, however has limited use due to non-specific interference by proteins, salts and the starch-iodine complex.

A number of pH-indicator dyes form specific inclusion compounds with CDs depending on their molecular size. This phenomenon forms the basis of several methods to quantitate CDs. These methods take advantage of the fact that formation of inclusion compounds with CDs and the guest molecule results in change (generally decrease) in absorbance of the dye. Thus, α-CD is estimated by methyl orange (Landert *et al.*, 1981; Makela and Korpela, 1988; Lejeune *et al.*, 1989), β-CD by phenolphthalein (Vikmon, 1981; Kaneko *et al.*, 1987; Makela *et al.*, 1987) or methyl orange (Makela and Korpela, 1988) and γ-CD by bromocresol green (increase in absorbance at 630 nm; Kato and Horikoshi, 1984a), calgamite (Hokse, 1983) or alizarin yellow (Aoki *et al.*, 1987).

Cyclodextrins can also be estimated fluorometrically as they can form clathrates with 2-p-toluidinylnapthalene-6-sulphonate (TNS) or 1-anilino-naphthalene-8-sulphonate (ANS) (Kondo *et al.*, 1976a, 1976b). Alpha-CD concentration can be monitored by its ability to hydrolyze dimethylphenylacetate (Lane and Pirt, 1973). An assay for α-, β-, as well as γ-CDs based on linoleate-CD complex in lipooxygenase reaction has also been reported (Laakso *et al.*, 1984). In this method the change in slope of reaction velocity as measured polarographically is proportional to the amount of CDs present in the reaction mixture.

CGTase activity can also be quantitatively estimated by separation and analysis of CDs produced by thin layer chromatography (TLC), gas chromatography(GC) or high performance liquid chromatography (HPLC).

Cyclodextrins can be resolved on a microcrystalline cellulose matrix by TLC using the solvent system n-butyl alcohol:ethyl alcohol:water (4:3:3, v/v) or on Kieselguhr G with the same solvent components in the ratio 4:3:2 and detected by keeping in an iodine chamber (Takeo, 1970). Similarly, CDs can also be separated on TLC with a solvent system n-propanol:water:ethyl acetate:ammonia (6:3:1:1) (Sicard and Saniez, 1987).

The reaction products of CGTase can also be accurately analysed by gas chromatography. However, the oligosaccharides require prior conversion to their volatile dimethylsilyl ethers (Beadle, 1969).

HPLC is the most accurate method to measure CDs. Separation of CDs has been widely described by Kitahata *et al.* (1978a) using Polygosil-10-NH_2 column and acetonitrile/water as solvent, Zsadon *et al.* (1979) for μ-Bondapack carbohydrate column with acetonitrile/water as solvent, Hokse (1980) for polystyrene based Aminex-50W-X4 ion exchange column with water or calcium salt of EDTA (Brunt, 1982) as solvent, Koizumi *et al.* (1986a) for Amino bonded silica with acetonitrile/water as solvent, Koizumi *et al.* (1986b) for vinyl alcohol polymer based Asaphiak GS-320 or Frijlink *et al.* (1987) for phenyl modified silica column. Though all these columns separate chemically uniform CDs satisfactorily, linear malto-oligosaccharides often interfere with their separation. The CGTase hydrolysates, therefore, have to be predigested by glucoamylase to remove these saccharides.

Sato *et al.* (1985) described a method to separate CDs using a 150 mm sulphonated polystyrene based Shodex-DC-613 column. When eluted at 60 °C with acetonitrile:water (65:35), CDs could be separated satisfactorily from maltodextrins within a detection limit of 5 μg/injection. Similarly Aoki *et al.* (1987) used a proprietary 'Cyclodextrin assay

column' (Astec) with deionized water as solvent for complete separation of CDs from linear dextrins.

2.5 Purification

CGTases have been isolated and purified from the culture supernate of a number of bacteria. The enzyme purification comprises of a number of steps. In the first step the enzyme is concentrated from broth by ultrafiltration, salt precipitation or adsorption. This is followed by fractionation using ion-exchange, hydrophobic interaction or gel permeation chromatography. CGTases have been purified using conventional chromatographic supports as well as by specifically adsorbing them onto affinity matrices (Table 2.1). These matrices are either the substrate i.e. native/modified starch or the product CDs attached to inert supports (e. g. Sepharose). Crosslinked polymers of CDs itself or immuno affinity matrices prepared by raising monoclonal antibodies against CGTase have also been used (Table 2.2). The enzyme yields vary from 7 to 75% and the purified enzymes have specific activities of 80 to 45,000 U/mg protein depending upon the assay method used and definition of a unit of enzyme activity. High enzyme yields with one step affinity purification of CGTase have been documented. Laszlo *et al.* (1981) purified *B. macerans* CGTase to homogeneity with 90-92% yield on an α-CD-Sepharose column. CGTase from a *B. stearothermophilus* mutant was purified in a one step affinity chromatography (β-CD-Sepharose) with 98% yield (Joong-Hoon *et al.*, 1991). On starch columns 40-100% yields of CGTase have been reported depending on the type of starch and desired purity of the enzyme (Table 2.2). A novel method for a single step purification of a recombinant CGTase by pseudo metal affinity has been reported (Berna *et al.*, 1994). The enzyme broth was passed through tandem columns of Cu(II) and Zn(II) linked Sepharose-6B matrices. The CGTase was selectively bound to Zn(II) and not Cu(II) and thus could be selectively eluted.

Sr. No.	Type of Matrix	Method of elution	Yield* %	Bacterial Strain	Reference
	STARCH				
1	Raw corn starch granules + Hyflo Super-cel (30 % $NH_4(SO_4)_2$ supernate	Phosphate Buffer (0.033M)	75	*B. macerans* IFO 3490	Kitahata *et al.*, 1974
2	Raw corn starch + 20 % $NH_4(SO_4)_2$	Phosphate Buffer 50 °C	63	*B. macerans*	Stavn and Granum, 1974
3	Raw corn starch + 20 % $NH_4(SO_4)_2$, 4 ° C	Phosphate Buffer 40 °C	62	*B. stearothermophilus*	Kitahata and Okada, 1982a
4	Raw corn starch + 20 % $NH_4(SO_4)_2$, 4 ° C	3M NaCl + 0.1 M maltose in phosphate buffer (10 mM) with 20 % $NH_4(SO_4)_2$	103	*B. circulans* ATCC 21783	Nakamura and Horikoshi, 1976b
5	Starch + 15 % $NH_4(SO_4)_2$	Distilled water	98	*B. licheniformis* IT 25	Aoki *et al.*, 1987
6	Corn starch + 22 % $NH_4(SO_4)_2$	Phosphate Buffer (33 mM)	79	*Bacillus sp.* A2-5a	Komentani *et al.*, 1994
7	Heat moisture treated corn starch (20 min at 120°)	Wash three times with 33 % ethanol, elute with distilled water 50 ° C	80	*B. macerans* IAM 1243	Kobayashi *et al.*, 1978
8	Potato starch load with 10 % ethanol, 4 ° C	25 °C water, 30 min	96	*B. circulans* ATCC 21783	Makela *et al.*, 1988
9	Starch (freeze thawed and crushed) 4 ° C	≥ 30 ° C buffer	70 ± 6	*B. macerans*	Gottvaldova, 1988
10	Corn starch	β-CD (10 mM) in Tris-HCl buffer 50 mM	42	*B. circulans*	Pongsawadi and Yagisawa, 1988

Table 2.2: Purification of CGTase by affinity chromatography (continued).

Sr. No.	Type of Matrix	Method of elution	Yield* %	Bacterial Strain	Reference
	CD-SEPHAROSE				
1	α-CD -	α-CD gradient	90-92	*B. macerans*	Laszlo *et al.*, 1981
2	β-CD -	10 mg/mL β-CD in acetate buffer	65	*B. stearothermophilus*	Joong-Hoon *et al.*, 1990
3	β-CD -	10 mg/mL β-CD in acetate buffer	95	*B. stearothermophilus* mutant	Joong-Hoon *et al.*, 1991
4	γ-CD -	γ-CD in buffer		*Bacillus* sp. 290-3	Englbrecht *et al.*, 1990
	CD-POLYMER				
1	β-CD polymer	1 % β-CD in phosphate buffer	69	*B. circulans* E 192	Villette *et al.*, 1991
	MONOCLONAL ANTIBODY				
1	Sepharose linked antibody				Arbatova and Reeben, 1991
	PSEUDO-METAL AFFINITY				
1	Tandem columns of Cu(II) and Zn(II) linked Sepharose-6B	Imidazole buffer		Recombinant gene product	Berna *et al.*, 1994

* In multistep purification procedures the yield in a particular affinity step is given.

Empty cells indicate data not available.

Table 2.2: Purification of CGTase by affinity chromatography.

2.6 Properties

2.6.1 Molecular weight

CGTases have molecular weights in the range of 68,000 to 170,000 Daltons (Table 2.3) as determined by SDS-PAGE or gel permeation chromatography. It may be a single polypeptide or a dimer of two subunits (*B. macerans* IAM 1243, Kobayashi *et al.*, 1978; *B. licheniformis* IT25, Aoki *et al.*, 1987 *Bacillus* sp. no. 562, Yan and Lin, 1995). Gel permeation chromatographic estimations often give lower molecular weights probably due to association of CGTase with the matrices (Makela *et al.*, 1989). CGTases from *B. macerans* IAM 1243 (Kobayashi *et al.*, 1978) and *B. licheniformis* IT25 (Aoki *et al.*, 1987) consist of two identical subunits of molecular weight 74,000 and 72,000 Da respectively. The molecular weight of the cloned gene product from *B. macerans* showing CGTase activity is 76,994 Da (Takano *et al.*, 1986). These results are contradictory to the earlier report where Kobayashi *et al.* (1978) indicated that individual subunits of CGTase do not have enzymatic activity. The amino acid sequences of other cloned CGTases also reveal the molecular weight to be around 70,000 to 78,000 (Kimura *et al.*, 1987; Kaneko *et al.*, 1989; Georganta *et al.*, 1991; Sin *et al.*, 1991; Paloheimo *et al.*, 1992; Lee and Tao, 1994). The CGTase from another *B. macerans* is a glycoprotein (Jeang *et al.*, 1992). It contains 12.3% carbohydrate. When this enzyme is digested with N-acetyl-β-D-glucosaminidase it loses its activity and ability to bind to α-CD-conjugated affinity matrices. There are no other reports of glycoprotein CGTases although a few amylases are known to be glycoproteins (Vihinen and Mantsala, 1989).

2.6.2 pH optimum and stability

The pH optimum of CGTase varies from 4.5–10.5 and is normally around 6.0 (Table 2.3). Alkalophilic bacteria have pH optima between 9 and 10.5 (*Bacillus* sp. AL-6,

Sr. No.	Organism	Optimum		Stability		Ca^{2+} effect	pI	Molecular weight (Daltons)	Km/Vmax (substrate)	α:β:γ ratio	Reference
		pH	Temperature °C	pH	Temperature °C						
1	*Bacillus amyloliquifaciens* AL 35	4.0-7.0 max 6.0	70	8-10	≥ 60° 2 h - 5% loss	+					Yu *et al.*, 1988
2	*Bacillus autolyticus* 11149	5-6	60	5-9	≤ 40° 1h at pH 6			68,000[2] 70,000[3]		56% βCD in presence of Triton-X-100 (α,γ < 1%)	Tomita *et al.*, 1993
3	*Bacillus circulans* ATCC 21783 acid CGTase	4.5-4.7	45 at pH 4.65	6-10	65° for 30 min	+		88,000	Km α5.88, β 0.39, γ 0.25 mM / Vmax α 133.4, β 23.4, γ 12.3 μmol glu/min/mg (CDs)		Nakamura and Horikoshi, 1976b
4	*Bacillus circulans* ATCC 21783 neutral CGTase		50	6-9	60° at pH 7.0	+	5.4	85-88,000	Km α 10, β 0.83 mM / Vmax α 417, β 70 μmol/min/mg glucose (CDs)		Nakamura and Horikoshi, 1976d
5	*Bacillus circulans* ATCC 21783						4.9 (main) 4.55 to 4.85 (others)	70,500[1] 58,000 ± 2000[2]			Makela *et al.*, 1988
6	*Bacillus circulans* C31	5.5	60	5.5-9	≤ 50° pH 7, 20 min			103,000[2]		1:10.5:0	Pongsawasdi and Yagisawa, 1988

Table 2.3: Properties of CGTase from various bacteria (continued).

Sr. No.	Organism	Optimum		Stability		Ca^{2+} effect	pI	Molecular weight (Daltons)	Km/Vmax (substrate)	α:β:γ ratio	Reference
		pH	Temperature °C	pH	Temperature °C						
7	*Bacillus circulans* E192	5.5-5.8	60	7-8	45	++	6.9 (90 %) 6.7 (10 %)	78,000	Km 0.57 w/v /Vmax 43μmol/mg/min CD (starch) & Km 0.7 w/v /Vmax 43μmol/mg/min CD (maltodextrin)	42:44:14 (24 h)	Bovetto *et al.*, 1992a
8	*Bacillus coagulans* A-147 FERM 9735	6.5	65 75 (Ca^{2+})	6-9 2 h, 40°	≤ 50, 15 min, pH 7.0	+		36,000[2]		2:2.6:1	Kaneko *et al.*, 1990
9	*Bacillus lentus*	6.5-8.5	45-55			+				1:67:1.6	Sabioni and Park, 1992a,b
10	*Bacillus licheniformis* IT 25	5.5	65-70	6-9.5	≤ 65, 15 min	+	4.3	72,000[1], 140,000[2]		1.6:4.0:1.0	Aoki *et al.*, 1987
11	*Bacillus macerans*	5.4-5.8	60	6.0	≤50		5.4	75,000[1] 67,000[4],	Km 5.7 mg/mL (starch)		Stavn and Granum, 1979
12	*Bacillus macerans* ATCC 8514	6.1-6.2						139,000	Km 3.33 mg/mL (starch)		DePinto and Campbell, 1968
13	*Bacillus macerans* IAM 1243	6.0 5.5-7.5	60	5.5-9.5	50°, 15 min			74,000[1], 1,45,000[2]	Km 2.5 mM (αCD)	5.7:1:0.4 (Initial) 2.1:1:0.3 (20h)	Kobayashi *et al.*, 1978; Kitahata and Okada, 1982b
14	*Bacillus macerans* WKMW 506	6	51	6-7	$t_{½}$=56 for 60 min	$t_{½}$=60		70,000	Km 3.8 (sol. starch)		Steighardt and Klein, 1993
15	*Bacillus obhensis*	5.5	60	6.5-9.5	55		<4	80,000[1]			Yagi *et al.*, 1986; Sin *et al.*, 1991

Table 2.3: Properties of CGTase from various bacteria (continued).

Sr. No.	Organism	Optimum		Stability		Ca^{2+} effect	pI	Molecular weight (Daltons)	Km/Vmax (substrate)	α:β:γ ratio	Reference
		pH	Temperature °C	pH	Temperature °C						
16	*Bacillus* sp. no. 5	5-5.7	55	7-10	≤ 55°		6.07, 6.80				Kitahata *et al.*, 1974
17	*Bacillus* sp. 290-3 (*B. firmus*/*B. lentus*)	6-8	60	6-8	50°, 16 h	-	4.1	75,000			Englbrecht *et al.*, 1990
18	*Bacillus* sp. A2-5a	5.5	50-55	6-10	≤ 60° I_2	+		80,000[1] 70,000[5]		0:7:1	Kometani *et al.*, 1994
19	*Bacillus* sp. AL-6	8 (I_2), 7.5-10.5 (HPLC)	55 (I_2), 60 (HPLC)	5-8	40°, pH 7.5, 20 min	+	3-4	74,000[1,2]		8% γ-CD, 22% with ehtanol	Fujita *et al.*, 1990
20	*Bacillus* sp. KC 201	6	60-65	6-10	<50°, pH 9, 30 min			75,000		0:6.2:1	Kitamoto *et al.*, 1992
21	*Bacillus* sp. HA3-3-2 ATCC 39612	6.5-8.0 (TCE) 5,9 (I_2)	70 (TCE) 60 (I_2)	6-11 (TCE)	< 60° (TCE) ≤ 70° (I_2)			68,000[1]			Pongsawasdi and Yagisawa, 1988
22	*Bacillus* sp. No. 562	7.0	65	7-11	≤ 70° 50% at 90°, ½ h	+	7.2	82,000[1] 1,70,000[2]			Yan and Lin, 1993 & 1995
23	*Bacillus stearothermophilus*	6.0	60	5.5-9.5	55°, 30 min 65° (Ca)	+	4.8	78,000[1]			Joong-Hoon *et al.*, 1990
24	*Bacillus stearothermophilus* TC-60	6	70	7-9.2	≤ 50° pH 7, 2 h		4.5	68,000[1]			Kitahata and Okada, 1982a

Table 2.3: Properties of CGTase from various bacteria (continued).

Sr. No.	Organism	Optimum		Stability		Ca^{2+} effect	pI	Molecular weight (Daltons)	Km/Vmax (substrate)	α:β:γ ratio	Reference
		pH	Temperature °C	pH	Temperature °C						
25	*Bacillus subtilis* no. 313	8	65		≤50°, pH 7	+	7.1	64,000	Km 6.67 mM (β-CD) Km 0.65 (γ-CD)	Only γ-CD 5% yield	Kato and Horikoshi, 1986
26	*Brevibacterium* sp. 9605	10	45	6-8	50	+	2.8	75,000[1] 86,000[2]		1.3:11.3:18.9 (20 h) γ-CD initially	Mori *et al.*, 1994a
27	*Klebsiella oxytoca* 19-1	5.5-7.5 max 6.0	30-45 max 40							96.5:3.5:0 (19 h)	Lee *et al.*, 1992
28	*Klebsiella pneumoniae* M 5 al (now called *K. oxytoca*)	5.2-7.0	50	6.0-7.5	45° 100% 50° 13%	+		68,000			Bender, 1977a,b
29	*Thermoanaerobacterium thermosulphurigenes* EM1	4.5-7.0	80-85		≥ 70° 5h no loss 10 % (80°), T½-30 min (100°)	+	5.0 major 4.3, 4.4, 4.6 others	68,000[1]		43:46:11 (2h) 33:54:13 (24h)	Wind *et al.*, 1995

Empty boxes indicate that data is not available. Molecular weight determined by GPC (1), SDS-PAGE (2), PAGE (3), ultracentrifugation (4), HPLC (5).

Table 2.3: Properties of CGTase from various bacteria.

Fujita *et al.*, 1990; *Bacillus* sp. HA3-3-2, Pongsawasdi and Yagisawa, 1988). As CGTase has many activities, the pH optima of these activities may not be the same, e.g. dextrinizing activity optimum (by iodine assay) of *Bacillus* sp. AL-6 is 8 while cyclization optimum is 7.5–10 (HPLC). *Bacillus circulans* 38-2 ATCC 21783 has two pH optima at 4.5–5.0 and 7–8.5 (Nakamura and Horikoshi, 1976b). CGTase is stable over a wide pH range of 5 to 11 depending upon the nature of producing bacteria. *B. macerans* enzyme is stable between pH 6 to 7 while alkalophilic *Bacillus* AL-6 is functional in the pH range of 6 to 11.

2.6.3 Temperature optimum and thermal stability

The temperature optima of most CGTases are in the range of 50–60 °C (Table 2.3). Among all the CGTases reported, *Klebsiella oxytoca* M5al (Bender, 1977a) CGTase has the lowest optimum of 35–40 °C. The two thermophilic organisms, *Thermoanaerobacter* sp. (Norman and Joergensen, 1992) and *Thermoanaerobacterium thermosulphurigens* (Wind *et al.*, 1995), have optima at 90–95 °C and 80–85 °C respectively. Quantitative studies giving half life and decay constants are available only for a few bacteria. CGTase is generally stable at 50–60 °C during the 15–30 min assay period. In *B. amyloliquifaciens* only 5% activity is lost on incubation at 60 °C for 2 h (Yu *et al.*, 1988). CGTase from *Thermoanaerobacter* retains 95-100% activity when incubated at 75 °C for 1 h at pH 5.5 (Norman and Joergensen, 1992). Similarly *T. thermosulphurigenes* CGTase lost only 10% activity at 80 °C for 5 h and none at 70 °C for same time (Wind *et al.*, 1995). CGTase activity is stabilized in the presence of its substrate, starch, or products (CDs) and Ca^{2+} ions. Stability in presence of starch is because of two reasons (Vihinen and Mantsala, 1989). When it binds to the enzyme, the conformation of the latter becomes more rigid and thus stable to denaturing conditions. Secondly, starch also contains some calcium ions as impurity which impart stability (Vihinen and Mantsala, 1989). CGTase is stabilized in the presence of moranoline and its derivatives (Maruo *et al.*, 1993). These

compounds, which are inhibitors of CGTase, also stabilize the enzyme against temperature.

2.6.4 Effect of ions

Metal ions, particularly heavy metals like Cu^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Pb^{2+}, are strong inhibitors of CGTase. Calcium ions stabilize CGTase against temperature denaturation. There are two binding sites for Ca^{2+} ions in CGTase (Klein and Schulz, 1991; Lawson *et al.*, 1994). One of the Ca^{2+} ion binds near the active site and probably participates in enzyme activity. In amylases there are reports where Ca^{2+} can be replaced by Zn^{2+}, Sr^{2+} or Mg^{2+} (Vihinen and Mantsala, 1989). Such studies involving direct replacement of Ca^{2+} ions have not been done in CGTase, but CGTase of *B. licheniformis* IT25 is stabilized by Mg^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} (Aoki *et al.*, 1987).

2.6.5 Inhibitors of CGTase

Besides heavy metals, CGTase is inhibited by malto-oligosaccharides and sugar analogues. Malto-oligosaccharides are competitive inhibitors. In *K. pneumoniae* M5al CGTase the degree of inhibition increased from maltose to maltotetrose and decreased with larger saccharides; maltotriose and maltotetrose were most effective inhibitors (Bender, 1985). Detailed studies on inhibition of *B. circulans* E192 CGTase were done by Bovetto and others (1992b). These workers found that glucose, maltose and salicin inhibited the CGTase competitively, deoxynojirimycin was a non-competitive inhibitor and acarbose was an uncompetitive inhibitor. Further, the acceptors glucose, maltose and salicin inhibited the cyclization activity and greatly enhanced the disproportionation of substrate, starch.

2.7 Isozymes of CGTase

Some strains of CGTase producing bacteria are known to produce a number of its subforms. *Bacillus circulans* E192 CGTase has two subforms of pI 6.9 (90%) and 6.7 (10%) (Bovetto *et al.*, 1992a). Bacillus sp. No.5 subforms (of pI 6.07 and 6.8) have similar pH and temperature optima and differ only in their isoelectric points (Kitahata *et al.*, 1974). The CGTase of *B. circulans* ATCC 21783 has a number of subforms. Makela *et al.* (1988) and Mattsson *et al.* (1990) analysed these subforms in detail. The major fraction has a pI of 4.9. Others have pI's ranging from 4.55 to 4.85. By studying the reaction product profiles they confirmed that the micro-heterogeneity in CGTase was only due to some post-translational enzymatic or chemical modification of the native protein.

2.8 Action of CGTase

The enzymology of CD production is quite complex. CGTase can act on multiple substrates and exhibits cyclization, transglycosylation and disproportionation activities simultaneously. The precise enzyme-substrate relationships are not fully understood, however, there are some studies carried on characterization of active site of CGTase using various maltooligosaccharides (native or 4-nitrophenyl derivatives) (Bender, 1990; Vetter and Thorn, 1992a; Bovetto *et al.*, 1992b).

Bender (1990) studied the mechanism of cyclization by α-CGTase of *K. pneumoniae* M5al, a truncated version of the same enzyme and *B. circulans* strain 8 β-CGTase. The maximum initial velocity of cyclization from G_1–G_{13} and the profile of cyclic and linear products obtained from short term digests of G_8–G_{13} revealed an active site of nine glucosyl residues for both α- and β-CGTases. The catalytic site of α-CGTase was found to be situated between subsite 3 and 4 whereas in β-CGTase it exists between 2nd and 3rd subsites on the active centre. In *K. pneumoniae* the α-CD was produced as follows

$$G_n \rightarrow CG_6 + G_{n-6}$$

where 'n' is the number of glucosyl residues.

Similar results were obtained with the α-CGTase of *B. macerans* (Vetter and Thorn, 1992a). These workers also observed that the glycosidic bond between the reducing end and the subsequent anhydrounit is not hydrolysed. On reaction with G7 some glucose was released probably due to the affinity of CGTase for G6 (Figure 2.1). The rate of preference for transfer of glycosyl residues was $G_1 << G_2 < G_3 < G_4 \leq G_5 \leq G_6 \geq G_7$.

However, in the case of β-CGTase from *B. circulans* strain 8, β-CD was produced from maltooligosaccharides containing more than nine glucose residues and maltose was the only major linear product formed. From G_8, α-CD was the major product. Vetter and Thorn (1992a) also studied the coupling (transglycosylation) products of α-CD with maltose. Using *B. macerans* α-CGTase, it was observed that maltooctaose was the primary product (45% of reaction mixture) followed by formation of G_{14} and G_{20}, other saccharides being formed later. Glucose and α-CD gave G_7, G_{13} and G_{19}. Whereas with α-CD, coupling was more prominent, β- and γ-CD had no preference for coupling or disproportionation. Maltooligosaccharides showed the following order of preference for the coupling reaction

$$G_2 > G_3 > G_4 > G_5 >>> G_6 \approx G_{7\text{-}11} > G_{12}$$

Maltose was the prefered saccharide while the maltohexaose was the poorest acceptor probably due to competition with α-CD for the donor site. Acceptor specificity studies revealed interaction of acceptor subsite with C-2, C-3 and C-6 of glucopyranosyl ring. The acceptor molecule in addition, required a free C-4 equatorial hydroxyl group on the non-reducing end (Bovetto *et al.*, 1992b; Vetter and Thorn, 1992b; Kitahata *et al.*, 1978b). The reactivity of the acceptor is enhanced if the hemiacetal group is blocked. Methyl α-D-glucopyranoside is prefered over glucose suggesting that the enzyme

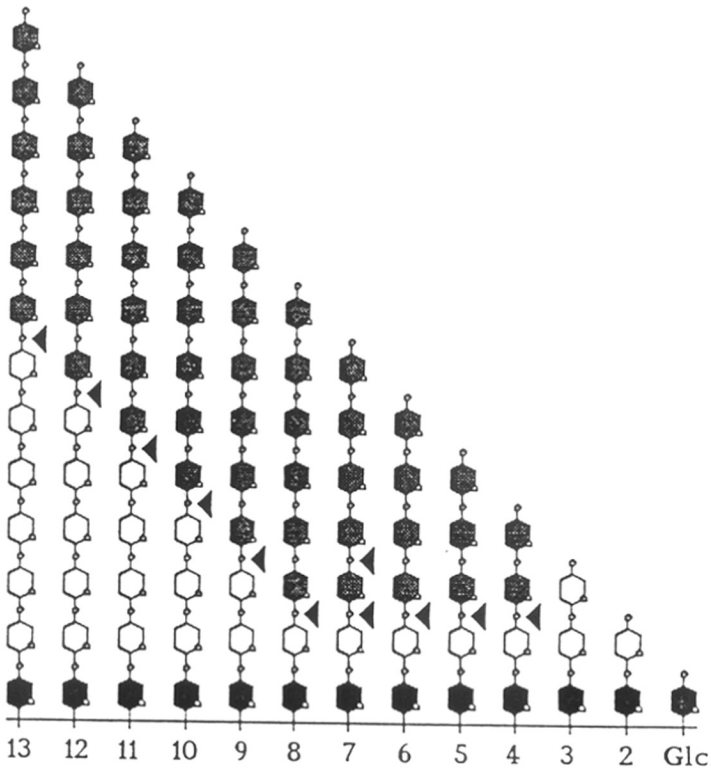

Figure 2.1: Schematic diagram of the prefered point of attack of CGTase on malto-oligosccharides of increasing chain length (from Vetter and Thorn, 1992a). The black glucose residue has the reducing end.

recognises the configuration at the anomeric centre and generally prefers the α configuration. Xylose can also act as an acceptor thus indicating that the acceptor site has a preference for the configuration rather than the glucopyranosyl ring as such.

The disproportionation activity of CGTase is also governed by the type and state of substrate. The saccharides G_1 to G_3 are hardly reactive. While G_5 is converted upto 50% into G_2 to G_{11} by *B. macerans* CGTase, more than 70% of G_{13} disappears in disproportionation reactions (Vetter and Thorn, 1992b). Maximum CDs are produced from amylose with a chain length of 60-80 glucose residues. For longer chain length amylose the disproportionation reaction predominates (Bender, 1981b). When the substrate concentration is low, cyclization predominates. Higher starch concentrations promote disproportionation.

2.9 Immobilization of CGTase

For continuous production of CDs and enzyme reusability, CGTase has been immobilized on a variety of matrices. Several techniques, including physical entrapment, adsorption as well as covalent linkage, have been used for immobilization. CGTase was entrapped in calcium alginate and polyacrylamide by crosslinking (Lee *et al.*, 1991a). It has been immobilized by ionic adsorption on weak basic Amberlite IRA93 (Hashimoto *et al.*, 1986), Amberlite IRA900 (Lee *et al.*, 1991c), on strong basic HN-11-32 (Hashimoto *et al.*, 1986), by simple adsorption on DIAION HP-20 (Kato and Horikoshi, 1984b) and on porous glass, aluminium silicate, hydroxyapatite, cellulose powder and sand (Steighardt and Kleine, 1993). CGTase has been immobilized covalently on a polyacrylamide support whose carboxyl groups were activated by water soluble carbodiimide (Ivony *et al.*, 1983), or on chitosan or chitin using glutaraldehyde (Yang and Su, 1989; Lee *et al.*, 1991b). Nakamura and Horikoshi (1977) immobilized chemically modified (succinylated) CGTase onto vinyl pyridine copolymer. Kleine *et al.* (1992) bound CGTase to glutaraldehyde

treated sillanized porous glass beads (Trisopearl). Arbatova and Reeben (1991) entrapped CGTase in a monoclonal antibody linked agarose carrier. Depending on the matrix and the source of enzyme, 6–80% of activity has been found to be retained on the matrix. The yields of CDs vary from 70% (1% starch, Su and Yang, 1990), to 46% (15% starch, Nakamura and Horikoshi, 1977). The pH and temperature optima of the immobilized enzyme were also found to be changed and the K_m value increased from 40 to 50 g/L of liquified starch (Yang and Su, 1989).

To improve the yield of CDs a few modifications in immobilized CGTase are documented. Coimmobilization of pullulanase increased yields of CD by 3–6% (Nakamura and Horikoshi, 1977; Lee *et al.*, 1991c). To facilitate the downstream separation of CDs, Su and Yang (1990) immobilized α-amylase and glucoamylase along with CGTase. Ninety-eight percent of the oligosaccharides produced were converted to glucose and this made purification of CDs easy.

When present in high concentrations, CDs inhibit action of CGTase on starch (Kim *et al.*, 1992). Thus, to increase CD yields two reactor configurations have been reported. Lee *et al.* (1991c) carried out the reaction in a two stage reactor consisting of CGTase immobilized on Amberlite IRA900. In first stage 1.5 U/g enzyme converted 10% starch to CDs and in second stage 0.47 U/g enzyme gave 39% yield of CDs. Okada *et al.* (1994a) prepared an immobilized enzyme bioreactor by coupling CGTase to the surface of a hollow fibre ultrafiltration membrane module. About 10-20% higher yields of CDs, as compared to those with free enzyme, were obtained. By increasing the enzyme loading, the same reactor could also be used for efficient transglycosylation reaction (Okada *et al.*, 1994b).

CGTase immobilized on DIAION HP-20 was stable for 2 weeks at 60 °C and pH 8.0 without significant loss of activity (Kato and Horikoshi, 1984b). The immobilized CGTase from *B. macerans* WKMW 506 (Steighardt and Kleine, 1993) could be reused

for 12 reaction cycles without loss of activity and even after 20 uses 65% activity was retained on Trisopearl beads. Maruo *et al.* (1993) reported a novel method to stabilize immobilized CGTase. They found that, in presence of the inhibitor glycosyl moranaline and dextrin, the CGTase immobilized onto chitosan beads retained its activity for over 600 days at 55 °C. It retained over 80% of activity, even after 700 days.

2.10 Cloning and overexpression of CGTase gene

The CGTase genes from a number of wild strains have been cloned primarily to overexpress and enhance the yields of this commercially important enzyme (Schmid, 1989; Tao, 1991). Though the genes have been cloned in *E. coli* as well as *B. subtilis*, the level of expression is low in *E. coli*. In *E. coli* the enzyme seems to accumulate more in periplasmic space (Sin *et al.*, 1991; Kimura, *et al.*, 1990; Kaneko, *et al.*, 1988) or as intracellular inclusion bodies (Lee and Tao, 1994). The CGTase gene has been expressed under the control of various promoters e.g. tac, trp, λ-pL, α-amylase, cellulase or T7 RNA polymerase (Kimura *et al.*, 1990; Paloheimo *et al.*, 1992; Sin *et al.*, 1993; Lee and Tao, 1994). Using a triple protease deficient *B. subtilis* host, Sin *et al.* (1993) introduced degQ gene in the plasmid containing CGTase gene resulting in 98% secretion of expressed CGTase. The yields of CGTase in recombinant strains vary form 2 mg/L to 6-7 g/L (Lee and Tao, 1994). In *E. coli* the yields of CGTase obtained were 2 mg/L (tac promoter, Bock *et al.*, 1989) to 140-300 mg/L (lac promoter, Hellman *et al.*, 1990; 1992). When placed under the control of α-amylase promoter of *B. amyloliquifaciens*, Paloheimo *et al.* (1992) obtained 170 mg/L of CGTase (from *B. circulans* ATCC 21783) in *B. subtilis*. The yield further increased to 1.2 g/L in an industrially feasible medium in a 10 L fermenter. Similarly, Hermann *et al.* (1990) reported 6-7 g/L CGTase produced from cloned gene of *Bacillus* no. 1-1 in a protease negative *B. subtilis* host under the control of subtilisin promoter.

The CGTase yield as well as the ratio of CDs produced have been modified by carrying out chemical mutations or site directed mutagenesis. With N-methyl-N'-nitro-nitrosoguanadine (MNNG) the secretion of CGTase in recombinant *E. coli* was increased from 2 mg/L to 400-500 mg/L (Schmid, 1989). In CGTase gene of *B. obhensis* cloned in *B. subtilis* replacement of tyrosine residue at position 188 of domain A with tryptophan resulted in twice the amount of γ-CD as compared to the wild type enzyme (Sin et al., 1994).

2.11 Structure and properties of cyclodextrins

The structure and properties of CDs have been extensively reviewed by a number of authors (Szejtli, 1982; Bender, 1986; Hedges, 1992). The following section is based on the work of these authors.

The ring shape of CDs is due to the C1-chair conformation of the D-glucopyranosyl units linked together by 1,4 α-D linkages. The wider side of the `truncated cone' has all the secondary hydroxyls where as the primary ones are placed on the other edge. The cavity is lined by hydrogen atoms and ether-like glycosidic oxygen bridges. The nonbonding electron pairs of glycosidic bond are directed towards the inside of the cavity producing a high electron density thus giving a hydrophobic character to it. The hydroxyl groups on the outside surface make it hydrophilic. The C 6 of glucose molecules is free to rotate which results in the tapering at one end of CD.

2.11.1 Crystal structure

A unit cell of α-CD has 24 glucose units in an orthorhombic unit cell of space group $P2_12_12_1$. The dimensions of unit cell are a = 14.856, b = 33.881 and c = 9.717 Å. The unit cell of β-CD contains 14 glucose units and its space group is $P2_1$. In γ-CD the tetragonal space group $P42_1$ is made up of 48 glucose residues. Table 2.4 shows some physical properties of the CDs.

Property	α	β	γ
No. of glucose units	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100 mL, 25 °C)	14.5	1.85	23.2
Specific rotation $[\alpha]_D^{25}$	150 ± 0.5	162.5 ± 0.5	177.4 ± 0.5
Cavity diameter (Å)	4.7–5.3	6.0–6.3	7.5–8.3
Height of torus (Å)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Diameter of periphery (Å)	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
Volume of cavity (approx.) (Å^3)	174	262	472
Cavity volume (approx.) per mole CD (mL)	104	157	256
per 1 g CD (mL)	0.1	0.14	0.20
Crystal water (wt %)	10.2	13.2–14.5	8.13–17.7

Table 2.4: Physical properties of CDs

2.11.2 Properties

The solubility of CDs in water is in the order β < α < γ. Among all solvents β-CD is most soluble in dimethyl sulphoxide and dimethyl formamide. CDs are insoluble in most solvents. In ethanol, solubility increases upto 30% solution in water after which it decreases. The CDs decompose at 300 °C. They are hydrolysed by strong acids like hydrochloric and sulphuric acid but slowly as compared to starch. Hydrolysis by organic acids is much slower. Exposure of CDs to bases does not hydrolyse them even at higher temperatures. Peroxide cleavage does not form formaldehyde as no reducing ends are present. Enzymic hydrolysis of CDs vary. They are not hydrolysed by glucoamylase and

β-amylase which require reducing ends for action. Most of α-amylases do not cleave α and β CDs, however γ-CD is readily hydrolysed. Cyclodextrins can be hydrolysed by a specific enzyme cyclomaltodextrinase and by CGTase in the presence of acceptors. Complexes of CDs are more resistant to enzymatic digestion.

Pure CDs show negligible toxicity to the animals tested. The minimum lethal dose to kill 50% animals tested (LD_{50}) for β-CD is > 12,500 mg/Kg (orally), 788 mg/Kg (intravenous), 700 mg/Kg (intraperitoneal) and > 900 mg/Kg (subcutaneous) (Hedges, 1992).

The chemical and physical properties of CDs can be varied by chemical modification. Hydroxypropyl, hydroxyethyl, methyl and sulphate groups increase the solubility. Hydroxyethyl β-CD (molar substitution 1.6) is 55 times more soluble than free CD. Crosslinking β-CD with epichlorhydrin results in a water insoluble polymer.

2.12 Production of CDs

Cyclodextrins are formed by intramolecular transglycosylation reaction in an endo attack of CGTase on sufficiently long chains of glucose linked together by α 1→4 glycosidic bonds. Thus CDs are produced from amylose, amylopectin, starch, glycogen and maltodextrins. CDs can also be formed from malto-oligosaccharides with chain length less than six as a result of interchain transglycosylation reaction of CGTase. However, glucose cannot be transglycosylated (Bender, 1981b). The ability of CGTase to form CDs from maltooligosaccharides (G_2–G_6) varies. No CDs were detected when the enzyme of *K. oxytoca* (Lee *et al.*, 1992) was incubated with saccharides of chain length G_1–G_7.

Commercially CDs are produced from raw or modified starch. Corn and potato are major starch sources although cassava, rice and wheat starches are also used. The best source of starch is probably dependent on its structure and the affinity of CGTase for it. Thus Hwang *et al.* (1990) found potato starch the best for CD production by alkalophilic

Bacillus sp., whereas maximum CDs were produced from cassava starch by *B. stearothermophilus* CGTase (Chen *et al.*, 1991).

Production of CDs depends on the concentration of starch in the reaction mixture. At low substrate concentration high yields of CDs are obtained. High starch concentration gives low CD yields. Though CD yield depends on the intrinsic nature of CGTase, as high as 90% conversion to CDs is obtained with 1% w/v starch (Jamuna *et al.*, 1994), whereas a solution of 30% w/v starch gives only 20% conversion to CDs (Matzuzawa *et al.*, 1975). Starch being a cheap raw material, economical CD production demands high starch concentrations, primarily to minimize the reaction volume and facilitate concentration of products. A concentration of 15% starch was found to be optimum (Matzuzawa *et al.*, 1975). Higher starch concentrations also promote its retrogradation (Szejtli, 1982) which decreases CD yields. Partially hydrolyzed starch promotes CD production. However, the degree of hydrolysis should not be more than dextrose equivalent (DE) of 2 (Horikoshi *et al.*, 1981) as excessive thinning decreases CD yields. Starch of DE $\approx$ 1 gave upto 35% CD yield whereas starch of DE $\approx$ 9 gave only 14-15% conversion to CDs (Horikoshi *et al.*, 1981). To circumvent the retrogradation of starch and stirring of large volumes of concentrated gelatinized starch solutions, CGTase is added to starch suspensions and the suspension is heated to 80 °C for both solubilization and liquifaction (Horikoshi and Akiba, 1982).

Cyclodextrin production can also be enhanced by physical modification of starch, e.g., extrusion or milling of raw starch. Extruded corn starch yielded 54 g/L CDs as compared to 45 g/L (liquified) or 6 g/L (raw) starch (Lee and Park, 1992).

Though CGTase can 'bypass' α-1$\rightarrow$6 linkages in the starch molecule, addition of a debranching enzyme, such as pullulanase, improves the CD yields (Sato and Nakamura, 1974; Suzuki *et al.*, 1977). The enzyme to starch ratio is very critical as it can change yield as well as ratio of CDs in the product (Sato and Yagi, 1991). The best results have

been obtained with enzyme substrate ratios (w/w) of 1:1000 (Sato *et al.*, 1985) to 1:5000 (Bender, 1983b).

Individual CDs have been produced commercially by fractionating mixtures of CDs produced by specific CGTases (Bender, 1986). Some CGTases, however, produce a particular CD in abundance. Therefore, such enzymes are preferable for CD production. Some prominent enzymes are those from *B. amyloliquifaciens* (α-CD, > 95%) (Yu *et al.*, 1988), *B. circulans* C31 (β-CD, > 90%) (Pongsawasdi and Yagisawa, 1988) and *Brevibacterium* sp. (γ-CD, > 55%) (Mori *et al.*, 1994a).

The equilibrium of cyclization reaction can be drastically shifted towards a particular CD by addition of compounds capable of forming specific inclusion complexes (clatharates) with that CD. These clatharates being insoluble in aqueous medium precipitate from the reaction mixture thus shifting the reaction equilibrium in favour of a particular CD. Using this method high yields of CDs can be obtained (Bender, 1986). Thus, α-CD is the main reaction product with yield of 50% in presence of 1-decanol (Armbruster and Jacaway, 1972; Flaschel *et al.*, 1984). Yields of β-CD are enhanced by the use of toluene (Cramer and Steinle, 1955; Vakaliu *et al.*, 1979), trichloroethylene (McClenahan *et al.*, 1942), limonene (Ammeraal, 1988) and bromobenzene (Raja and Ramakrishna, 1994). For γ-CD production Bender (1983b) employed combination of bromobenzene and sodium acetate to obtain 18.7% yield. A mixture of butanone and α-naphthol (Seres *et al.*, 1985), glycyrrhizic acid or stevioside (Sato and Yagi, 1991), 13-24 ring cyclic compounds (Schmid *et al.*, 1988) and C_{12} cyclic compounds (Rendleman, 1992) have been used to enhance γ-CD production. Cyclodextrin production is also increased by addition of solvents like aliphatic alcohols and acetone (Shiraishi *et al.*, 1989b; Lee and Kim, 1991a) and surfactants like sodium dodecyl sulphate and Triton-X-100 (Kobayashi *et al.*, 1983; Tomita *et al.*, 1993). Different workers used ethanol (10-30%), resulting in 2-2.75 fold increase in CD yields (Shiraishi *et al.*, 1989a; Tomita

et al., 1990; Fujita *et al.*, 1990; Lee and Kim, 1991a; Mattsson *et al.*, 1991; Tomita *et al.*, 1993). Depending upon the CGTase used the yield of a particular CD is enhanced at the expense of others (α-CD, Shiraishi *et al.*, 1989a; β-CD, Tomita *et al.*, 1993; γ-CD, Tomita *et al.*, 1990). Though the exact mechanism of the ethanol effect is not known, it is believed that solvents like ethanol and DMSO decrease the water activity of the reaction medium thus decreasing the probability of an occasional hydrolysis reaction that might be taking place at the active site (Mattsson *et al.*, 1991). Ethanol can also inhibit the transglycosylation reaction (Tomita *et al.*, 1990). On the contrary, Lee and Kim (1991a) have indicated enhanced starch hydrolysis in the presence of ethanol. However these workers reported an increase in CD production as well. Surfactants increase CD yields and modify product profiles. Kobayashi *et al.* (1983) observed that linear surfactants enhance α-CD production whereas those with a bulky hydrophobic moiety selectively produced β-CD. They ascribed this effect to substrate modification (helix formation in starch). Tomita *et al.* (1993) tested a number of surfactants like Triton X-100, Span and SDS for their effect on CD production with *B. autolyticus* CGTase. Triton X-100 (4% v/v) gave best results converting 56% starch (10% w/v) into CDs. Beta-CD was the only product formed.

Enzymatic production of CDs is severely inhibited by CDs themselves. Kim *et al.* (1993) found 50% inhibition of CGTase activity in the presence of 2 g/L of α/γ-CDs. Similarly Bergsma *et al.* (1988) reported CD mediated inhibition of CGTase activity. It was demonstrated that at higher starch concentrations CDs inhibited *B. circulans* BE101 CGTase. This inhibition could be reversed either by precipitating the β-CD as a sodium benzoate complex or removing CDs by coupled ultrafiltration. Kim *et al.* (1993) also observed 57% increase in yield in an ultrafiltration membrane recycle reactor when compared to a batch run.

2.12.1 Fractionation of CDs

The CGTase reaction mixture often contains linear oligosaccharides along with CDs. To purify and fractionate the CDs a number of approaches are possible depending upon the end use (Bender, 1986).

1. β-CD, which has very low solubility in water (18.5 g/L, 25 °C) can be crystallized directly from concentrated solutions at low temperatures. Such process can produce approximately 98% pure β-CD (Hedges, 1992).

2. Using specific complexing agents (as described in the previous section) α-, β- or γ-CD can be selectively precipitated. These are recovered by filtration / centrifugation. The complexing agent is separated by steam distillation or solvent extraction (Rendleman, 1992) and CDs crystallized from solutions after concentration.

3. Cyclodextrins can be separated by adsorption onto strong cation-exchange resins (Hokse, 1984; Horikoshi *et al.*, 1981), hydrophobic polymeric resins (Rikagaku, 1985) or gel permeation chromatography (Horikoshi *et al.*, 1981). Specific tailor made adsorbent resins have also been prepared to separate CDs (Yamamoto and Horikoshi, 1981; Makela *et al.*, 1989; Tsuchiyama *et al.*, 1991a,b).

4. The starch digested with CGTase is treated with α-amylase / glucoamylase to completely hydrolyze residual dextrins aiding further purification of CDs. In another method for α-CD production the contaminating β- and γ-CDs are broken down with a mixture of CGTase from *B. obhensis* (having high affinity for β-CD) and α-glucosidase. α-CD in the resulting solution is separated by conventional techniques (Sato *et al.*, 1994).

2.12.2 Commercial processes

Two types of processes are known for commercial production of CDs (Bender, 1986; Hedges, 1992). The first is the a solvent based recovery process (1-decanol for α-CD,

toluene for β-CD, bromobenzene for γ-CD) using *K. pneumoniae* or *B. macerans* CGTase. The other process is based on alkalophilic *Bacillus sp.* or *B. macerans* CGTase where CDs are purified chromatographically.

2.13 Application of CDs

The ability of CDs to form inclusion compounds finds a lot of applications in research and industry. Cyclodextrins are used in foods, cosmetics, pharmaceutical, chemical and agricultural industries, in analytical chemistry, and biology mainly because of two reasons.

1. Their ability to disperse hydrophobic molecules in a hydrophilic environment.
2. Their ability to mask the guest molecule from the environment thereby changing its physical and chemical properties.

These applications have been widely reviewed (Horikoshi, 1979; Szejtli, 1982 and 1984; Bender, 1986; Hedges, 1992). There are large number of applications of CDs and their derivatives as testified by numerous publications and patents filed (Szejtli, 1995). A few of the representative applications compiled from the above publications are described here.

2.13.1 Pharmaceutical Industry

Complexation of drugs with CDs has been one of the approaches to enhance the solubility of oily suspensions and poorly water soluble compounds. There is increase in rate of dissolution of drugs when administered intravenously or orally. Hence the bioavailability of drugs increases. Some of these drugs are vitamin A, progesterone, ibuprofen, hydrocortisone and diazepam. The prostaglandin E_2-β-CD complex was the first cyclodextrin complexed drug to be marketed (Szejtli, 1984). CDs protect the drugs from hydrolysis (e.g. aspirin, atropine, procaine and prostacycline), oxidation (chlorpromazine, epinephrine, aldehydes), photo-decomposition (phenothiazines, ubiquinones, vitamins) and dehydration (prostaglandin E group). CDs convert liquid drugs to crystalline powders

(vitamins A, D and K, phenols, benzaldehyde, nitroglycerine, essential oils). Volatile compounds are rendered non-volatile (iodine, 1-menthol, chlorobutanol, salicylic acid). CDs also have organoleptic properties. They improve or mask the undesirable smell and taste of drugs (chloral hydrate, prostaglandins, non steroidal anti- inflammatory drugs, thymol, chloramphenicol, ginseng extract etc.). Sometimes the complexed drugs have reduced side effects (e.g. reduced gastric mucosal irritation with drugs like indomethacin, aspirin, naproxen, phenylbutazone, etc.). Some complexes prevent haemolysis induced by their corresponding free drugs like phenothiazines, flufenamic acid, antibiotics, menadione etc. Drug-CD complexes can be administered through a wide range of routes which are not feasible otherwise. CDs can be used as binders and fillers in tabletting and thus they can have multipurpose presence in drug formulations. CDs have been directly used in drug detoxification, treatment of shock and wound cleansing formulations (Szejtli, 1994).

2.13.2 Foods, Cosmetics and Consumer items

The most important uses of CD in foods, cosmetics and consumer products are based on preservation of aroma, flavours, masking of undesirable tastes and odours, improvement of quality and presentability of products.

2.13.2.1 Stabilization of food flavours

Volatile flavouring compounds, fragrances and aromas are often lost during cooking, processing and storage of food items. CD complexes with these compounds (e.g. essential oils, vegetable extracts etc.) can retain them in prepared items for a long time. Besides it makes their dispensing, storage and utility much easier and convenient. The complexes remain stable to oxidation, chemical decomposition and heat denaturation. CD-flavours can be used for invalids in hospitals where normal food is not advised. The quality of beverages like tea and coffee can be improved by blending with CDs. Natural flavours in fruit juices, butter, chewing gum are preserved using CDs.

2.13.2.2 Elimination of objectionable tastes and odours

The compounds responsible for giving prolonged or intermittent objectionable taste when mixed with saliva while eating can be masked by CD encapsulation. The bitter taste of tea, coffee and protein hydrolysates, the characteristic odour of mutton or fish and the grassy smell of soyabean products can be eliminated.

2.13.2.3 Improvement of food quality

CDs prevent precipitation of chalcone and dihydroxychalcone in soft drinks, neohesperidin and narangin in citrus juices and white precipitates in tinned bamboo shoots. The bitterness of citrus juices due to narangin is reduced. Narangin can also be separated from juice using CD polymers. CDs prevent staling of prepared foods. They help in emulsification of oil-water mixtures in bakery products. Addition of CDs to emulsified foods and cheese increase their storage life. CD-I_2 complex can be incorporated into marine food products, and packaging material for preventing putrefaction and mould growth in packaged foods. In the tobacco industry CDs preserve volatile aroma substances in cigarettes during storage. They can also be incorporated into cigarette filters to remove nicotine.

2.13.2.4 CDs in cosmetics and toiletries

CDs find use in perfumes, detergents and laundry items for ensuring prolonged fragrance. CDs are incorporated into mouth-wash and deodorant preparations. CDs also act as defoaming agents in detergents. CDs are also used to impart perfume to fabrics and make them soft after washing.

2.13.3 Application in chemical industry

2.13.3.1 Separation by selective CD complexation

A host of separations can be carried out by complex formation with CD monomers or polymers. The simplest use of CD monomers is to form complex with a guest molecule

and cause it to precipitate from solution. As CDs have an affinity for molecules of a particular structural configuration, differential precipitation can be achieved. e.g. separation of xylene isomers and ethyl benzene, isomeric alkyl phenols, straight as well as branched chain hydrocarbons and removal of free fatty acids from oils. Toxic vapours of solvents can also be removed from workplaces using CDs. Another application that has recently been commercialized involves separation of cholesterol from egg and dairy products. Mixture of enantiomers can also be resolved by CD complexation.

2.13.3.2 Chromatography on CD polymers

Cyclodextrins are used for selective chromatographic separations. They are either bonded onto conventional polymeric and silica supports or alternately they are crosslinked with foam or block polymers. CD polymers can also act as molecular sieves. Thus o- and m-dichlorobenzene, cinnamic alcohol and eugenol can be separated. By selective complexation on a β-CD polymer based column, nucleotides and nucleosides can be separated. Aromatic amino acids can be separated from each other and from aliphatic ones. Crosslinked β-CD polymer can be used to separate R(-) and S(+) isomers, e.g. mandelic acid.

2.13.4 CDs in analytical chemistry

Sprays containing CDs enhance iodine staining of TLC plates. Phenolic and naphtholic compounds can be separated and identified on TLC using dissolved α-CD in the mobile phase. Similarly prostaglandins PGE, PGA, PGB, PGF can be resolved. Racemic mixtures are separated on HPLC with β-CD as the mobile phase. Selective titration of different organic acids and phenols can be done in the presence of CDs. Substrates modified with CDs are helpful in the assay of amylase, and the estimation of serum uric acid and glucose. Dansylated amino acids at concentrations as low as 0.2 nmolar can be detected due to increased fluorescence in the presence of CDs.

2.13.5 Agricultural applications

Molecular encapsulation of volatile, inflammable, liquid insecticides and herbicides is carried out with CDs. e.g. stabilization of O,O-dimethyl-2,2dichlorovinylphosphate, methylparathion, chloropicrin, natural pyrethrins and pyrethroids etc. Complexation with CDs also results in prolonged activity, reduced evaporation losses and resistance to heat and light. Toxic liquids can be converted to powdered form which ensures safe handling. Cyclodextrin complexes of bird- repellents are also added to insecticides to save them from intoxication. Ripening of fruits and vegetables can be enhanced by α-CD-ethylene complex. A spray containing CDs was found to enhance the leafy content of vegetables by 15–30%. Similarly, the yields of wheat and triticale plants also increased by 20–30%. Fat soluble vitamins can be properly mixed into animal feeds if they are available as CD complexes. Uniform distribution within a great mass of fodder becomes easier. Cyclodextrins are known to improve the palatability of microbial cell mass and bone powder.

2.13.6 Cyclodextrins in biotechnology

Biotransformation of hydrophobic compounds is accelerated in the presence of CDs. Conversion of hydrocortisone to prednisolon increased by more than 300%. Similarly 95% of cholesterol is converted to androst-4-ene-3,17-dione in the presence of β-CD. Yeasts convert aromatic aldehydes to alcohols faster in the presence of CDs. Fatty acid synthesis is stimulated in *Mycobacterium phlei* in the presence of CDs. The production of lankacidin group of antibiotics by *Streptomyces* is enhanced in the presence of β-CD. In vaccine preparation, growth of *Bordetella pertusis* is enhanced in the presence of 0.5 mg/ml dimethyl or trimethyl β-CD while toxin production is enhanced 100 fold. Similarly CDs stimulate *in vitro* growth of *Mycobacterium lepraemurium*. CDs are used to solubilize the substrate oil in lipase assay. Cyclodextrins are used to stabilize lipophilic substrates in many diagnostic kits. CDs are used to dissolve hydrophobic substrates in

fermentation. In animal tissue culture fatty acids complexes of CDs are used as serum substitute. CDs also enhance solubility of lipophilic substrates and act as carriers of nutrients. Complexes of antifungal antibiotics (e.g. nystatin) are widely used in tissue culture. In industrial waste treatment, CDs and their derivatives are used to sequester toxic molecules in a pretreatment step for higher loading of degradable compound in the waste stream.

2.13.7 CDs as catalysts

Reactions catalysed by CD are of two types. Covalent catalysis, where a covalent intermediate is formed and the noncovalent type, where the cavity of the CDs provides a nonpolar or restricted reaction field. The reactions catalysed include cleavage of esters, amides, organophosphates, carbonates and sulphates, intramolecular acyl migration, decarboxylation and oxidation. CDs and their derivatives also act as models for enzymes. CD derivatives of 1,4-diaminoethane and polyamine act as metalloenzymes. Biomimetic models of ribonuclease, tryptophan synthetase, carbonic anhydrase, aminotransferase and phosphate transferase (kinase) have also been prepared from CD conjugates.

2.13.8 Miscellaneous applications

Patents have been filed for CD applications in textile (imparting perfume to fabrics, retarders in dyeing process), paint (improvement of thickener), plastics (expandable plastic composites), printing (dye for inkjet printer, transfer of ink to polar polymers), polymers (stabilization of catalysts), fire fighting equipment, photography, solid fuels and many more.

3. Materials and Methods

3.1 Chemicals and growth media

Chemicals and dehydrated culture media of highest grade, procured from local suppliers, were used in this study.

3.2 Microorganisms

The microorganism producing CGTase was isolated from garden soil. A 25 g sample of soil was suspended in 100 mL distilled water (DW) containing 1% soluble starch and 1% sodium carbonate in a 500 mL conical flask. The flask was incubated overnight on a rotary shaker (200 rpm) at 30 °C. One mL aliquots from the flask were then serially diluted and plated on alkaline medium containing phenolphthalein and methyl orange (Table 3.1; Park *et al.*, 1989). The plates were incubated at 30 °C for 24-48 h. The colony forming units (CFU) with yellow halo around them were selected as CGTase positive cultures. These cultures were isolated to purity on fresh plates containing dye and the Horikoshi medium II (Table 3.2; Nakamura and Horikoshi, 1976a). Their ability to produce CGTase was confirmed by means of a spectrophotometric / HPLC based assay. Besides these isolates a number of *Bacillus circulans* and *B. macerans* cultures from culture collections (NCIM and others; Appendix 1) were also tested for CGTase activity.

The culture selected for the work was a soil isolate which was found to give best results. This organism was identified as *Bacillus firmus* by standard procedures (Gordon, 1977; Sneath, 1986).

3.3 Detection and assay of CGTase activity

All enzyme assays were carried out with same batch of soluble starch (DE 8) purchased from Qualigens. The α, β, and γ cyclodextrin standards were procured from Sigma, Aldrich or Fluka.

Component	Concentration (g / 100 mL)
Soluble starch	1.0
Peptone	0.5
Yeast extract	0.5
$MgSO_4.7H_2O$	0.02
K_2HPO_4	0.1
Phenolphthalein	0.03
Methyl orange	0.01
Agar agar	2.0
Na_2CO_3 (Autoclaved separately).	1.0

Table 3.1: Composition of medium used for culture isolation.

Component	Concentration (g / 100 mL)
Soluble starch	1.0
Peptone	0.5
Yeast extract	0.5
$MgSO_4.7H_2O$	0.02
K_2HPO_4	0.1
Agar agar (for solid medium only)	2.0
Na_2CO_3 (Autoclaved separately)	1.0

Table 3.2: Horikoshi medium II composition.

3.3.1 Tilden and Hudson microscopic tests

The Tilden and Hudson test for estimation of CGTase activity was performed as described by Tilden and Hudson (1942).

3.3.1.1.1 Reagents

1. Soluble starch, 3% in Tris-HCl buffer 0.05 M, pH 7.0
2. Iodine 0.1 M in 0.1 M KI made in DW.

3.3.1.1.2 Procedure

To 1 mL starch solution, 0.5 mL of appropriately diluted enzyme was added. The mixture was incubated at 60 °C. At five minute time intervals, three drops of reaction mixture were mixed with one drop of iodine solution and a loopfull was examined under microscope under conditions of evaporation. In the initial stages of reaction, when the colour of the starch-iodine complex was blue-violet, small blue hexagonal crystals of α-CD-I_2 complex were observed. At the end point, when the colour turned brown-violet, long, dichoric crystals, extending from the border of drop, were observed. One Tilden and Hudson unit was defined as the amount of enzyme that converted 1 mL of starch to the end point in 30 min.

3.3.2 Trichloroethylene method

The CGTase activity by trichloroethylene (TCE) method (Nomoto *et al.*, 1984) was determined as follows.

3.3.2.1.1 Reagents

1. Tris-HCl buffer, pH 8.5, 0.05 M
2. Soluble starch, 2% in buffer
3. Trichloroethylene (TCE)

3.3.2.1.2 Procedure

The enzyme was serially diluted in buffer. One mL of enzyme was incubated with 5 mL of starch solution at 60 °C. After 48 h, 2.5 mL of TCE was added to the reaction mixture. The contents of the tube were shaken vigorously and the resulting suspension was kept undisturbed overnight. White precipitates of CD-TCE complex confirmed the presence of CGTase. The maximum enzyme dilution which gave distinct precipitation was defined as the enzyme activity.

3.3.3 Methyl-orange assay

The CGTase assay based on complex formation of CDs with methyl orange was used (Makela and Korpela, 1988).

3.3.3.1.1 Reagents

1. Imidazole-HCl buffer, pH 7.0, 0.1 M
2. Maltotriose / Soluble starch
3. $CaCl_2$
4. Methyl orange

3.3.3.1.2 Procedure

Imidazole buffer containing 0.3 mM methyl orange and 5 mM $CaCl_2$ was filtered through 0.45 μ membrane. To this solution 1% maltotriose or soluble starch was added to prepare the substrate for CGTase.

One mL of substrate was mixed with 0.5 mL enzyme solution and incubated at 60 °C for 20 min. Absorbance change at 546 nm with respect to control (containing buffer instead of enzyme) was calculated. The amount of β-CD produced was calculated from a standard curve of 0-500 mg/mL β-CD. One unit of CGTase was defined as the amount of enzyme that produced 1 μmol β-CD/min.

3.3.4 Phenolphthalein assay

3.3.4.1.1 Reagents

1. Phenolphthalein (PHP) stock 4 mM in ethanol
2. Na_2CO_3 solution 125 mM in DW
3. Tris-HCl buffer 0.05 M, pH 7.0 or 8.5
4. Soluble starch 1% in buffer
5. β-CD in buffer 200 μg/mL and 500 μg/mL

The working phenolphthalein solution was made by adding 1 mL of PHP stock to 100 mL of Na_2CO3 solution containing 4% v/v ethanol just before starting the experiment.

3.3.4.2 Standard assay

One hundred μL of appropriately diluted enzyme was incubated with 1 mL starch solution at 60 °C for 20 min. The reaction was stopped by quickly cooling the tubes in ice. Four mL of working PHP solution was added, the tubes vortexed and the absorbance of the mixture immediately measured at 550 nm. Percent decrease of sample with respect to control, containing 1.1 mL of buffer in place of enzyme and starch solution, was calculated.

$$\% \text{ decrease in absorbance} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

The amount of β-CD produced was estimated from a standard curve of 0-200 μg/mL concentration. One CGTase unit was defined as the amount of enzyme that produced 1 μmol β-CD per min.

3.3.4.3 Stability studies for PHP reagents

The absorbance of duplicate tubes containing 1 mL buffer or 100 μg β-CD was measured every 5 min after adding 4 mL of PHP solution. Parallel experiments, according to the procedure of Makela *et al.* (1989) were also performed.

3.3.4.4 Linearity studies

The standard procedure was used to test the linearity of assay upto 500 μg/mL β-CD concentration. Effect of PHP concentration on linearity was also studied.

Throughout the work, PHP assay was routinely used to determine CGTase activity.

3.3.5 Iodine assay

The dextrinizing activity of CGTase was measured by a modification of iodine method reported by Fuwa (1954).

3.3.5.1.1 Reagents

1. Tris-HCl buffer, pH 7.0, 0.05 M
2. Soluble starch, 1% in buffer
3. Iodine 0.01 M in 0.25 M KI

3.3.5.1.2 Procedure

One mL starch solution was mixed with 0.1 mL of appropriately diluted enzyme solution and incubated at 60 °C for 20 min. The tubes were then cooled and the reaction stopped by adding 0.1 mL of 0.5 N HCl. An aliquot of 0.1 mL of reaction mixture was mixed with 0.8 mL of iodine solution and 10 mL of distilled water. The absorbance of this solution was measured at 660 nm. One enzyme unit was defined as the amount of enzyme that caused 1% decrease in absorbance of iodine, per min, under the test conditions.

3.3.6 HPLC based assay

CGTase activity was determined by HPLC by estimating the amount of CDs produced during the assay according to the method of Sato *et al.* (1985).

3.3.6.1.1 Reagents

1. Tris-HCl buffer, pH 7.0, 0.05 M

2. Soluble starch, 1% in buffer

3. Eluent Acetonitrile:water in the ratio 65:35

3.3.6.1.2 Procedure

One mL of starch solution was mixed with 0.1 mL of appropriately diluted enzyme solution and incubated at 60 °C for 20 min. The reaction was stopped by cooling the tubes in ice cold water and adding 0.1 mL of 0.5 N HCl.

3.3.6.1.3 Sample preparation

Two hundred μL of reaction mixture was mixed with equal amount of acetonitrile in a microfuge tube. The contents were thoroughly mixed and centrifuged at 10,000 rpm in Remi microcentrifuge for 10 min.

3.3.6.1.4 HPLC

Ten μL sample was loaded onto a Shodex DC-613 column maintained at 60 °C. The mobile phase flow rate was maintained at 1.5 mL/min. The reaction products were detected on-line by Waters 410 refractive index detector. The CDs were quantified by an external standardization method. Under these conditions 1 CGTase unit was defined as the amount of enzyme that produced 1 μmol of β-CD per min.

3.4 Cell mass estimation

3.4.1 Dry cell weight (DCW)

A sample from the fermentation broth was centrifuged in Remi C-24 cooling centrifuge at 16,000 g, 4 °C, for 20 min. Minimum sample size was 10 mL. The centrifuged cells were dried in a vacuum oven at 55-60 °C and the dry cell weight measured till constant weight was obtained.

3.4.2 Optical density

The cell broth was appropriately diluted with 0.85% NaCl solution in distilled water such that the absorbance at 600 nm was between 0.1-1.0.

3.4.3 Viable count

The cell broth (0.1 mL) was serially diluted in 9.9 mL sterile blanks and plated onto Horikoshi medium II (HM II, as described earlier). After 48 h of incubation the number of colony forming units were counted.

3.5 Protein estimation

Total protein in the samples was estimated by Lowry's method (Lowry *et al.*, 1951) or by absorbance at 280 nm using bovine serum albumin (Fraction V, Sigma) as standard.

3.6 Total sugars

Total carbohydrate content of samples was measured by Anthrone's method (Jermyn, 1971) using glucose as standard.

3.7 Reducing sugars

The total reducing sugar content of samples was estimated by the DNSA method (Miller, 1959) or Somogyi-Nelson method (Nelson, 1944) with glucose as standard.

3.8 Elemental analysis

The broth samples were treated with dilute HCl to remove inorganic carbon (in the form of carbonate and bicarbonate). The samples were then freeze dried for analysis. The cell mass samples were dried in a vacuum oven at 55-60 °C before analysis to remove moisture. The elemental analysis (Carbon, hydrogen, nitrogen) was done using an automated elemental analyzer (Model Carlo Elba).

3.9 Amylase assay

The amylase activity of the broth was determined by estimating the reducing sugars produced from starch. This assay was a modification from that reported by Bernfeld (1955).

3.9.1.1.1 Reagents

1. Tris-HCl buffer, pH 7.0, 0.05 M
2. Soluble starch, 1% in buffer
3. Dinitrosalicylic acid (DNSA) reagent (Miller, 1959).

3.9.1.1.2 Procedure

To 1 mL of soluble starch, 0.1 mL of appropriately diluted enzyme was added. The mixture was incubated at 60 °C for 30 min. The reaction was stopped by adding 1 mL DNSA reagent and the amount of reducing sugars produced was measured as described earlier. One unit of amylase was defined as the amount of enzyme that produced 1 μmol of maltose per min.

3.10 Protease assay

The protease activity was determined by the method of Keay and Wildi (1970).

3.10.1.1.1 Reagents

1. Tris-HCl buffer, pH 8.5, 0.05 M
2. Trichloroacetic acid (TCA), 10%
3. Casein

3.10.1.1.2 Procedure

One gram of casein was dissolved in minimum quantity of 0.1 N NaOH. The pH of this solution was adjusted to 8.5 with HCl and its volume made to 50 mL with distilled water. The final volume was made to 100 mL with Tris buffer.

One hundred µL of enzyme was mixed with 1 mL of casein solution and incubated at 37 °C for 30 min. The reaction was terminated by adding 2 mL of TCA and the mixture was further incubated for 20 min. The precipitated protein was removed by centrifugation and the amount of soluble peptides was monitored by absorbance at 280 nm. One unit of protease was defined as the absorbance units produced per min from a 1% casein solution.

3.11 Shake flask studies

B. firmus was cultivated aerobically in HM II at 30 °C on rotary shaker (200 rpm). Culture was transferred from stock slants to 50 mL medium in 250 mL conical flask and grown for 24 h. This broth was used to inoculate fresh flasks for further experiments. The ratio of flask capacity to the culture volume was maintained at 5:1.

3.11.1 Cell growth and enzyme production

A number of flasks containing HM II were inoculated with 1% of fresh inoculum of *B. firmus*. The flasks were incubated at 30 °C on shaker. After regular time intervals, two flasks were harvested and DCW and CGTase in supernate were determined.

3.11.2 Induction by carbohydrates

The bacillus was grown in basal medium (HM II) in which 1% starch, dextrin or glucose was used as the carbohydrate source. The cell growth and CGTase activity were determined as described earlier.

3.11.3 Effect of cell mass

The culture was grown in 250 mL flask containing 50 mL HM II. The cell mass was monitored by measuring wet cell weight. After the cells had reached end of log phase they were harvested aseptically by centrifugation (16,000 g) and resuspended in 50 mL fresh medium. The reconstituted broth was further incubated at 30 °C on shaker. The growth and enzyme activity during the two phases were monitored as described earlier.

3.11.4 Growth at various temperatures

B. firmus was grown in 250 mL flask with 50 mL HM II on a New Brunswick Scientific shaker at 250 rpm. The flasks were incubated at 28, 31, 34, 37, 40 and 45 °C. The dry cell weight and enzyme activity were determined at regular time intervals.

3.12 Fermenter studies

B. firmus growth and enzyme production were studied in 1 L Gallenkamp (working volume 700 mL) and 12 L New Brunswick Scientific (working volume 10 L) automated fermenters. The fermenter vessel containing HM II was sterilized at 121 °C for 20 min (1 L vessel) or 30 min (12 L vessel). The temperature was controlled at 30 °C. The pH was controlled by addition of 5 N NaOH and 2 N HCl using a pH-stat (NBS). The dissolved oxygen was measured by a polarographic oxygen probe (Ingold). On-line vent gas analysis was also performed. Carbon-dioxide and oxygen concentrations in the off-gas were measured with an infrared analyser (NBS) and a paramagnetic analyser (Beckman), respectively. A schematic representation of fermenter assembly is shown in Figure 3.1.

3.12.1 Fermenter inoculum preparation

The *B. firmus* culture was transferred from slant to 50 mL HM II broth in a 250 mL flask. The flask was incubated at 30 °C for 24 h on a shaker. A small portion of this broth (1% v/v) was added to fresh 100 mL medium in 500 mL flasks. After 10 h incubation at

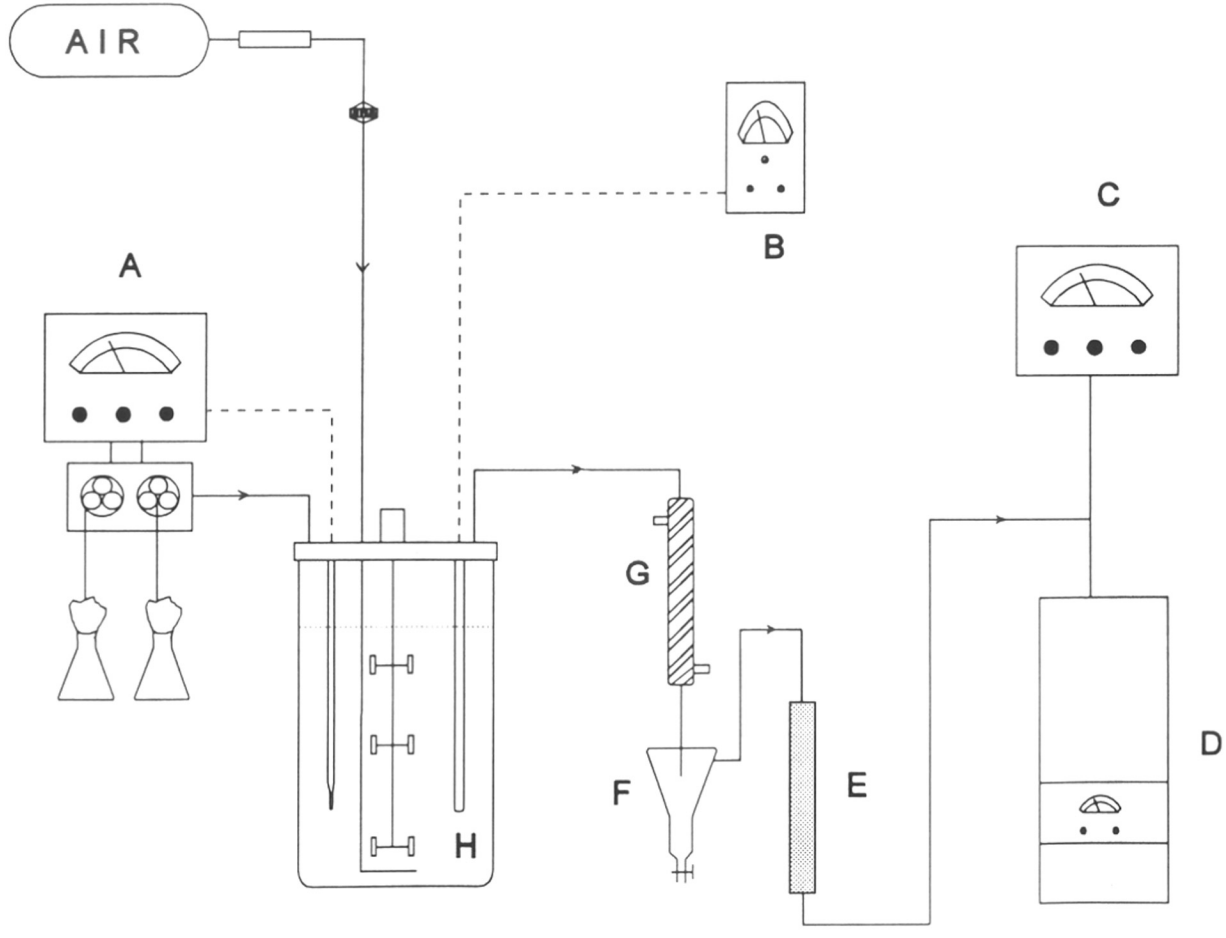

Figure 3.1 : Schematic diagram of the batch fermenter assembly. A: pH controller B: dissolved oxygen meter C: paramagnetic oxygen analyser E: zeolite moisture trap F: water trap G: water condensor G: fermenter.

30 C, this culture was used to inoculate the fermenter. The inoculum size was maintained at 5% v/v.

3.12.2 Sampling

Broth samples were withdrawn from fermenter using a sampling device as described by Jogdand and Karanth (1983). The sample size for the Gallenkamp and NBS fermenters was 10 mL and 50 mL respectively.

3.12.3 Effect of pH on growth

B. firmus was cultivated in HM II at different pH in 1 L Gallenkamp fermenter. The pH was controlled at 6.0, 6.5, 6.75, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 using 5 N NaOH. The cell mass estimated at various time intervals. The maximum specific growth rate (μ_{max}) was calculated at each pH run. CGTase activity was also determined at different time intervals.

3.12.4 Batch culture studies

The effect of aeration and agitation on *B. firmus* growth and enzyme production was measured. Experiments were carried out in a 12 L NBS fermenter. *B. firmus* was grown in buffered HM II. The agitation speed was varied from 400 to 600 rpm and its effect on pH, DO, vent gases (CO_2, O_2), DCW, CGTase activity, total sugars and proteins was studied. Elemental analysis of broth and cell mass was performed.

3.12.5 Continuous culture

The experiments were done in a 1 L Gallenkamp fermenter. The temperature was maintained at 30 C. The pH and DO were monitored. *B. firmus* was grown in batch mode as described earlier for 12 h. Sterile buffered HM II was then fed to the fermenter using a peristaltic pump. The medium volume in the fermenter was maintained at a predetermined level by pumping out excess broth through a dip tube in the vessel. Sterile

polypropylene glycol antifoam was used to prevent excessive gas holdup and foaming. The inlet feed rate was suitably adjusted to achieve various dilution rates ranging from 0.1 to 0.7 h^{-1}. The actual feed rates were calculated from the volume of broth collected over a large time interval. To obtain a steady state, constant feed flow rate was maintained for at least five residence times. Three samples of 10 mL each were then taken at hourly intervals to establish steady state and estimate DCW, sugars, proteins, CGTase activity and elemental composition of broth and biomass.

3.13 Cell recycle reactor

The bacillus was grown in a 1 L Gallenkamp fermenter coupled to a microfiltration membrane module (Figure 3.2) through a pump. The microfiltration unit contained a 0.45 μ tubular zirconium based membrane (Carbosep Micro 40, Tech-Sep, France) element (6 mm ID) with an area of 80 cm^2 and hold up volume of 26 mL. The cells were recycled using a gear pump (Micropump 130 with Watson-Marlow 503 U drive) having flow rates of 0-5 L/min at a pressure of 2 bar. The fermenter was sterilized along with the medium (HM II) at 121 C for 20 min. The membrane loop including the pump was sterilized by the following procedure. First, the membrane module and the pump were cleaned by passing sterile distilled water. Then the system was sterilized by passing live steam for 30 min. The bacillus was initially grown in batch mode for 11 h. Later sterile medium (HM II), with pH adjusted by 1% Na_2CO_3, was fed to the fermenter via a dual channel pump (Watson-Marlow, 505 L pump head) at desired flow rates. The other channel of the pump was coupled to the permeate coming out from the membrane module. A fast speed pump was used to bleed the fermenter to maintain its volume. However, the flow in dual channel pump was matched and the bleeding was very infrequent. Decreased volume in fermenter was compensated by stopping the permeate. Polypropylene glycol antifoam was added when required. The dissolved oxygen concentration and pH of the

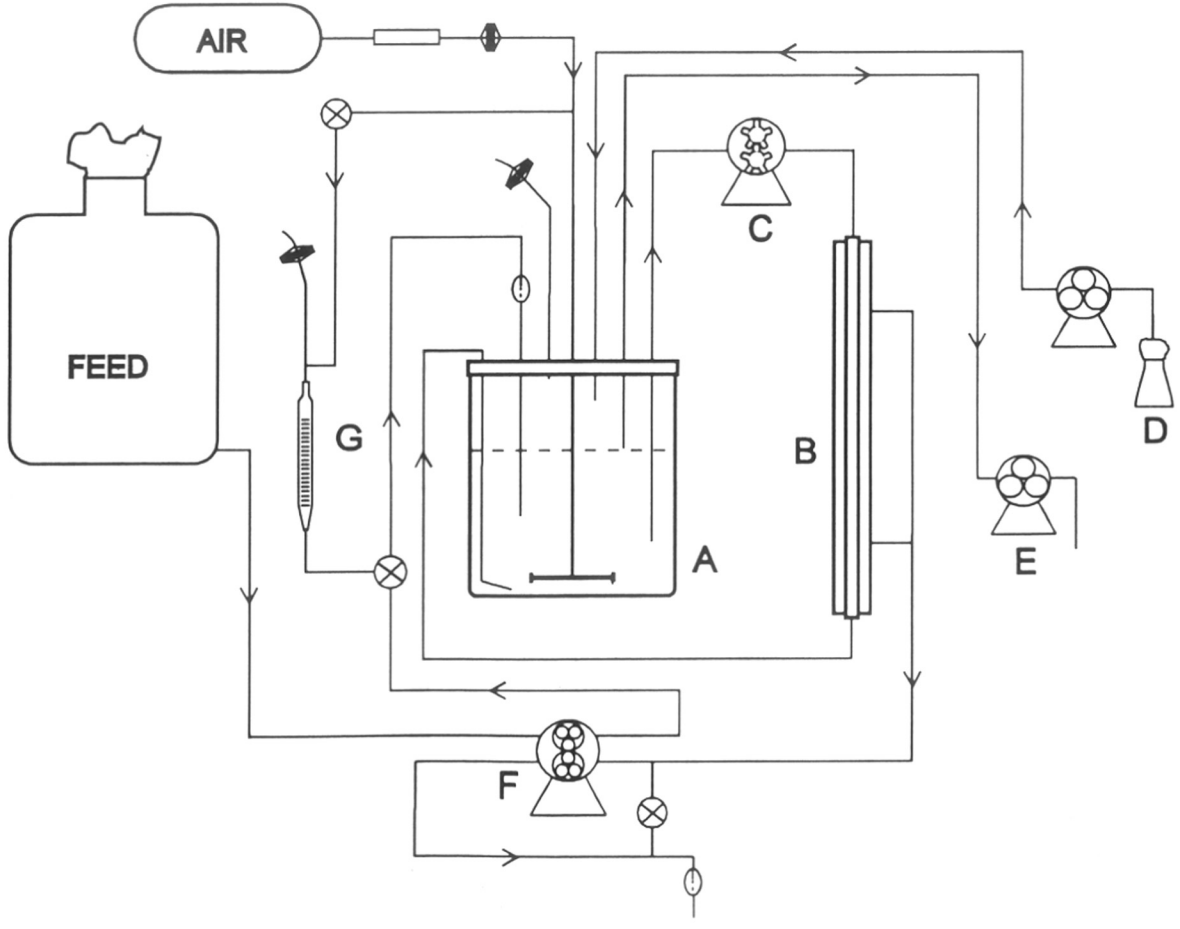

Figure 3.2 : Schematic diagram of a membrane recycle bioreactor. A: fermenter B: membrane module C: recycle pump D: antifoam addition E: cell bleed F: dual channel feed/permeate pump G: assembly to measure flow rate.

broth were monitored. Samples of retentate and permeate were taken to estimate DCW and CGTase activity respectively.

3.14 CGTase purification

CGTase was secreted into the broth by *B. firmus*. The cells were removed from the broth by centrifugation (at 16,000 g in Remi C-24 cooling centrifuge at 4 °C, for 20 min). CGTase was purified from this broth (termed as crude enzyme) according to the procedure described below.

3.14.1 Adsorption on starch

3.14.1.1 Preparation of modified starch

Physically modified starch was prepared as described by Hanus *et al.* (1974). To 100 mL distilled water 10 g raw corn starch was added. The mixture was placed in a boiling water bath and stirred till complete gelatinization took place. It was then cooled and kept in a freezer overnight. The following day, it was thawed to room temperature and further frozen for 24 h. The gel was thawed again and crushed in a laboratory mixer to fine particles. The crushed material was passed through a 1 mm sieve to remove large particles. Fines were removed by repeated decantation. This gel was used for CGTase adsorption.

3.14.1.2 Affinity adsorption of CGTase

The starch gel was packed in a jacketed chromatography column (XK 26/40, Pharmacia, Sweden) of dimensions 40 cm x 2.6 cm. The gel was sequentially washed with 1% Na_2CO_3, DW and 0.1% Na_2CO_3. The column temperature was adjusted to 4 °C with chilled water. Crude enzyme broth (950 mL) was then loaded onto the column at flow rate of 1 mL/min. The gel was then washed with 250 mL of 0.05 M Tris-HCl buffer, pH 7.0 to remove unadsorbed material from broth. The temperature of the column was then

raised to 45 °C. After holding the temperature for 10 min, the enzyme was eluted from the starch gel by passing 0.05 M Tris-HCl buffer, pH 7.0, at a flow rate of 0.5 mL/min in the reverse direction. The eluted enzyme was collected in 3 mL fractions on a fraction collector (Frac 100, Pharmacia). CGTase activity and protein content of the fractions was determined as described earlier.

3.14.2 Ion-exchange chromatography

The fractions containing most of the CGTase activity eluted from the starch column were pooled together and loaded onto a DEAE-Sepharose anion exchange resin (Pharmacia). The resin was packed in a 4 cm x 1.5 cm column (4.5 mL volume). The column was equilibrated with Tris-HCl buffer, pH 7.0, 0.05 M. The enzyme was loaded at a rate of 1 mL/min. The column was washed with Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl. The enzyme was eluted with 10 mL of 0.2 M NaCl in buffer.

3.14.3 Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of protein samples was performed according to the method of Ornstein (1964) and Davis (1964). The gels were stained as recommended by Neuhoff et al. (1988).

3.14.3.1.1 Reagents

a) Acrylamide 30 g; N,N-methylene bis acrylamide 0.8 g; DW 100 mL

b) N,N,N',N'-tetra methylene diamine (TEMED)

c) Ammonium per sulphate 1.5 g; DW to 5.0 mL, prepared fresh.

d) Stacking gel buffer: Tris-HCl buffer 0.0625 M, pH 6.8

e) Running gel buffer: Tris-HCl buffer, 0.375 M, pH 8.9

f) Electrode buffer: Tris-glycine buffer, 0.05 M, pH 8.3

g) Tracking dye: Bromophenol blue 5 mg; Ethanol 10 mL.

h) Fixing solution: Methanol 40% v/v in DW.

i) Staining solution: Coomassie Brilliant Blue G-250 100 mg; H_3PO_4 2 g; $(NH_4)_2SO_4$ 10 g; Methanol 20 mL; DW to 100 mL

j) Destaining solution: Methanol 25% v/v in DW.

k) Storing solution: $(NH_4)_2SO_4$ 25 g; DW 100 mL

To make gel the reagents were mixed in the following proportion.

Nature of gel	Reagent				
	a	b	c	d	e
Stacking	1.0 mL	10 µL	10 µL	9 mL	-
Running	2.65 mL	10 µL	10 µL	-	7.35 mL

3.14.3.1.2 Procedure

A 1 mm thick gel prepared and was loaded with protein samples (5-20 mL) containing 10% glycerol and 5% tracking dye. The separation was carried out at 100 volts on an electrophoresis power supply. When the tracking dye had reached 1 cm from the bottom of gel, the gel was removed and put into fixing solution overnight. The gel was washed twice with DW and stained in reagent (i) for 8 h. It was then briefly washed in solution (j) and stored in solution (k).

3.14.4 Molecular weight determination

The molecular weight of CGTase was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) method (Laemmeli, 1970).

3.14.4.1.1 Reagents

All reagents were prepared as described for PAGE except that solutions (a), (d), (e) and (f) contained 1% SDS as well.

3.14.4.1.2 Sample preparation

The samples were pretreated to denature all proteins.

Sample buffer was composed of 1.51 g Tris and 20 mL glycerol dissolved in 35 mL distilled water and pH adjusted to 6.75 with concentrated HCl. To this buffer 4 g SDS, 10 mL 2-mercaptoethanol and 0.002 g bromophenol blue was added. The volume was made to 100 mL with DW.

Equal volumes of sample and buffer were mixed together and the mixture was incubated at 100 °C for 60 sec. This sample was loaded onto the gel and processed as described before. The molecular weight was then calculated by plotting the relative mobility of proteins of known molecular weight against logarithm of molecular weight, and interpolating values for the unknown protein.

The standard proteins used in SDS-PAGE were supplied by Sigma and consisted of a mixture of carbonic anhydrase (29,000 Da), egg albumin (45,000 Da), bovine albumin (66,000 Da), phosphorylase B (97,400 Da), β-galactosidase (116,000 Da) and myosin (205,000 Da).

3.15 Optimum pH for enzyme stability and activity

3.15.1 Stability vs. pH

The purified enzyme was incubated with buffers of various pH at 10 °C and 60°C. The residual activity was assayed by PHP assay or HPLC. For the experiment at 10 °C the buffers used were

Citrate phosphate	pH 4-8
Diethanolamine-HCl	pH 8-11
Sodium phosphate (dibasic)-sodium hydroxide	pH 11-12

The buffer used at 60 °C was a mixture of citric acid, sodium phosphate (monobasic), Tris and glycine. The pH of this cocktail was adjusted by NaOH in the range 2-12. The exact composition of the buffers is given in Appendix II.

3.15.2 Activity vs. pH

Soluble starch (2 g) was dissolved by heating in 100 mL DW. One volume of starch solution was mixed with equal volume of cocktail buffer in the pH range 2-12. The pH of the mixture was adjusted with HCl or NaOH. One hundred μL of CGTase and 1 mL of starch solution were incubated at 60 °C for 20 min. The amount of CDs produced were estimated by PHP assay and HPLC.

3.16 Optimum temperature for enzyme activity and stability

3.16.1 Stability vs. temperature

Different aliquots of pure enzyme were incubated at 30°C-90°C for 20 min. The tubes containing the enzyme were then immediately cooled down by keeping in ice and the residual enzyme activity was determined by the standardized assay procedure (PHP, HPLC).

3.16.2 Activity vs. temperature

One hundred μL of pure CGTase was added to 1 mL of 1% starch solution in Tris-HCl buffer (pH 7.0, 0.05 M). The mixture was then incubated at 30-60 °C for 20 min. The amount of β-CD produced was estimated by HPLC and PHP assay.

3.17 Action on starch

3.17.1 Viscosity measurements

A solution of gelatinized tapioca starch was prepared by boiling 1 g native starch in 100 mL Tris-HCl buffer (pH 7.0, 0.05 M) with constant stirring. One mL of this solution was mixed with 1 unit of CGTase and the mixture was placed in the cone of a "Plate and Cone" type viscometer (Brookfield, Model DV-I) equilibrated at 60 °C. The change in viscosity (as the reaction progressed) was measured at a constant shear (100 rpm in the viscometer). A control containing starch and buffer was also examined similarly.

3.17.2 Action on different starches

Hydrolysis of raw and gelatinized tapioca, potato, corn and hydrolyzed potato (available commercially as soluble starch, DE 8) starches by CGTase was studied. The starch was solubilized or gelatinized by heating in boiling water bath. Five mL of 10 g/L raw starch powder suspended in Tris-HCl buffer (0.05 M, pH 7.0) was incubated with CGTase (0.015 U/mL of reaction mixture) in a reciprocating water bath at 65 °C. Samples were withdrawn periodically and analysed by HPLC.

3.17.3 Scanning electron microscopy

Raw tapioca starch was subjected to CGTase action and the structure of treated granules was examined by scanning electron microscopy (SEM). Two hundred mg starch was suspended in 10 mL Tris-HCl buffer (pH 7.0, 0.05 M) containing 1 U of CGTase. The mixture was incubated at 60 °C in a reciprocating water bath. After 2 and 4 h of incubation, the contents of the flask were harvested and the starch was separated by centrifugation (15,000 g, 10 min). The granules were washed with DW and transferred to an aluminium foil for drying. The starch was dried completely under vacuum.

A fine layer of starch powder was prepared by placing a drop of starch suspension in isopropanol on the sample stub. The stub was air dried and coated with a layer of gold in gold sputtering unit. The grid was loaded onto the SEM (Lieca, Model Stereoscan 440) sample chamber. The structure of starch granules was examined at a magnification of 900 X. Starch control samples, without enzyme treatment, were also examined similarly.

3.18 Yield of CDs on starch

Cyclodextrin yields were determined by incubating 10, 50, and 150 g/L gelatinized tapioca starch with 0.6 U CGTase per gram starch at 65 °C. The reaction was monitored for 24 h and the amount of α, β and γ-CDs produced were determined by HPLC at regular time intervals.

4. Results and Discussion

4.1 Isolation of culture

CGTases from alkalotolerant species of the genus *Bacillus* appear to have a distinct advantage over other bacterial CGTases for two reasons. First, the CGTases from neutrophilic *Bacillus* mainly produce α-CD whereas those from alkalotolerant strains of *Bacillus* produce β- and γ-CDs in the initial phase of reaction (Nakamura and Horikoshi, 1976a; Kobayashi *et al.*, 1978). Since β- and γ-CDs form inclusion complexes with a large number of bulky and complex chemicals owing to their larger cavity size, they are of greater value (Szejtli, 1982). Second, cultivation of bacteria at high pH in fermenters offers some protection against microbial contamination. By the same token alkaline CGTase functional at pH ranging from 9-11 need minimal sterile conditions during hydrolysis of starch to CDs. Moreover, at a pH above 8.0, retrogradation of starch is minimal (Yu *et al.*, 1988), allowing the use of high starch concentrations for the enzymic conversion. This was the rationale for isolating alkalototerant strains that produced CGTase.

A large number of soil isolates and *Bacillus* cultures from different culture collections were screened for CGTase activity. A rapid screening method with medium containing phenolphthalein and methyl orange dyes (Park *et al.*, 1989) was used to screen isolates in petri-dishes for formation of a yellow halo around the colony (Figure 4.1). Other plate screening methods reported for isolation of CGTase producing bacteria were also tested. These methods are based on the ability of a microorganism to degrade starch around its periphery when grown in a petri-dish in a starch containing medium. Exposure to iodine vapours, turned the plate blue-violet with a light coloured halo around the microbial colony. The other procedure employed a growth medium containing Remazole brilliant blue R starch (Kaneko *et al.*, 1990), where CGTase producing cultures degraded starch and thus form a halo around their colonies. Recently Hamaker and Tao (1994) reported

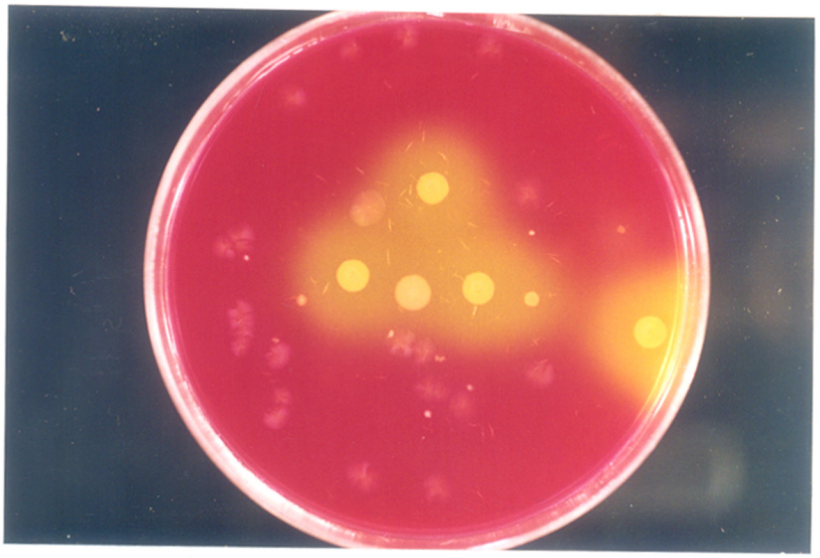

Figure 4.1: Photograph showing isolation of CGTase producing bacteria in a phenolphthalein-methyl orange containing medium. Yellow halos around the bacterial colonies indicate CGTase production.

use of a solid medium containing congo red dye for screening CGTase positive recombinant clones. However, it was observed that all these procedures gave a halo with any starch degrading enzymes as well and were therefore nonspecific for CGTase. Reports indicate that, infact, iodine and remazole brilliant blue assays have been used to screen amylase producing microbes (Rinderknecht *et al.*, 1967). The phenolphthalein-methyl orange method was found to be the only specific technique for detecting CGTase positive microbes. Moreover, this was the only method for rapid screening of alkalophilic cultures.

Of the cultures isolated, a select number of strains were tested for their ability to grow fast and produce CGTase at the earliest, in shake-flasks. Using these criteria, the soil isolate, identified as *B. firmus* (Gordon, 1977; Sneath, 1986; Table 4.1) and designated as SI4, was selected for further work. The culture produced CGTase within 12-16 h of incubation as compared to 2-4 days in earlier reports (Table 2.1). Other positive features of this culture were inability to produce α-amylase and the ability to degrade raw starch (Goel and Nene, 1995a). α-Amylase breaks down the starch into oligosaccharides of low DS which make their separation from CDs difficult.

4.2 Assay of CGTase

Selection of a suitable assay for CGTase activity and determining the distribution of α-, β- and γ- CDs in hydrolysed starch required extensive standardization even for techniques reported in the literature. This is primarily because CGTase enzyme shows three different activities (cyclization, transglycosylation and disproportionation activities) which vary from strain to strain. Moreover, the concentration of substrate, type of buffers and reaction time have a strong influence on the proportions of the three CDs formed.

Morphology	Fast growing bacilli are filamentous, long rods in stationary phase of growth.
Growth on agar	Convex, circular, spreading colonies with regular margins. Yellow pigment after 2 days of incubation.
Gram reaction	Gram positive
Spores	Subterminal, sporangia not swollen
Motility	+
Catalase	+
Anaerobic growth	-
Acetion production	+
pH of VP broth	6.8
Maximum temperature for growth	40 °C
Growth	
in lysozyme	-
in media of pH 5.7	-
in 7 % NaCl	+
Acid from Glucose	+
Hydrolysis of starch	+
Citrate utilization	-
Reduction of NO_3 to NO_2	+
Decomposition of casein	+

Table 4.1: Characteristics of *Bacillus firmus*.

Assays based on microscopic examination of I_2-CDs clatharates i.e. Tilden and Hudson method, (Tilden and Hudson, 1942), Trichloroethylene (TCE) precipitation method (Nomoto *et al.*, 1984) and the dextrinizing assay based on reduction of iodine staining power (Nakamura and Horikoshi, 1976b) were all tried but eventually rejected for different reasons. The Tilden and Hudson and TCE precipitation methods are specific for α-CD and are qualitative assays. The iodine based assay, although a sensitive method, measures the dextrinizing rather than cyclizing activity. Complexing of CDs with dyes like methyl orange (Landert *et al.*, 1981; Makela and Korpela, 1988; Lejeune *et al.*, 1989), phenolphthalein (Vikmon, 1981; Kaneko *et al.*, 1987; Makela *et al.*, 1987) and bromocresol green (Kato and Horikoshi, 1984a) have formed the basis of CGTase assays. Though these assays measure cyclizing activity, the complexing dyes are sensitive to pH changes. Moreover, each of these dyes show different affinities of complexation for α-, β- and γ- CDs. Therefore extensive standardization of assay methods was necessary.

4.2.1 CGTase detection by clatharate formation

Starch digests of *B. firmus* were precipitated both with TCE and toluene. With TCE stable emulsions were formed and it took 10-12 h for the precipitated CDs to settle down. TCE is also known to be more specific for complexation with α-CD (Nomoto *et al.*, 1984). Solvents are known to form precipitates with residual starch after action of CGTase and the TCE-CD complex forms stable emulsions (Szejtli, 1982). Abundant precipitates were formed with toluene on vigorous shaking. These results were quite expected as *B. firmus* CGTase was found to produce large amounts of β-CD that selectively forms complexes with toluene.

4.2.2 Maltotriose-methyl orange method

This assay is based on production of CDs from maltotriose and their detection by complexation with methyl orange (Makela and Korpela, 1988). Though methyl orange is

specific for α-CD it has been used to assay β-CGTase. However it was found that CGTase from *B. firmus* was unable to form CDs from maltotriose even after 1 h of incubation.

The assay is based on the principle that maltotriose is first disproportionated to produce maltooligosaccharide chains of sufficient length which are then cyclized to produce CDs. The rate of disproportionation must necessarily be faster than the cyclization velocity and the concentration of long chain oligosaccharides must be enough for the cyclization reaction to take place (Lee and Tao, 1995). This was probably true for the CGTase of *B. circulans* (ATCC 21783) for which Makela and Korpela (1988) proposed maltotriose based CGTase assay. However some CGTases are either incapable of disproportionating maltotriose or not fast enough (Kobayashi, 1975; Kitahata and Okada, 1982b; Vetter and Thorn, 1992a; also refer to section 'Action of CGTase' in the review of literature). Not all CGTases can use maltotriose as substrate for CD formation e.g. *B. macerans* CGTase (Yagi *et al.*, 1986; Lee and Tao, 1995). Even Makela and Korpela (1988) reported that production of CDs by *B. circulans* CGTase from maltotriose was promoted by methyl orange. Our own results also confirm the view that maltotriose-methyl orange assay is not a universal assay. Considering these facts Lejeune *et al.* (1989) and Lee and Tao (1995) have also recommended use of starch or dextrins to assay α-CGTases by this method. The CGTase from *B. firmus* is a β-CGTase and we found that the methyl orange based assay was not suitable for its estimation.

4.2.3 Phenolphthalein method

The phenolphthalein (PHP) method is based on the decrease in absorbance due to complexation of phenolphthalein with β-CD. The percent reduction in absorbance can be directly correlated to the concentration of CDs. As CGTase from *B. firmus* produces β–CD this assay was the method of choice for routine work. A number of modifications of this assay have been reported in literature (Vikmon, 1981; Kaneko *et al.*, 1987; Makela

et al., 1987). Vikmon (1981) originally reported the use of PHP for spectrophotometric estimation of CDs. But Makela *et al.* (1987) found spontaneous decolourization of PHP solutions on keeping or on addition to complex biological samples. They tested a number of possible factors like the effects of dissolved CO_2 on pH change or decolorization, effect of bivalent cations by chelating them with EDTA or effect due to adsorption to cuvette surface. None of these were found to be the causative agents for decrease in absorbance. Increasing the solubility by addition of ethanol or detergents (Triton-X-100, Brij 58) did not remove the anomalies. These workers later reported that the time dependent PHP reagent instability could be eliminated by firstly making the PHP stock solution in ethanol which could be later diluted with distilled water and by increasing the concentration of buffer Na_2CO_3 in the assay mixture. On replicating their method we observed that the instability problem persisted. Moreover using concentrated solutions of PHP gave errors while transferring solution to assay tubes. Therefore the method had to be modified and standardized.

By incorporating PHP in Na_2CO_3 solution a single reagent assay was developed (Goel and Nene, 1995b). The modified method thus eliminated variations caused by use of concentrated PHP solution. PHP in solution precipitates with time resulting in error in CD estimations. As shown in Figure 4.2B the absorbance of PHP shows a linear decrease with time, i.e., from 0.964 to 0.870 within a period of one hour. The results of repeating the method of Makela *et al.* (1987) showed similar instability of PHP reagent (Figure 4.2A). As the percent decrease in absorbance is calculated with respect to the absorbance of the control, there is a constant increase in error with time. To investigate this, the absorbance of samples containing 100 μg/mL of β-CD were measured at intervals of five minutes along with control. Figure 4.3 shows that, on calculating percent decrease in absorbance with respect to a single control gave a continuous increase in error which was as high as 80 percent after one hour. Individual controls for each test sample gave

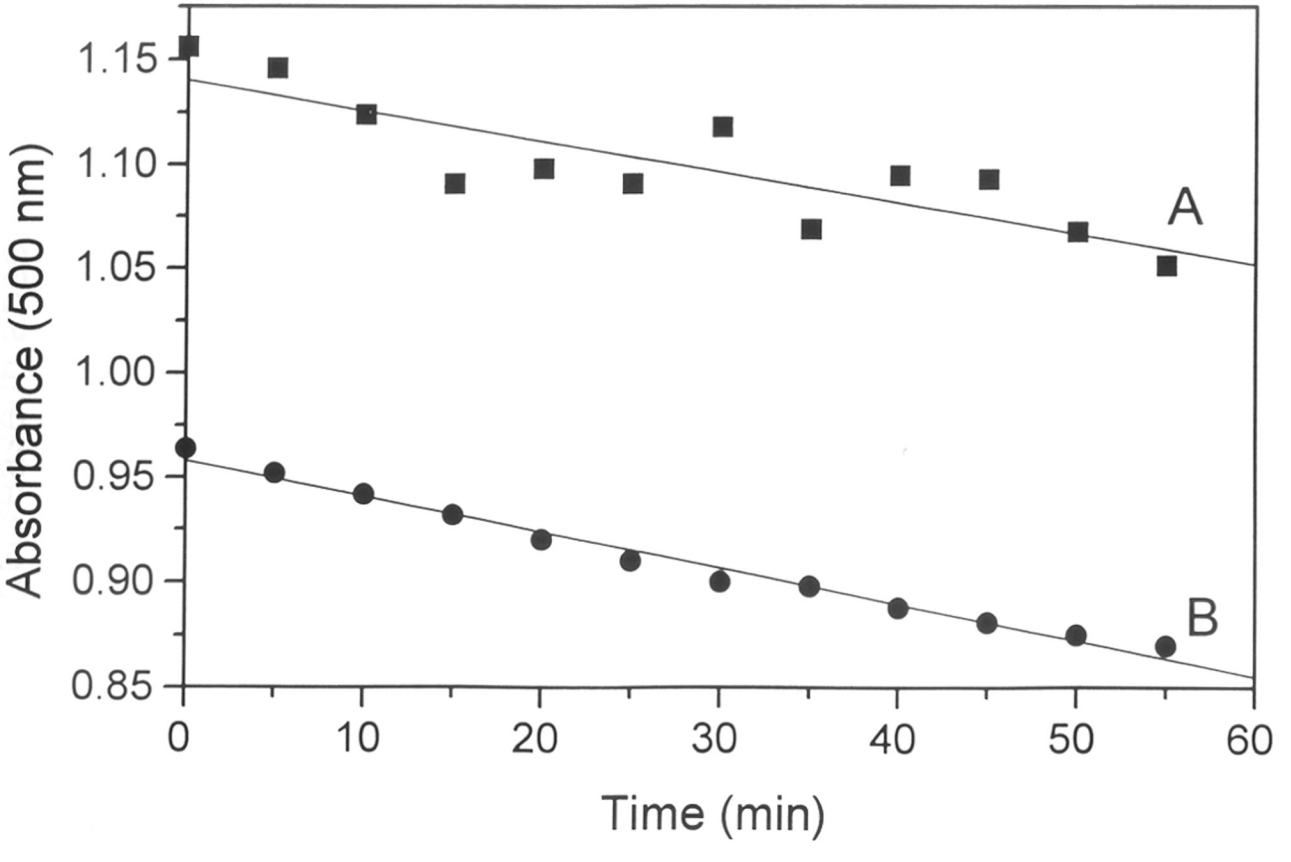

Figure 4.2: Spontaneous decrease in absorbance of Phenolphthalein solution. Profile of control readings observed by: ■ reproducing the method of Makela et al. [1989] and ● present method.

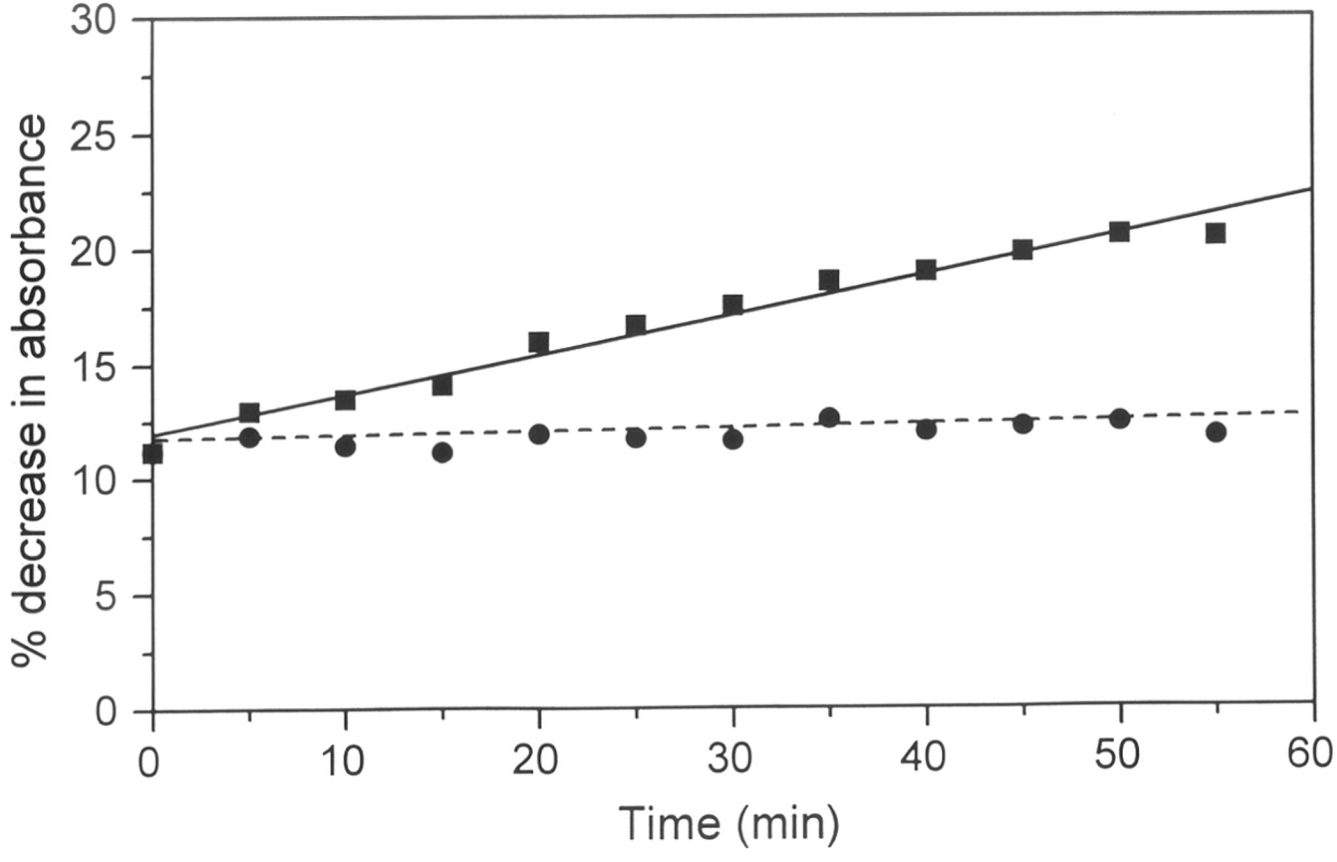

Figure 4.3: Percent decrease in absorbance for sample containing 100 μg/ml β-CD: ——using only one control reading taken at the start of experiment, ● using a control for every test sample and (- - -) based on calculation described in the text.

fairly constant and reproducible results. It was discovered that multiple sample errors due to decrease in absorbance of PHP could be compensated mathematically. Absorbance of the control was measured at the start (C_i) and at the end of the experiment (C_f). If the total number of readings taken was ' N ', then the decrease in absorbance in control per tube (assuming that absorbance of all the tubes was measured in equal amounts of time) was:

$$\Delta C = (C_i - C_f) / (N-1)$$

A new set of control readings were generated per sample (S) as follows:

For any sample S_n : $C_n = C_i - n\,\Delta C$

where 'n' is the sample serial number

Percent decrease in absorbance was calculated with respect to the new controls. These calculations matched well with actual experimental values (Figure 4.3). Thus, this method compensated for the instability of PHP reagent.

Whereas Vikmon *et al.* (1981) reported linearity up to 50 μg/ml β-CD and Makela *et al.* (1987) up to 68 μg/ml (60μM), the present modifications also resulted in an improved linear absorbance of PHP in proportion to β-CD concentrations up to 200 μg/ml (Figure 4.4). Thus a vast improvement was observed both in the linearity and range of the assay. Using these modifications in PHP assay it was possible to determine CGTase activity for larger number of samples. Thus the speed as well as reliability of the assay was vastly improved.

4.2.4 Starch-Iodine method

The iodine based assay of CGTase that measures the dextrinizing power of the enzyme is one of the most commonly used assay (Table 2.1) when no amylase is present along with CGTase. As *B. firmus* CGTase was found to be free of amylase (Goel and Nene, 1995a) this assay gave true CGTase activity. However, the phenolphthalein assay was always

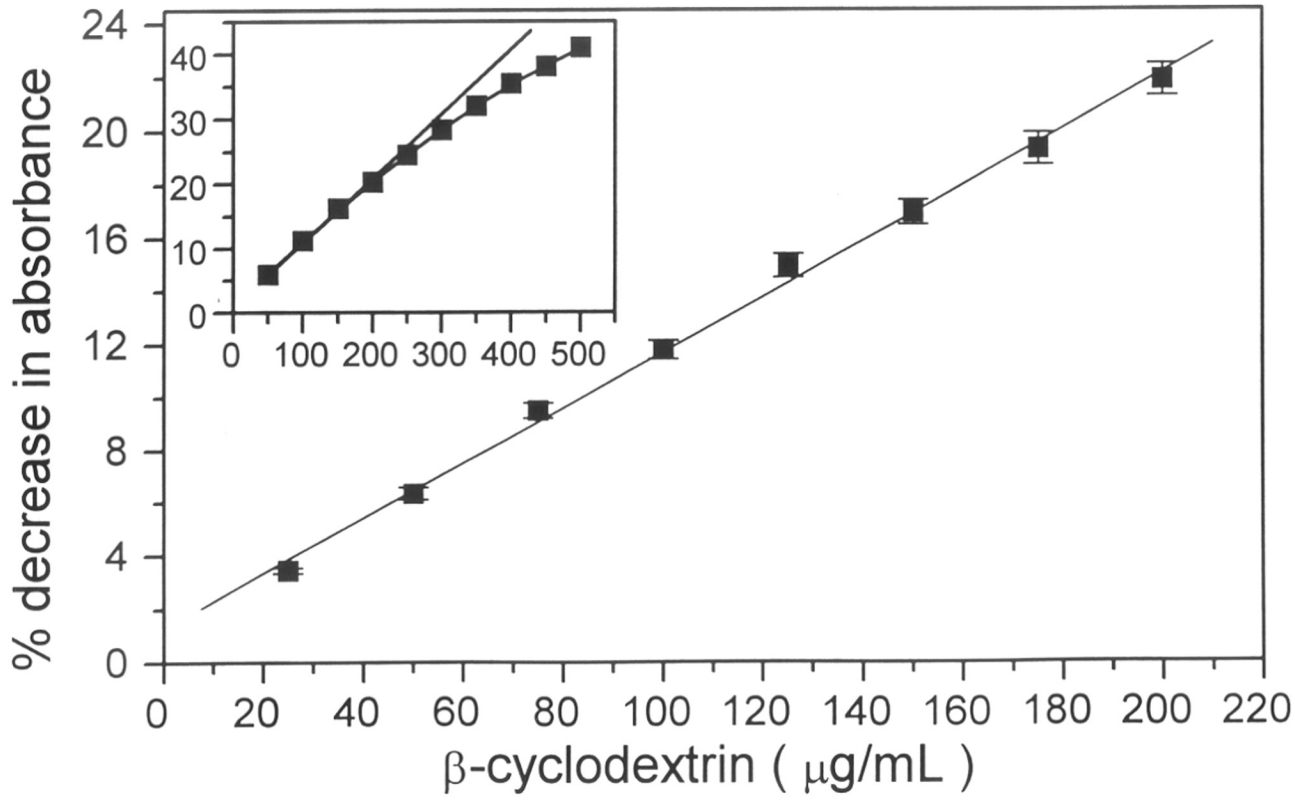

Figure 4.4: Percent decrease in the absorbance (550 nm) of phenolphthalein at various concentrations of β-CD. Inset shows the decrease in absorbance at higher concentrations. Error bars indicate 95 % confidence intervals calculated by repeating the experiment three times.

used for routine work as it actually measured the CDs produced. Though the dextrinizing activity *of B. firmus* CGTase showed a linear correlation to the cyclization activity the ratio of the two activities was not constant as was reported by Bovetto and coworkers (1992a). The exact reason for this behaviour is not known but this may be due to the fact that the velocity of disproportionation and cyclization reactions of CGTase showed a critical dependence on enzyme to substrate ratios (Bender, 1981b). This ratio could not be consistently maintained during an assay.

4.2.5 HPLC method

The HPLC method was found to be the most reliable method for determination of CGTase activity. Two types of columns were used in the present study. One was a sulphonated polystyrene based Shodex DC-613 column (Sato *et al.*, 1985) and other was a proprietary 'Cyclodextrin assay column' from Astec (Aoki *et al.*, 1987). The Shodex column was found to be ideal for the work as it could easily separate CDs and maltooligosaccharides completely. The Astec column, though it separated the CDs very well from linear oligosaccharides and residual starch in the reaction mixture, there was often interference from proteins and amine buffers.

The results of HPLC and phenolphthalein methods showed very good correlation. The major advantage of the HPLC method was in the accurate measurement of distribution of the three CDs in the reaction mixture. The phenolphthalein method was routinely used for bulk samples because of its simplicity. The HPLC method was used as a confirmatory test and in those studies where distribution of CDs formed was required to be estimated.

4.3 Cell growth and CGTase production

The cell growth and enzyme synthesis in *B. firmus* was studied in complex medium containing starch, peptone and yeast extract in shake flask and 10 L fermenter.

4.3.1 Estimation of Cell Mass

Cell mass in a culture broth can be estimated either by direct means such as optical density measurement, dry cell weight or bacterial count or by indirect means (by measuring cellular ATP, DNA or RNA). Estimations based on optical density, dry cell weight and bacterial count are the most commonly followed techniques. All these three methods were tried to measure *B. firmus* cell mass. Optical density measurements and bacterial counts gave highly inconsistent values. This was primarily due to the filamentous nature of *B. firmus* cells and their variable morphology at different pH, salt concentration and different phases of growth. As can be seen in Figure 4.5 bacterial cells were highly filamentous during the log phase of growth (A) and they progressively decrease in length as the cell growth proceeded (B and C). Thus, dry cell weight (DCW) based estimation were found to give the most reliable results.

4.3.2 Effect of temperature on cell growth and CGTase production

The growth and CGTase production in *B. firmus* were studied over a range of incubation temperatures (28-45°C) as shown in Figure 4.6. Maximum activity of 1.1 U/mL was observed at 28°C incubation temperature. There was a progressive decline in enzyme activity on increasing the incubation temperature. It was fifty percent of maximum (0.54 U/mL) at 37°C, whereas at 40°C only 27% of CGTase was produced. The cell mass (DCW) was almost constant at ≈ 4.0 g/L between temperatures 28 to 31°C. However, at higher temperature the maximum cell mass obtained decreased rapidly and no growth was observed beyond 40 °C.

Optimum temperatures for CGTase production in bacteria vary. While most of the mesophilic bacteria produce CGTase between 30 and 40 °C, thermophiles like *Thermoanaerobacter* sp. (Novo, 1987) and psycrophilic *Bacillus* sp. 3-22 (Georganta *et al.*, 1993) produced the enzyme at 67 °C and 4 °C respectively. *B. firmus* is a mesophilic organism and thus produces CGTase around 30 °C. Studies on temperature

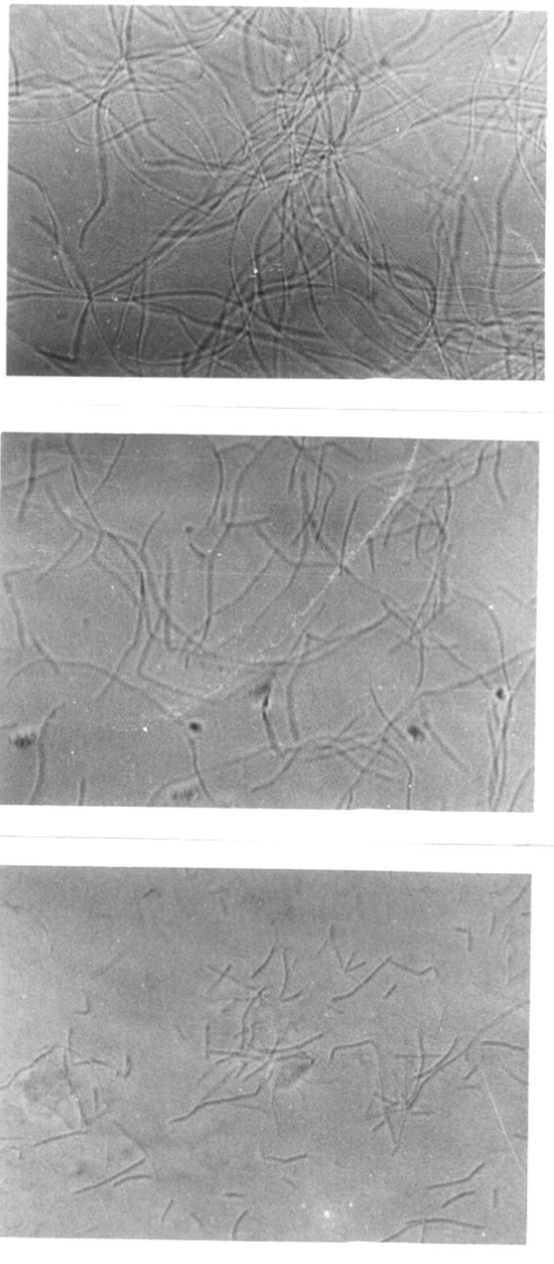

Figure 4.5: Morphology of *B. firmus* at different stages of growth cycle. A, B and C during early, late log and stationary phase of growth.

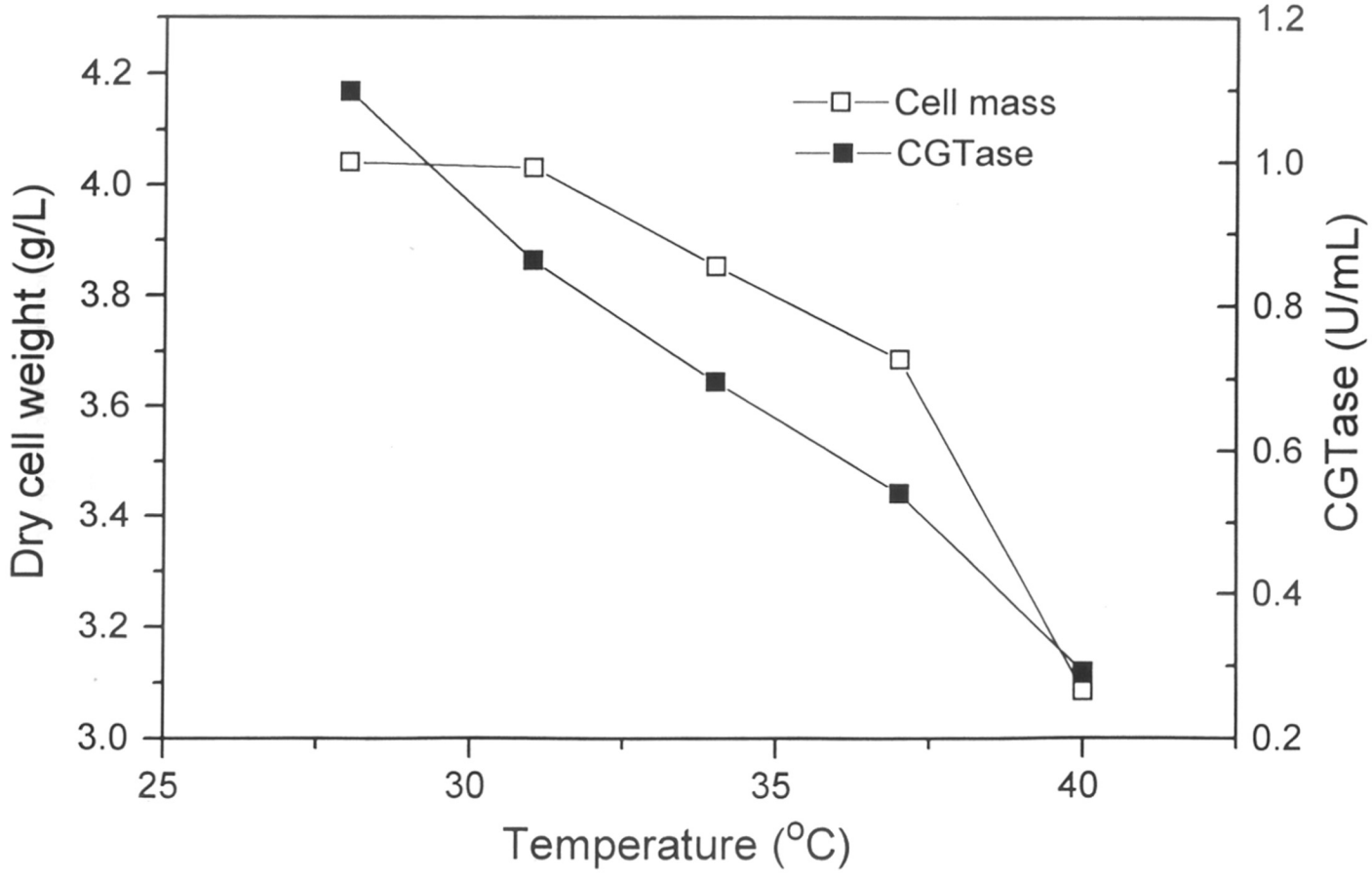

Figure 4.6: Effect of Temperature on growth and CGTase production in *B. firmus*

effects on cell growth and CGTase production have been reported in a few bacterial strains. *B. circulans* ATCC 21783 produces maximum CGTase at 37 °C, though there is negligible effect on cell mass within the temperature range 30-42°C (Makela *et al.*, 1990). Another alkalophilic *Bacillus* sp. no. 562 grows and produces maximal CGTase between temperatures 45-55 °C (Yan and Lin, 1993).

4.3.3 Effect of pH on growth and CGTase production

The effect of hydrogen ion concentration was studied by carrying out separate experiments in 1 L Gallenkamp fermenter over a pH range of 6.5 to 9.5 using 5 N NaOH to accurately control pH. It was observed that *B. firmus* could grow well over a wide range of pH between 7.0 and 9.5, thus indicating that this organism is an alkalotolerant and not an obligate alkalophile. It was found that the bacterium could not grow at all below pH 7.0. This character is also a diagnostic feature used to classify *B. firmus* at a species level. Maximum specific growth rate (μ_{max}) was almost the same between pH 7.5 and 9.0 ($\approx 0.4\ h^{-1}$). The μ_{max} at pH 7.0 and 9.5 declined to 0.258 and 0.30 h^{-1}, respectively (Figure 4.7). Though maximum enzyme was secreted at pH 8.5, the enzyme production between pH 8.0 and 9.0 was almost the same. In another experiment where the organism was grown in a medium (HM II) buffered with 1% Na_2CO_3, it was observed that values for biomass and CGTase production were virtually same as those of a parallel run under a controlled pH of 9.0 (Figure 4.8). These results indicated that precise control of pH was unnecessary during the fermentation as a buffered medium gave comparable results.

Alkalophilic microorganisms producing CGTase are generally cultivated in media buffered with Na_2CO_3 (Nakamura and Horikoshi, 1976a; Makela *et al.*, 1990, Yan and Lin, 1993). Only a few reports are available on controlled pH runs. Chen *et al.* (1991) found maximum CGTase production in alkalophilic *Bacillus* sp. no. 35 at pH 8.0 as compared to a buffered medium. These results are contrary to our findings for *B. firmus*. Nakamura and Horikoshi (1976a) and later Makela *et al.* (1990) observed maximum

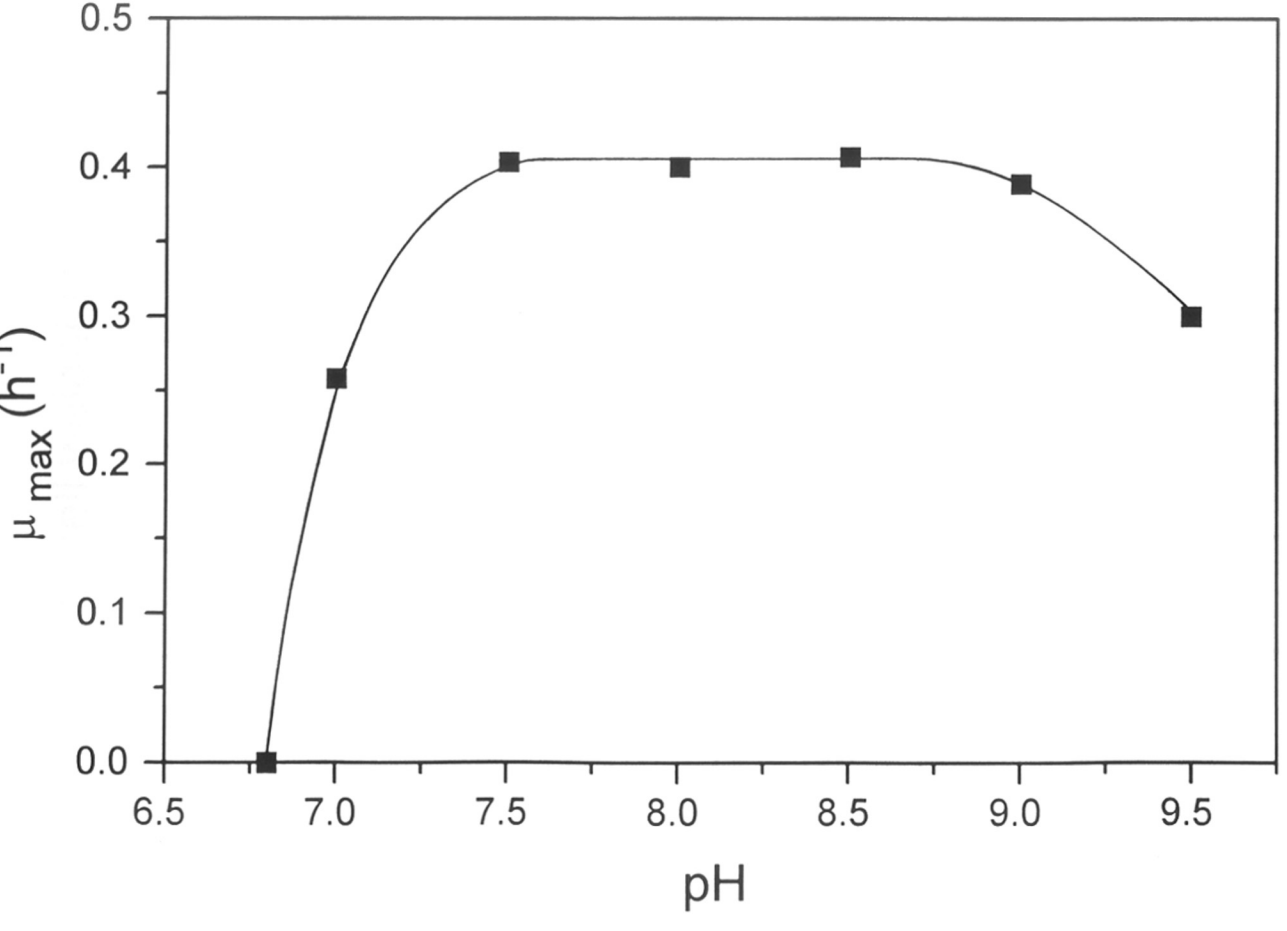

Figure 4.7: Effect of pH on maximum specific growth rate of *B. firmus*

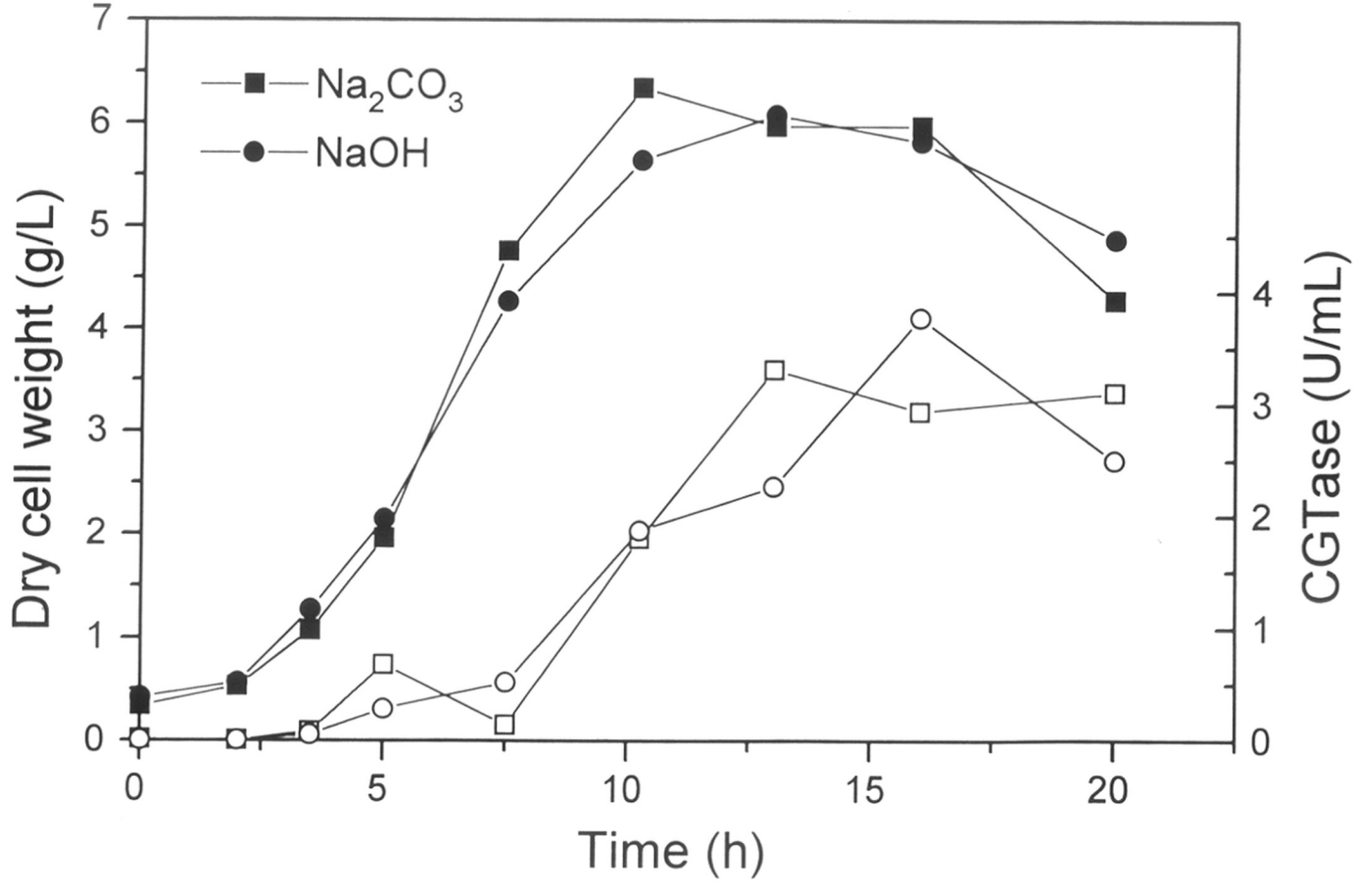

Figure 4.8: Growth profile of *B. firmus* in buffered (Na_2CO_3) and controlled pH (NaOH) fermentation Open and closed symbols represent CGTase activity and cell mass, respectively.

CGTase production in *B. circulans* ATCC 21783 buffered with 1% Na_2CO_3. On testing Na_2CO_3 concentrations from 0.25 to 1.75 % Makela *et al.* (1990) obtained similar yields of CGTase over Na_2CO_3 concentration of 1-1.5% w/v. Compared with CGTase production at 1% Na_2CO_3, these workers found about 3-fold increase in the lag phase with 1.5 % carbonate, hence 1% concentration was chosen for further experimentation. These observations were corroborated by shake flask data for *B. firmus* where maximum CGTase was produced using 1 % Na_2CO_3 in the growth medium.

4.3.4 Induction of enzyme by different carbohydrates

B. firmus was cultivated in a basal medium containing different carbohydrates in shake flasks and the cell mass and CGTase activity determined during the incubation period. The CGTase was found to be induced by starch or dextrins (Figure 4.9). Negligible amount of activity was detected in the presence of glucose. The maximum cell mass produced from glucose and dextrins was $\approx$ 3.5 g/L whereas that from starch was 2.8 g/L.

Nakamura and Horikoshi (1976a) studied CGTase production in the presence of various carbon sources. These workers also found maximum enzyme activity in media containing starch or dextrins. Sugars like glucose, fructose, lactose, maltose and sucrose gave only 5-10 % CGTases activity. However, maltitol - a sugar alcohol gave 60% CGTase compared to soluble starch. Similarly, Sabioni and Park (1992b) could not obtain any CGTase activity in the presence of sugars. *B. firmus* CGTase, as reported in the present study, is an inducible enzyme and only 10% of CGTase is produced in presence of glucose. Thus, it appears that a part of this enzyme is constitutive in nature and is secreted in the presence of glucose. The work of Nogrady *et al.* (1995) on *B. macerans* also supports this assumption. The CGTase production in *B. cereus* RJ 30 (Jamuna *et al.*, 1993) appears to be different. In this bacterium, formation of CGTase does not require the presence of a specific inducer as the CGTase is formed in the absence of starch, or its hydrolysed products and in the presence of simple sugars like glucose. These authors

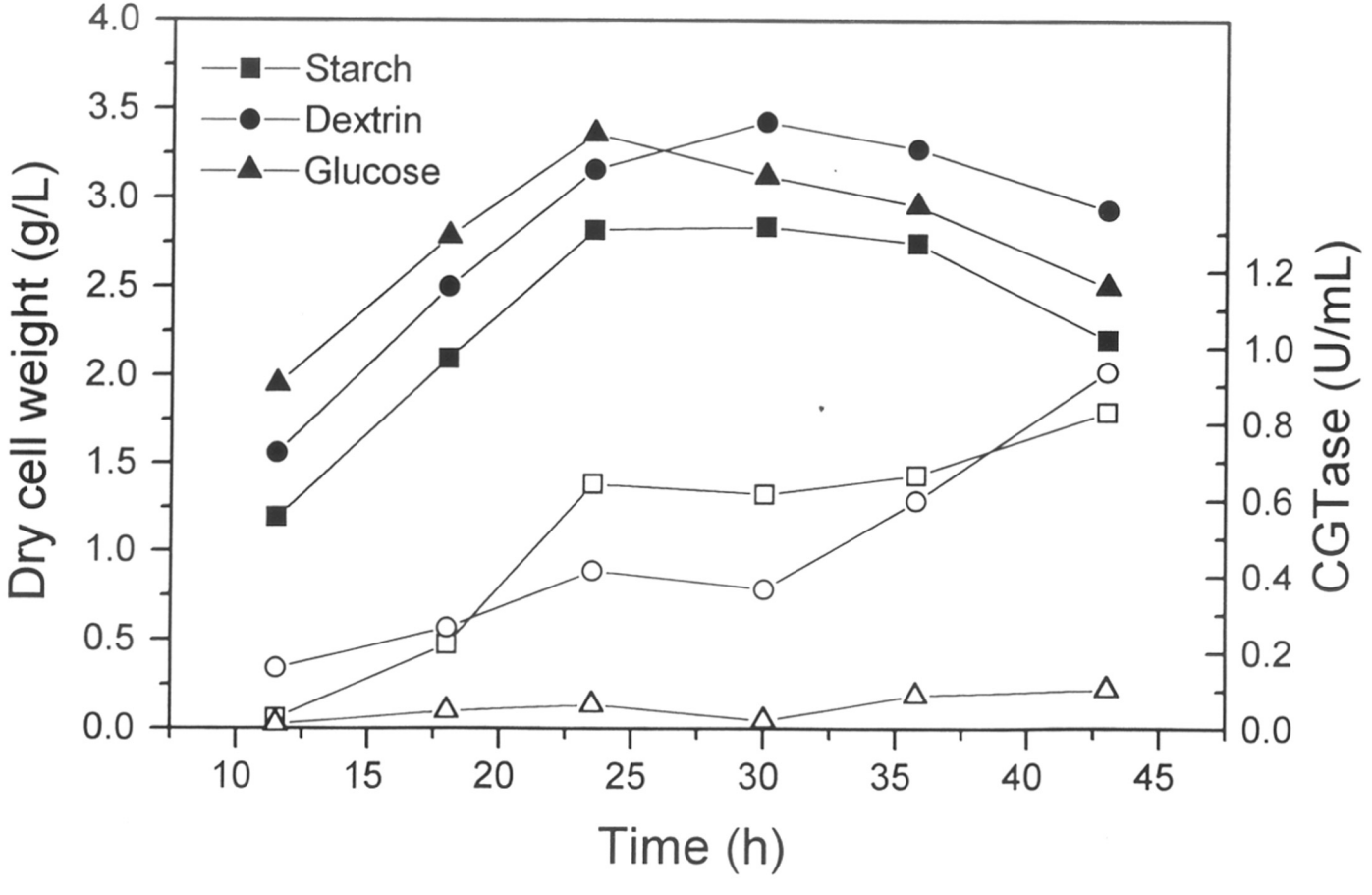

Figure 4.9: Production of CGTase on different carbohydrates. Open and closed symbols represent CGTase activity and cell mass, respectively.

found that xylose and wheat bran extract enhanced the CGTase production. The production of CGTase in the present study was inhibited in the presence of simple sugars.

4.3.5 Growth in Shake-flask

The time course profiles of cell growth and CGTase secretion are depicted in Figure 4.10. After an initial lag of ≈ 5 h *B. firmus* cells entered log phase. Onset of stationary phase occurred at 15 h. The dry cell weight (DCW) reached a maximum of 2.94 g/L after 20 h of incubation. There was a marginal decrease in DCW on further incubation. CGTase synthesis appeared to be growth linked. It was produced as the cell growth progressed. The maximum CGTase activity detected was 0.23 U/mL after 20 h of incubation. The activity remained constant till 30 h.

4.3.6 Growth in Fermenter

Growth associated parameters and CGTase synthesis in *B. firmus* were studied in an aerated fermenter. Samples were periodically removed and analysed for biomass, CGTase activity, total sugars, total protein and elemental composition of cells and broth (C, H and N). Hydrogen ion concentration (pH), dissolved oxygen and vent gas composition (O_2 and CO_2) were recorded on-line. It was observed that the bacterium grew rapidly under these conditions with no detectable lag phase (Figure 4.11). The cell mass continued to increase upto 10-12 h of incubation. The maximum DCW obtained was 6.4 g/L after 12 h incubation. The stationary phase was minimal and the growth quickly entered the lytic phase indicating exhaustion of growth limiting nutrients. This growth pattern was unlike that in the shake flask where considerable lag and stationary phases were observed. This could be mainly due to better aeration in fermenters than flasks. However, it was observed that the lag period in flasks could be reduced by increasing the inoculum size. There was negligible enzyme production in fermenter upto 4.5 h. The enzyme production increased rapidly after 7 h of growth. In contrast to the shake-flask experiments, the

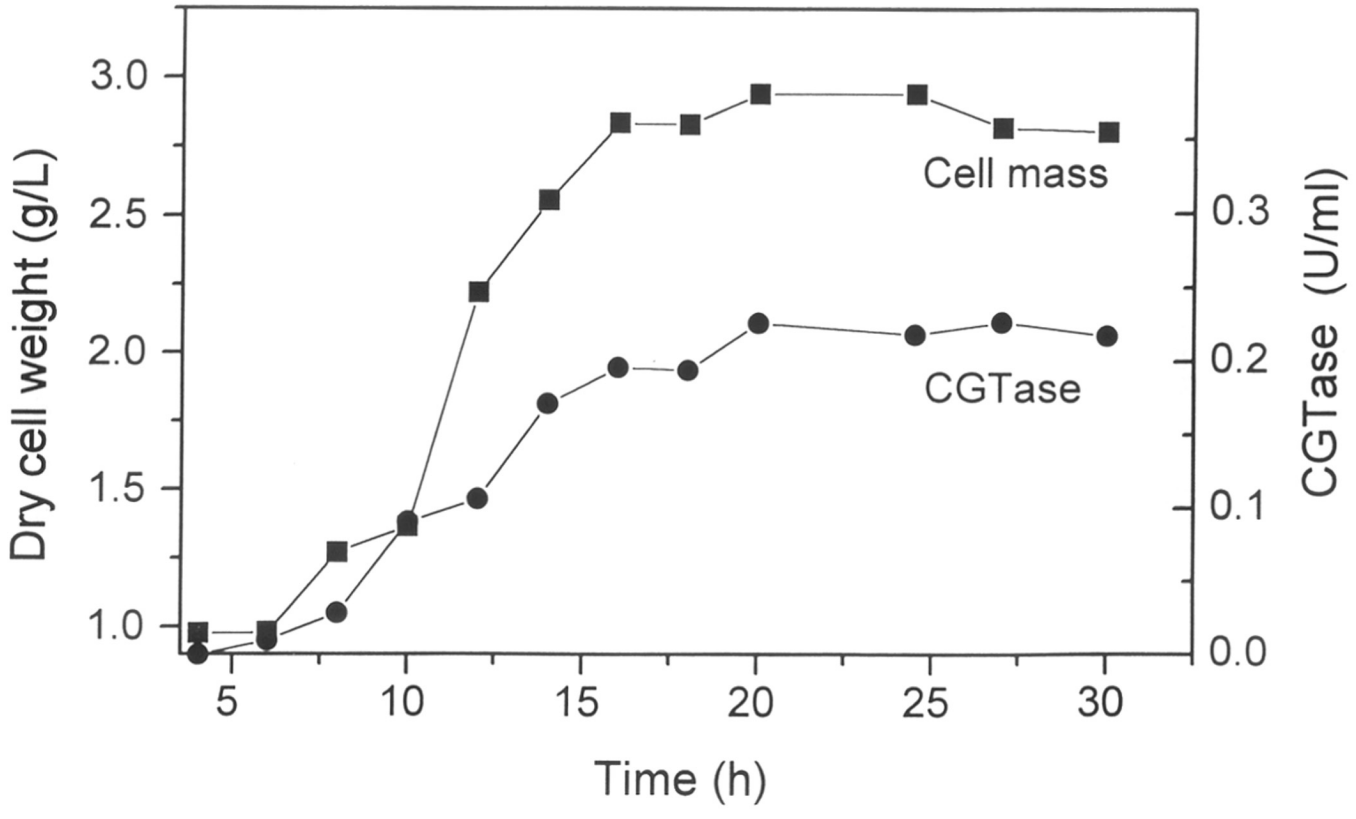

Figure 4.10: Production of CGTase in shake-flask.

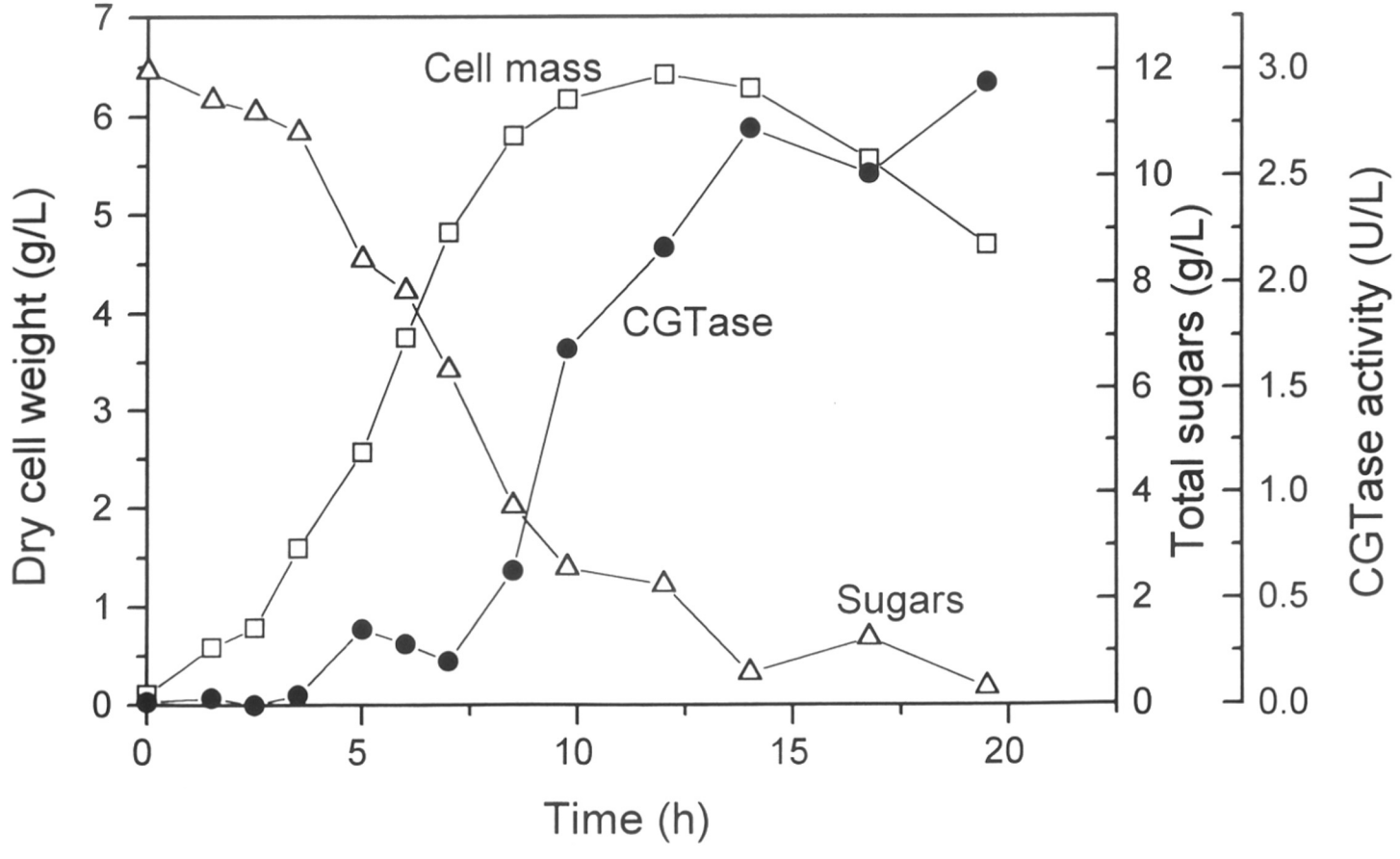

Figure 4.11: Cell growth and CGTase production at 600 rpm in 12 L fermenter.

CGTase production in the fermenter occurred after most of the growth was over. After 9 h of incubation more than 90% of total growth had occured whereas only 20% of CGTase was secreted into the culture broth. Small amount of enzyme was produced in the initial phase (around 5 h) of growth. Though the exact reasons for this behaviour are not known, it was reproducible. Similar observations have been recorded by Yan and Lin (1993) in CGTase production by alkalophilic *Bacillus* sp. no. 562. The maximum CGTase activity detected was ≈ 3 U/mL around 15-18 h of incubation.

Bacteria show considerable diversity in the onset of their synthesis of extracellular enzymes during growth. Enzyme synthesis in the genus *Bacillus* has been reviewed by Priest (1977). Some enzymes were produced during log phase, e.g. laminarinase (Eka and Fogarty, 1975), protease (Fogarty and Griffian, 1973) and amylase (Griffin and Fogarty, 1973) by *B. polymyxa*. Alpha-amylase was produced by *B. licheniformis* (Saito and Yamamoto, 1975), *B. amyloliquifaciens* and *B. subtilis* (Coleman, 1967) during the transition between log and stationary phase. Protease by *B. subtilis* Marburg (Millet, 1970) and subtilisin by the same species (Valle and Ferrari, 1989) were produced during the stationary phase.

The CGTases have been reported to be produced either along with cellular growth (*K.. oxytoca*, Lee *et al.*, 1992), in late log phase (Yan and Lin, 1993) or in a biphasic manner (Makela *et al.*, 1990). In *B. macerans* CGTase is produced intracellularly (De Pinto and Campbell, 1964). In the present study, CGTase production in *B. firmus* appears to be growth linked in shake-flask experiments whereas the enzyme is produced in the late log phase in the fermenter. This observed difference in enzyme production can be explained on the reported relationship that exists between enzyme production and the specific growth rate. Meers (1972) has reported a similar growth rate dependence of α-amylase production in *B. licheniformis*. In *B. firmus* we found that the maximum specific growth rate in shake flasks was one-third of that obtained in fermenter. Similarly,

in continuous culture experiments (described later) more enzyme was produced only at lower dilution rates.

4.3.6.1 Effect of dissolved oxygen

Dissolved oxygen (DO) concentration is one of the most important parameters affecting cell growth. The fermentation runs at 400, 500 and 600 rpm (Figures 4.12, 4.13 and 4.11 respectively) were performed to study the effect of DO on cell mass and CGTase concentration. Figure 4.14 shows the profile of dry cell weights obtained and the dissolved oxygen concentration. It was clear that due to a limitation of DO in the 400 rpm run the maximum DCW was only 4.44 g/L as compared to 6.34 g/L and 6.42 g/L in 500 and 600 rpm runs (Table 4.2). At 400 rpm percent oxygen saturation reached 5.3% within 4 h whereas at higher rpm run it reached minimum of 19% in 6 h (500 rpm) and 48% in 6 h (600 rpm). Under oxygen limitation the maximum specific growth rate (μ_{max}) also decreased to 0.34 h^{-1} as compared to $\approx$ 0.44 h^{-1} at 500 and 600 rpm. The maximum cell yield based on total sugars available at 400 rpm was about 69% of that at higher rpm runs. Since CGTase production is cell growth dependent, much lower enzyme yields were obtained at 400 rpm (0.74 U/mL at 23 h) than 500 rpm (3.1 U/mL at 20 h) and 600 rpm (2.94 U/mL at 19.5 h) (Figure 4.15).

Molecular oxygen has a multifarious influence over microbial cell physiology (Harrison, 1972) and is the final electron acceptor in the cellular metabolism. Hence in an oxygen limited environment the yield of bacterial growth is highly reduced due to incomplete oxidation of substrates available in the growth medium. Since enzyme production is influenced by cell growth, it is also affected. Roychoudhury *et al.* (1989) studied production of α-amylase by *B. amyloliquifaciens* in oxygen limited environment. In oxygen limited conditions the maximum specific biomass yield based on glucose decreased to 0.139 g/g as compared to 0.33 g/g in glucose limited fermentation. The amylase production was also marginally impaired from 5500 units/mL (Glucose limited) to 4200

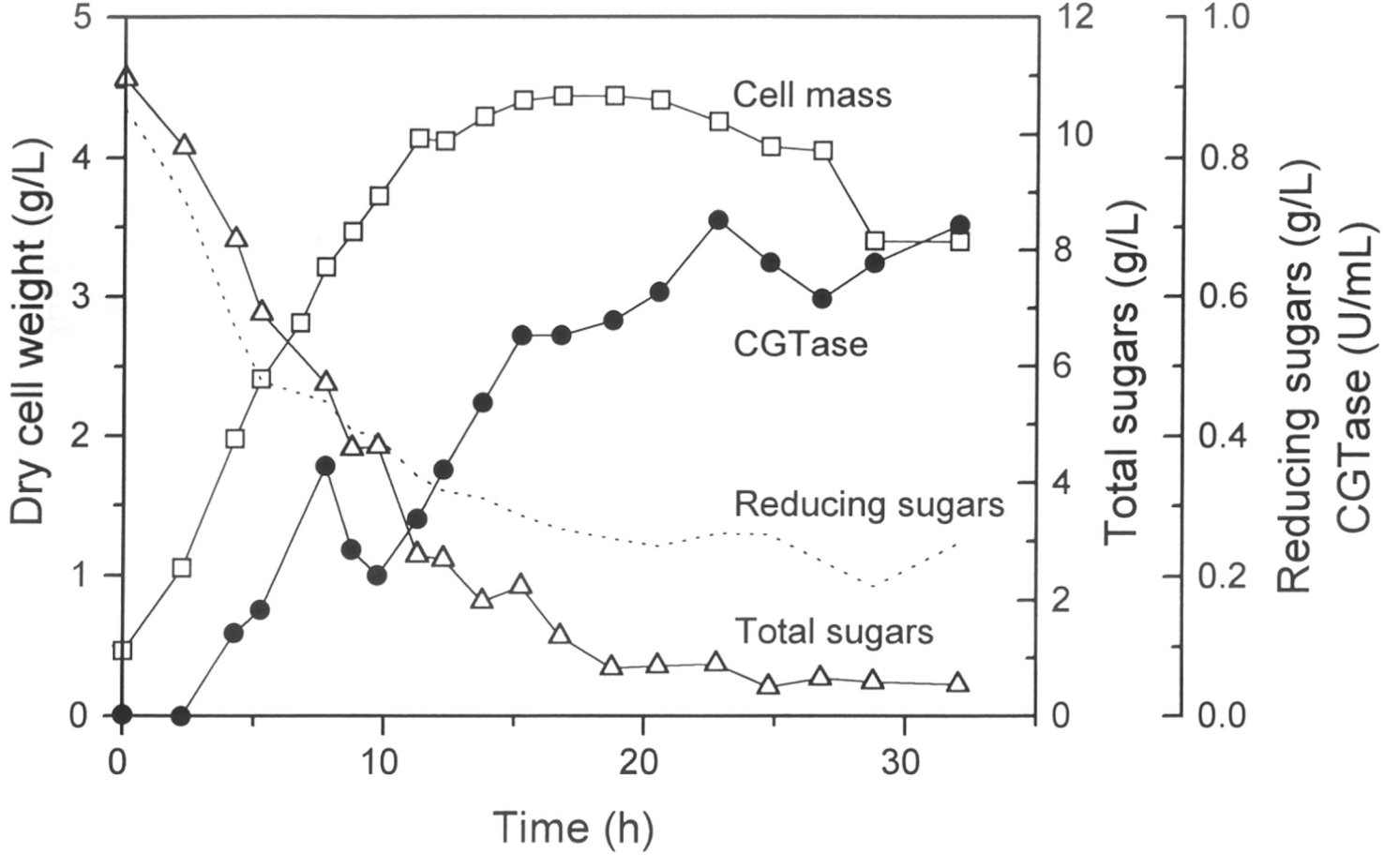

Figure 4.12: Cell growth and CGTase production at 400 rpm in 12 L fermenter.

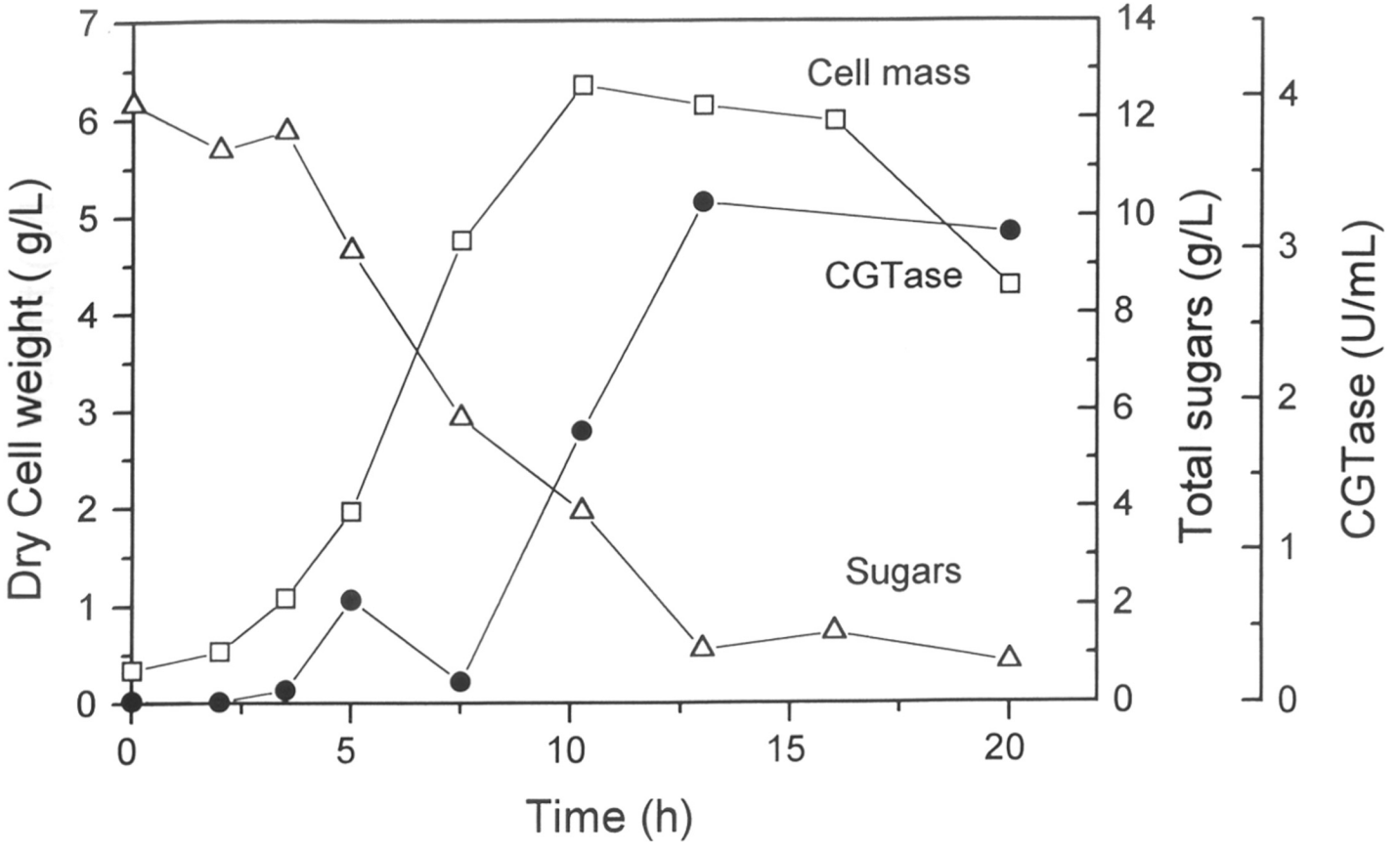

Figure 4.13: Cell growth and CGTase production at 500 rpm in 12 L fermenter.

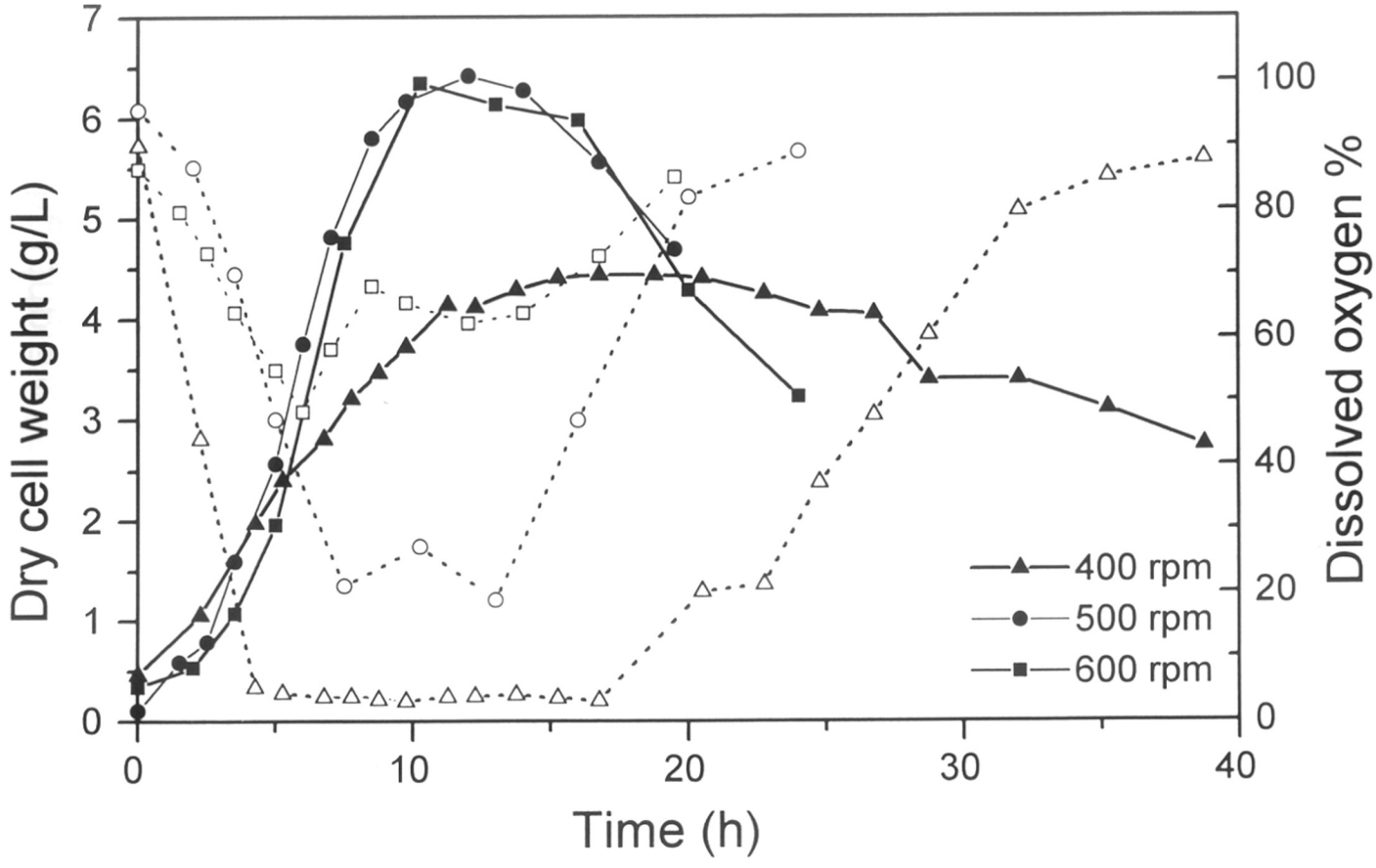

Figure 4.14: Effect of agitation speeds on cell mass. Open and closed symbols represent dissolved oxygen and cell mass respectively.

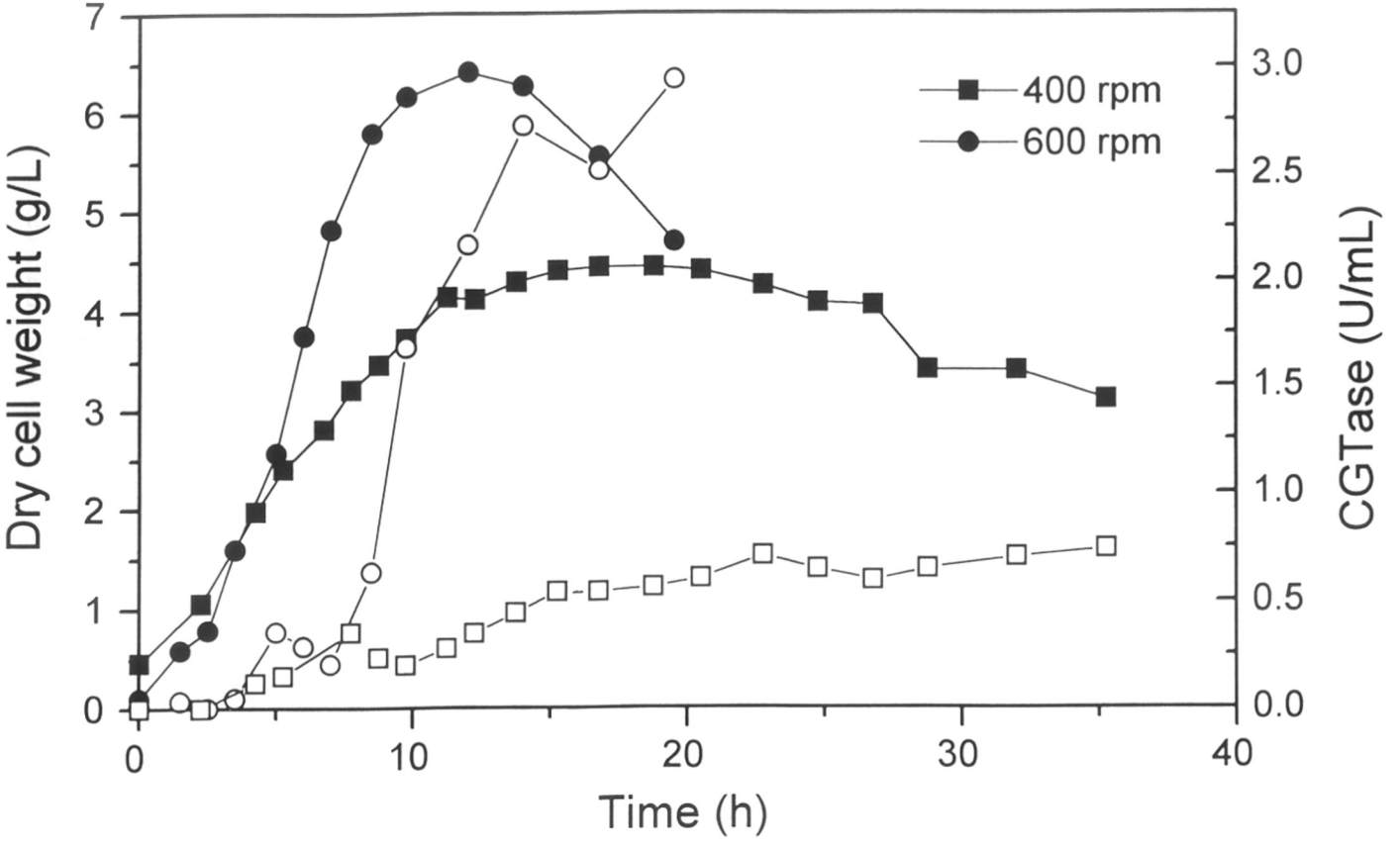

Figure 4.15: Comparison of growth and CGTase profiles at different rpm. Open and closed symbols represent dissolved oxygen and cell mass respectively.

RPM	Cell mass X_{max} (g/L)	CGTase Activity, P_{max} (U/mL)	Specific growth rate μ_{max} (h^{-1})	Cell yield X_{max} / Total sugars (g cells / g sugars)	Specific enzyme productivity P_{max}/X_{max} (U/g)
400	4.44	0.74	0.34	0.36	166.67
500	6.34	3.31	0.43	0.51	520.5
600	6.42	2.94	0.44	0.52	457.9

Table 4.2: Cell mass and CGTase yields in different rpm runs in batch culture.

Units/mL (oxygen limited). These workers observed that even though cell mass yields were drastically decreased, enzyme production was not much affected, probably because most of the enzyme was secreted during cell lysis and not during the growth phase. However, in the present study only 25% enzyme was produced under oxygen limited conditions as compared with those under adequate oxygen availability. There is another report on CGTase production at different agitation speeds in a fermenter (Jamuna *et al.*, 1993). In a starch based medium, a μ_{max} of 0.32 h^{-1} at 500 rpm was reported as compared to 0.29 h^{-1} at 300 rpm. The CGTase production also improved from 30 U/mL (300 rpm) to 50 U/mL (500 rpm). However, on further increasing the agitation to 700 rpm, though μ_{max} increased to 0.36 h^{-1}, the enzyme activity fell down to 42 U/mL. Further, these authors reported that the enzyme profile was similar in all cases and that peak activity was observed at 16-20 h. CGTase production in *B. firmus* was severely affected by available oxygen concentration. Whereas at low rpm a broad peak of enzyme activity was obtained around 23-38 h of growth, under favourable conditions maximum enzyme was produced much faster (within 20 h).

4.3.6.2 Vent gas analysis

The profiles of vent CO_2 and O_2 for fermenter runs at 400 and 600 rpm are shown in Figures 4.16 and 4.17. It was observed that during the initial phase of fermentation (upto 7 h at 400 rpm and 5 h at 500 rpm) CO_2 could not be detected in the vent gas. This is mainly due to the high solubility of CO_2 at alkaline pH. Noorman *et al.* (1992) have evaluated CO_2 evolution in alkalophilic bacteria. These authors have concluded that there is a major interaction between metabolic CO_2 evolved and the carbonate and bicarbonate ions present in the media between pH 7–9. Further, the ammonium ions present in the medium also react with CO_2 and bicarbonate. These workers have concluded that proper estimation of CO_2 in batch fermentation would require measurement of concentration of bicarbonate ions in the broth. From our observations it appears that CO_2 is detected in vent gas only after the above-mentioned reactions reach an equilibrium and the broth gets saturated with CO_2. Nevertheless further trends of evolved CO_2 showed a gradual decline in CO_2 concentration as the cell growth declined. The maximum CO_2 concentration detected in 400 and 500 rpm runs was 1.4% v/v and 2.5% v/v respectively. The vent O_2 profile matched well with the growth pattern in the fermenter runs.

4.3.6.3 Stoichiometeric analysis

During batch growth an effort was made to estimate the distribution of C, H, O and N (supplied in the medium) between biomass, unutilized substrate/product(s) and CO_2. Elemental analysis of carbon and nitrogen sources in the growth medium were performed. Similar analyses was done for biomass and unutilized substrate/product(s). Normally bacilli are known to produce a wide range of small molecular weight products (Sonenshein, 1993), the analysis of which would be beyond the scope of this work. Any product formed by *B. firmus* during aerobic growth was therefore clubbed with the unutilized nutrients in the medium for convenience of stoichiometry. Values of elemental analyses are given in table 4.3. From this data the elemental composition of carbon source

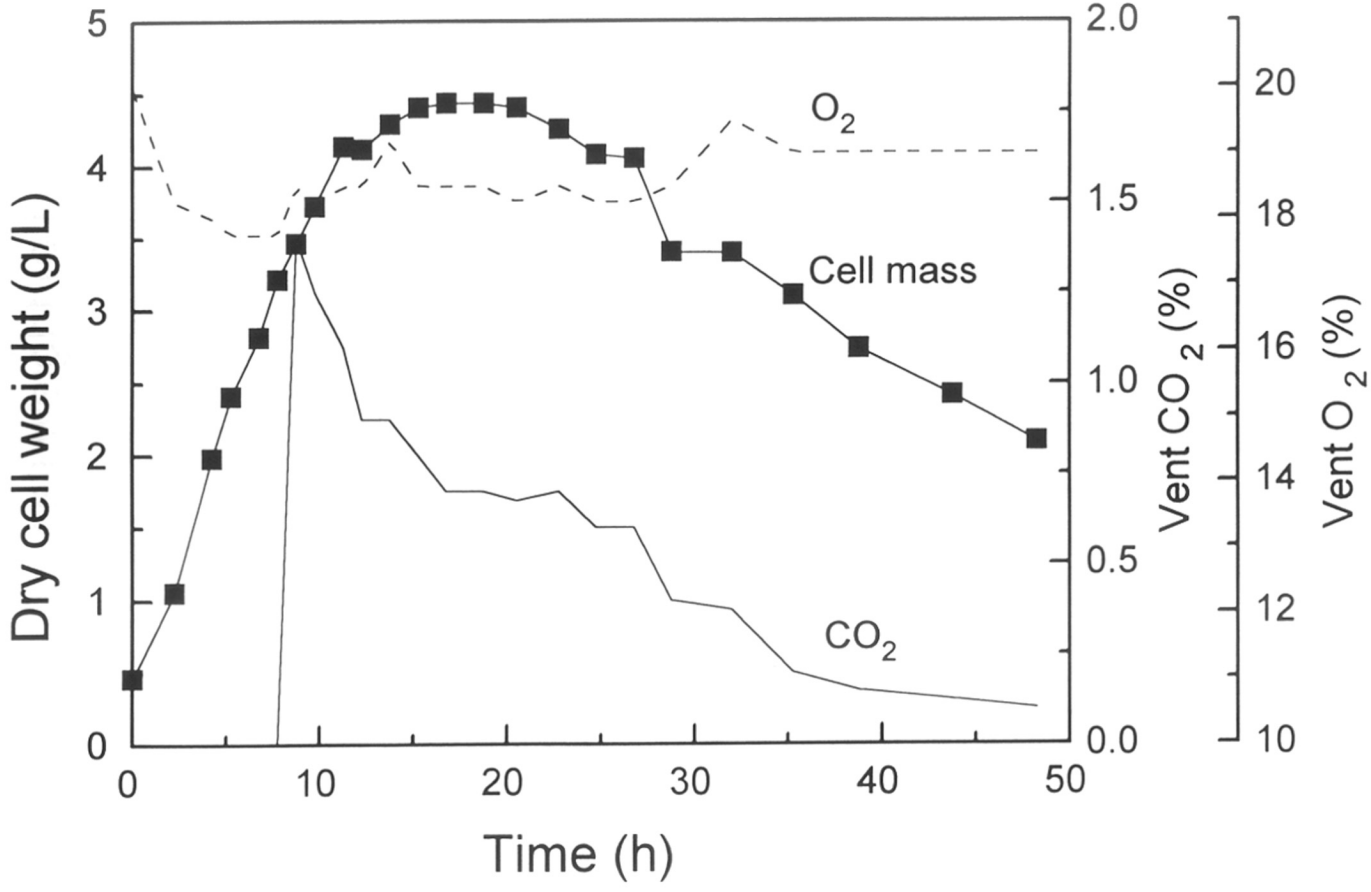

Figure 4.16: Profile of vent oxygen and carbon dioxide in a 400 rpm fermentation run.

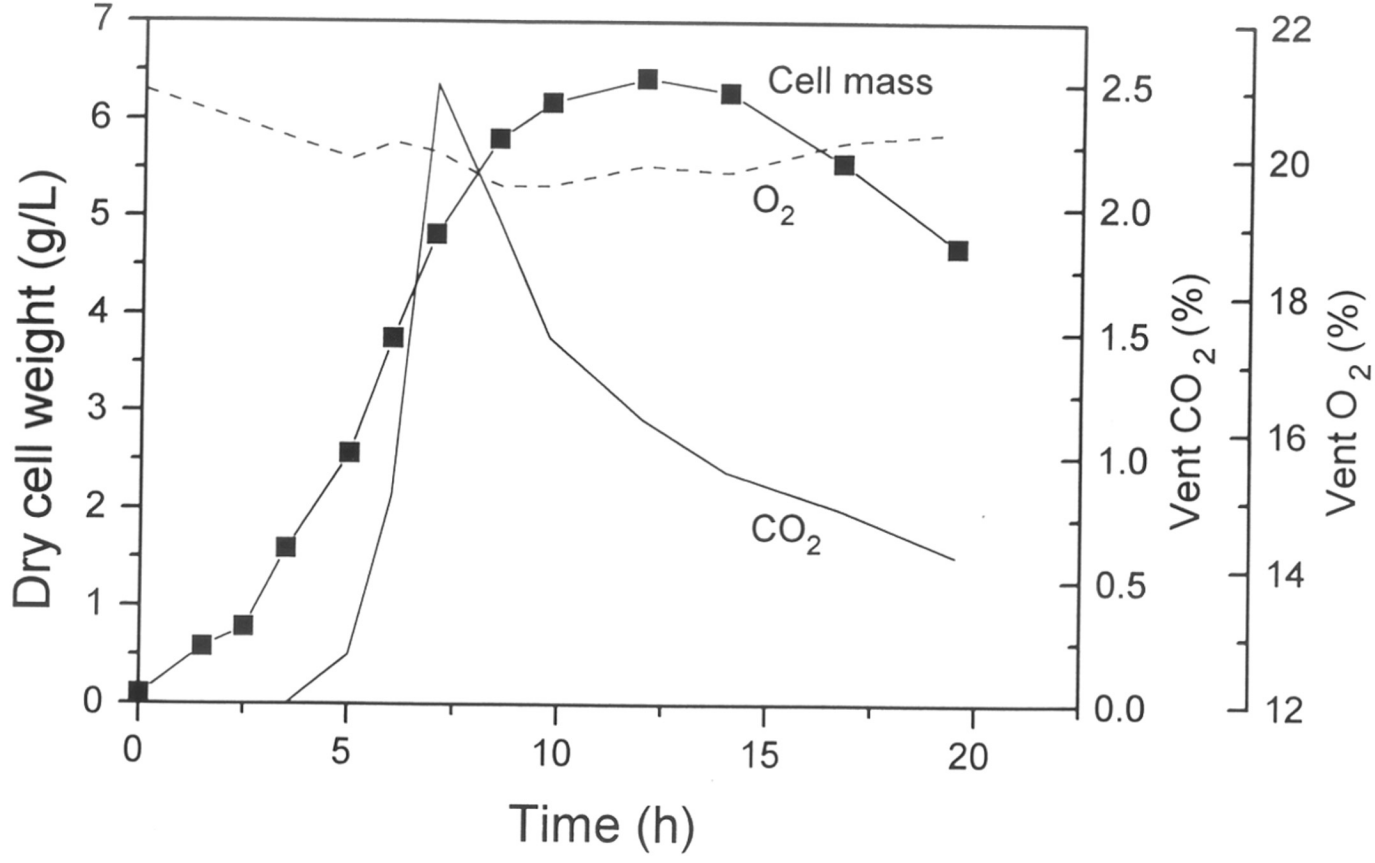

Figure 4.17: Profile of vent oxygen and carbon dioxide in a 600 rpm fermentation run.

Time (h)	Cell mass (g/L)	Broth (g/L)				Cells (%)			
		C	H	N	O	C	H	N	O
0	0.11	8.46	1.45	1.24	8.85	29.35	4.46	3.71	57.48
1.5	0.59	5.53	0.94	1.17	7.40	31.45	4.78	5.04	54.03
2.5	0.79	6.40	1.01	1.05	8.50	33.68	5.4	6.09	49.83
3.5	1.60	4.71	0.75	1.00	6.32	35.31	4.84	6.94	47.91
5	2.57	5.56	0.92	0.99	7.4	37.16	5.63	7.93	44.28
6	3.76	4.64	0.68	0.77	6.1	-	-	-	-
7	4.82	4.11	0.65	0.73	5.4	38.38	5.78	8.77	42.07
8.5	5.81	3.16	0.48	0.60	4.04	38.4	5.97	8.68	41.95
9.75	6.17	3.13	0.44	0.56	4.05	39.47	5.55	9	40.98
12	6.42	2.64	0.37	0.49	3.37	39.03	5.66	9.1	41.21
Starch						40.86	6.69	-	52.45
Peptone						45.35	7.33	13.99	28.33
Yeast extract						25.89	4.33	6.24	43.54

Table 4.3: Elemental analysis of growth medium components and various samples during the time course of fermenter run.

(starch), nitrogen source (peptone and yeast extract), biomass and unuitilized substrate were estimated. Ash contents of various samples were estimated and deducted from their weights. Values for oxygen gas consumed and water formed were calculated from the material balance. The elemental composition of the biomass was estimated by averaging the instantaneous composition for nine samples during the growth phase. Since the nitrogen source was a complex organic material it also contained assimilable carbon which was incorporated into the total carbon.

The elemental compositions were as follows:

Starch:	CH_2O
Nitrogen source:	$CH_{1.96}O_{0.75}N_{0.25}$
Biomass:	$CH_{1.78}O_{0.99}N_{0.16}$
Unutililized substrate/ product:	$CH_{1.68}O_{0.96}N_{0.16}$

The values for biomass and nitrogen source compare well with those mentioned in the literature (Atkinson and Mavituna, 1991). Based on actual values for weight (g/L) of the above, an over all equation for aerobic growth of *B. firmus* in batch culture was derived viz.

$$10.05\ CH_2O + 10\ CH_{1.96}O_{0.75}N_{0.25} + 7.04\ O_2 =$$

$$6.31\ CH_{1.78}O_{0.99}N_{0.16} + 8.69\ CH_{1.68}O_{0.96}N_{0.16} + 5.05\ CO_2 + 6.93\ H_2O + \Delta G$$

The above equation is representative of batch growth for a particular set of conditions which were optimized during 10 L fermentation runs. These results indicate that 55.5% of carbon utilized is incorporated into the biomass and 44% in CO_2. Reported values for percentage C incorporated into biomass when grown on carbohydrates are 45.1% for *B. subtilis* (Solomon and Erikson, 1981) and 66% in *Pseudomonas fluorescens* (Atkinson

and Mavituna, 1993). For percentage carbon incorporated into CO_2 values range between 35 and 44% (Atkinson and Mavituna, 1993).

4.3.7 Metabolism of Starch

When *B. firmus* was cultivated in batch cultures using HM II medium, it was observed that there was a gradual decrease in the starch content of broth (as estimated by total sugars) as well as reducing sugars as the growth progressed (Figure 4.11 and 4.12). In addition there was a transient production of CDs during the initial phase of growth (Figure 4.18). These CDs, however, disappeared later during the log phase of growth.

There are a few reports on the substrate metabolism among CGTase producing bacteria. Makela *et al.* (1990) studied CGTase production in *B. circulans* ATCC 21783. The growth of the bacterium was studied in the same medium as that in the present study. These workers found that most of the carbohydrates disappeared prior to bulk growth in contrast to our findings. This was simultaneously followed by a transient peak of reducing sugars which reached a maximum of about 1.4-2.0 mg/mL. This peak of reducing sugars was attributed to a concomitant pulse of amylolytic activity in the culture broth. There was also a production of acid metabolites as evident by a sharp decrease in pH. It was thought the bacterium utilized these metabolites for growth. Yan and Lin (1993) also observed similar results in *Bacillus* sp. no. 562. These results are quite different from those recorded in the present study. We found a gradual decrease in sugars concentration and pH as the growth progressed. As noticed in the present work, Yan and Lin (1993) also observed a peak of CDs during the initial growth phase. These CDs also disappeared as the growth of bacteria progressed.

At the onset of growth the cell produces hydrolytic enzymes to break down complex carbohydrates. Starch needs to be hydrolysed to yield mono or disaccharides for their uptake by *B. firmus*. Steinmetz (1993) reports that *Bacillus* cannot utilize

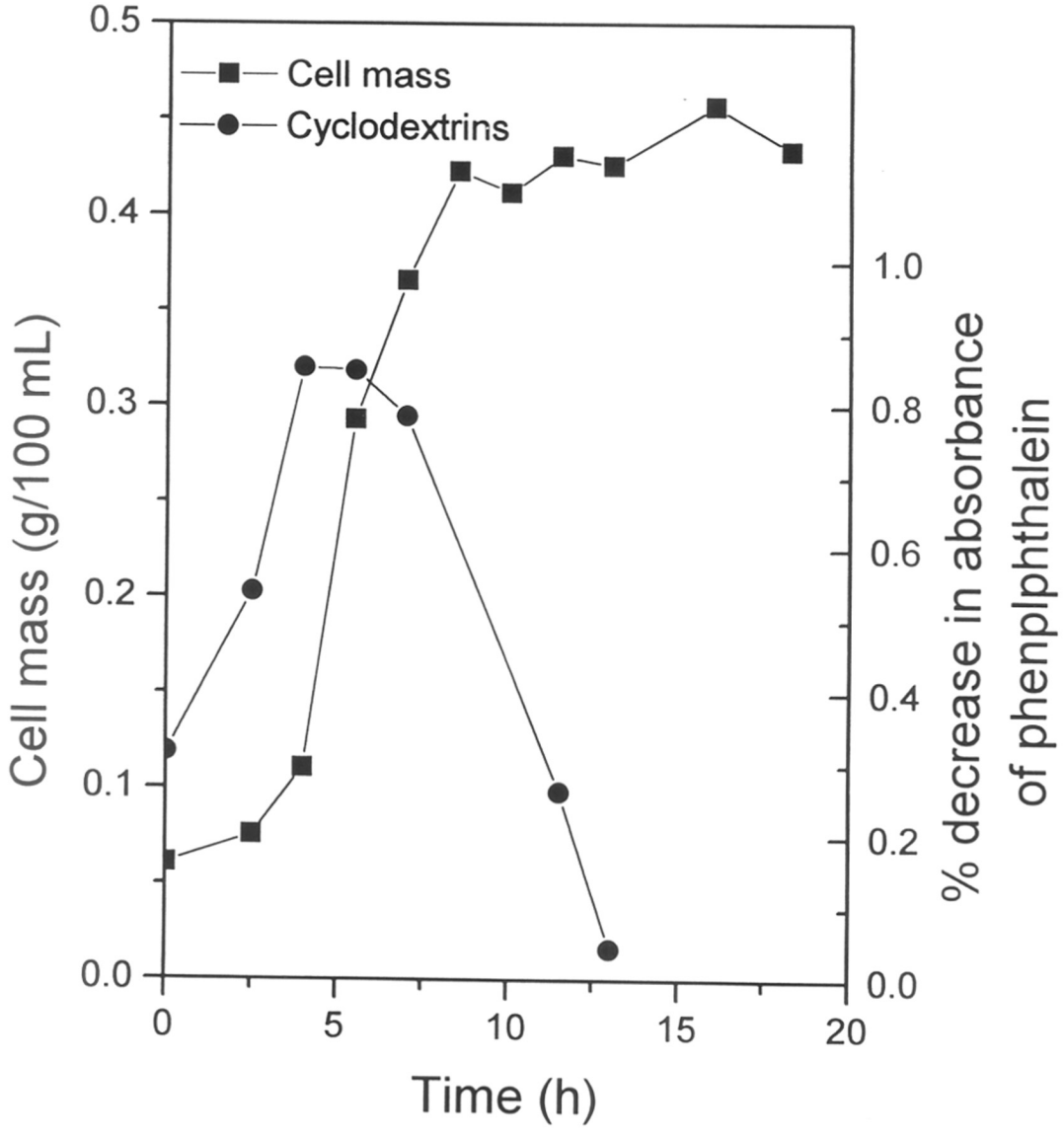

Figure 4.18: Cyclodextrins in broth during growth of *B. firmus*.

maltooligosaccharides of chain length more than two. It was found that starch digested with *B. firmus* CGTase did not contain any malto-oligosaccharides of small chain length as determined by HPLC (described in detail in a later section). To explore this further, another experiment was conducted in which *B. firmus* CGTase was incubated with different concentrations of soluble starch (DE 8) at 30 °C (the temperature at which cell growth studies have been done) and the formation of reducing sugars was monitored spectrophotometrically. The amount of reducing sugars actually decreased with incubation time (Figure 4.19). This behaviour is not completely unexpected as during intra- and inter-molecular transglycosylation reactions the total number of reducing ends (corresponding to maltooligosaccharide chains) are expected to decrease due to the formation of nonreducing CDs. If an amylase activity was to be present, the reducing sugars would have increased. When the same experiment was repeated with gelatinized native tapioca starch a slight increase in the reducing power of starch was observed (Figure 4.20). This behaviour further supports the observation that no true amylase is present in *B. firmus* and this minor increase in reducing power of starch was only a manifestation of a rapid disproportionation of starch by CGTase. Thus, these experiments have prompted us to propose a hypothesis for consumption of starch in the strain of *B. firmus* under study. We believe that minor amounts of glucose or maltose might be produced during the transglycosylation reaction of CGTase. These sugars are consumed by the bacillus thus pushing the reaction equilibrium towards production of more sugars. Because of rapid uptake of sugars produced at any time the amount of reducing sugars is never large. This behaviour explains the observed decrease in reducing sugars as the bacillus grows. The CDs formed during the initial phase of growth are degraded later by intermolecular transglycosylation and consumed. Though Bender (1977b) has indicated that CDs might be taken up directly by the bacterial cells, these findings require further work for confirmation as direct uptake of sugars, with chain length greater than 2, by bacteria has not been documented.

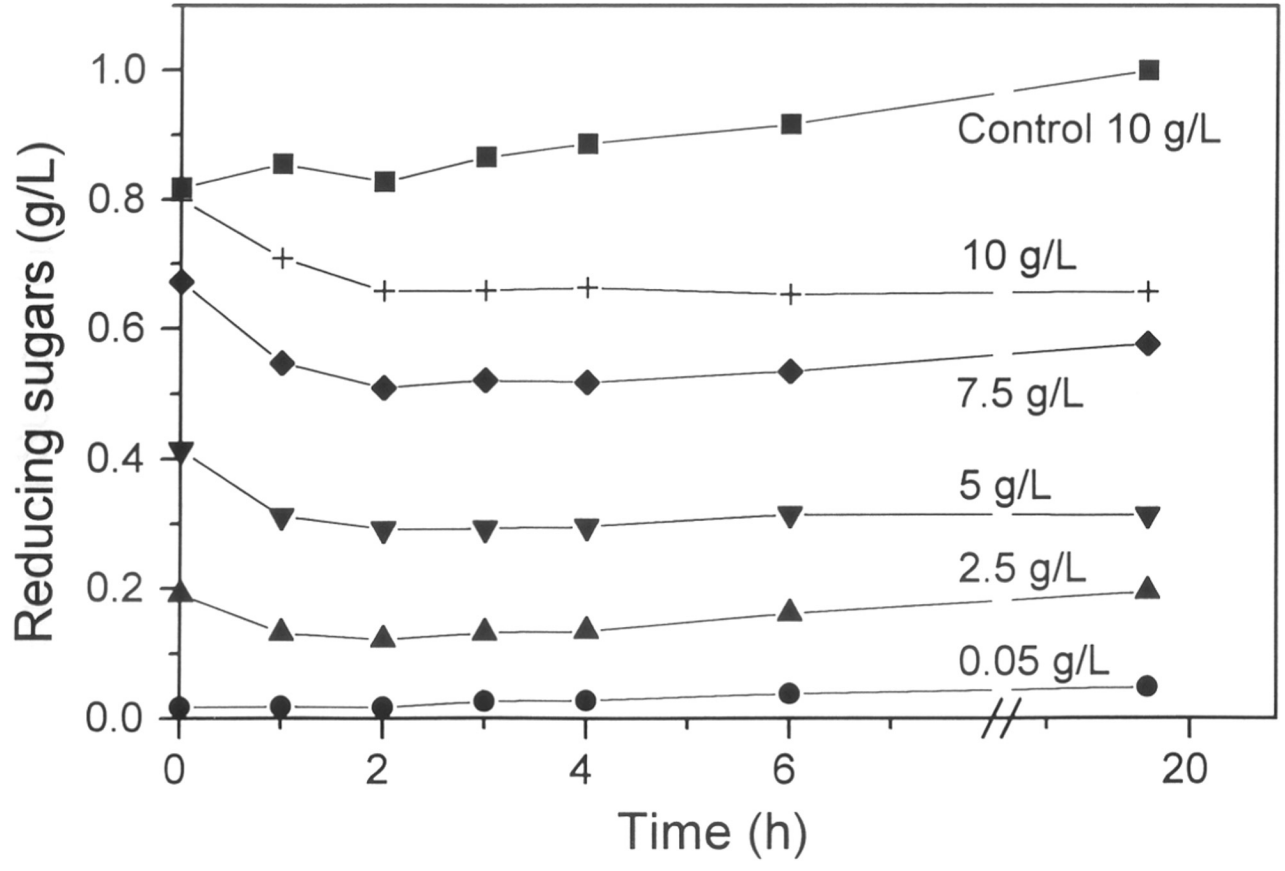

Figure 4.19: Production of reducing sugars from soluble starch by action of CGTase on soluble starch at 30 °C.

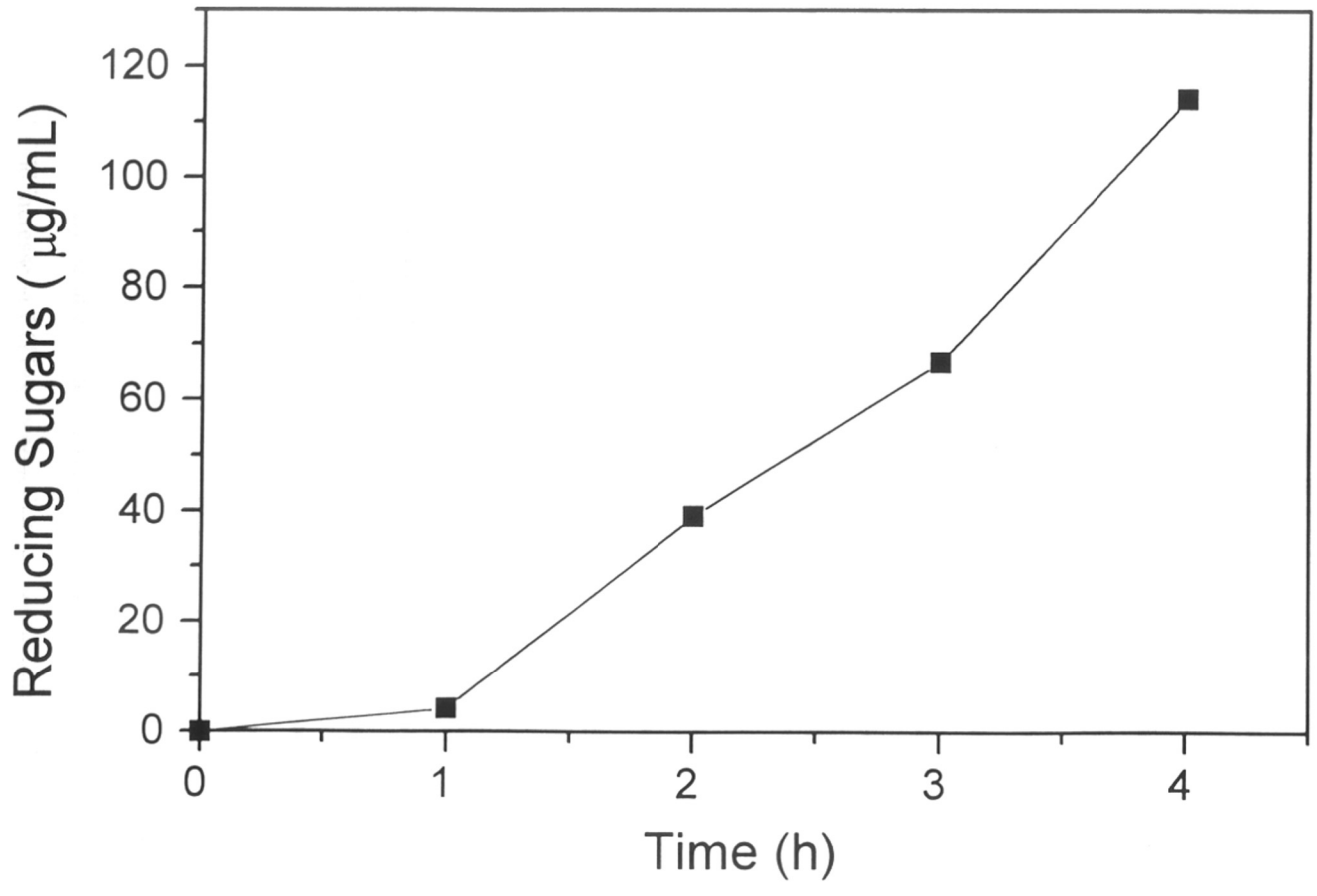

Figure 4.20: Production of reducing sugars from gelatinised starch by action of CGTase at 60 °C.

4.3.8 Continuous culture studies

Continuous culture studies were performed in a Gallenkamp fermentor (700 mL working volume) between dilution rates (D) 0.098 h^{-1} to 0.642 h^{-1}. The steady-state profiles of cell mass, enzyme activity, total sugars and proteins at different D are shown in Figure 4.21. The CGTase activity and cell mass decreased rapidly with increasing D. The pH of the broth also increased from an initial 8.3 (D = 0.098 h^{-1}) to a final 9.9 (D = 0.642 h^{-1}). As shown in Figure 4.22 the volumetric productivity of CGTase gradually declined from 80.6 U/L-h to 64 U/L-h over the D 0.098 h^{-1} to 0.485 h^{-1}. On increasing the D further there was a steep fall in volumetric productivity. The maximum volumetric productivity of cells (0.65 g/L-h) was obtained at a D of 0.323 h^{-1}. The amount of unconsumed sugars and proteins in the broth also increased with increasing D. Only 14% and 22% of sugars and proteins were consumed at D = 0.64 h^{-1} as compared to 87% and 57% utilization at D = 0.098 h^{-1} respectively. The volumetric productivity of CGTase in continuous culture was only 60% of that in batch culture. At the lowest D the residual sugars and proteins (1.88 and 1.28 g/L, respectively) approximated the values obtained in the batch culture at the end of log phase.

There are a few reports on production of CGTase in continuous culture. Lane and Pirt (1973) studied the production of *B. macerans* CGTase in a starch-containing minimal medium. These authors observed about 2.75 times increase in enzyme productivity in continuous culture as compared to the batch. Maximum CGTase activity was observed at D = 0.05 h^{-1} (270 U/mL) which rapidly decreased to 5 U/mL at D = 0.5 h^{-1}. The main reason for low enzyme activity at higher dilution rates was thought to be substrate repression due to high residual starch concentration. In our studies, the low enzyme activity at high D may be due to similar reasons. In another study Jamuna *et al.* (1993) obtained 3.1 fold increase in CGTase productivity in continuous culture of *B. cereus* on a

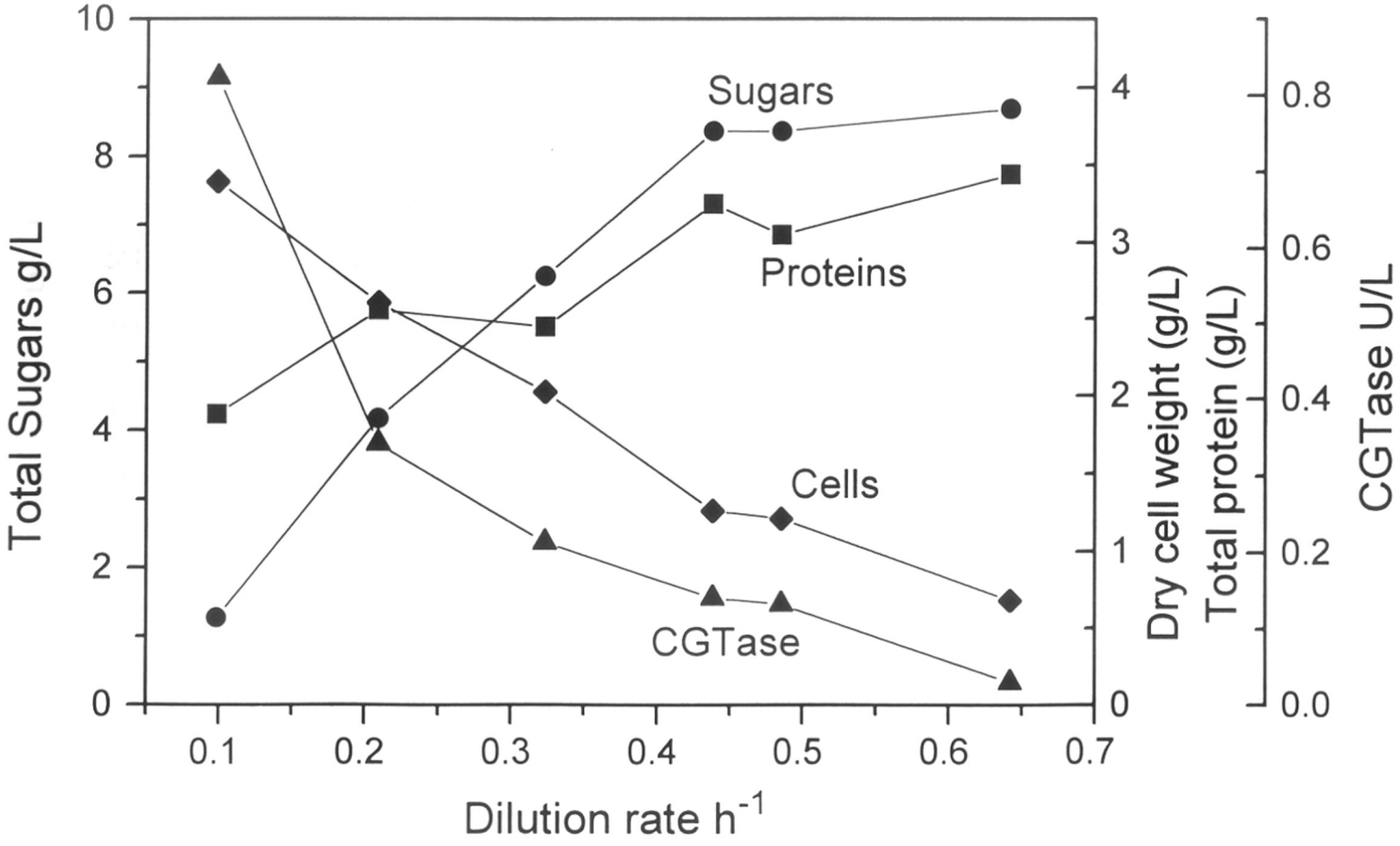

Figure 4.21: Cell growth and CGTase production in continuous culture in a 1 L fermenter.

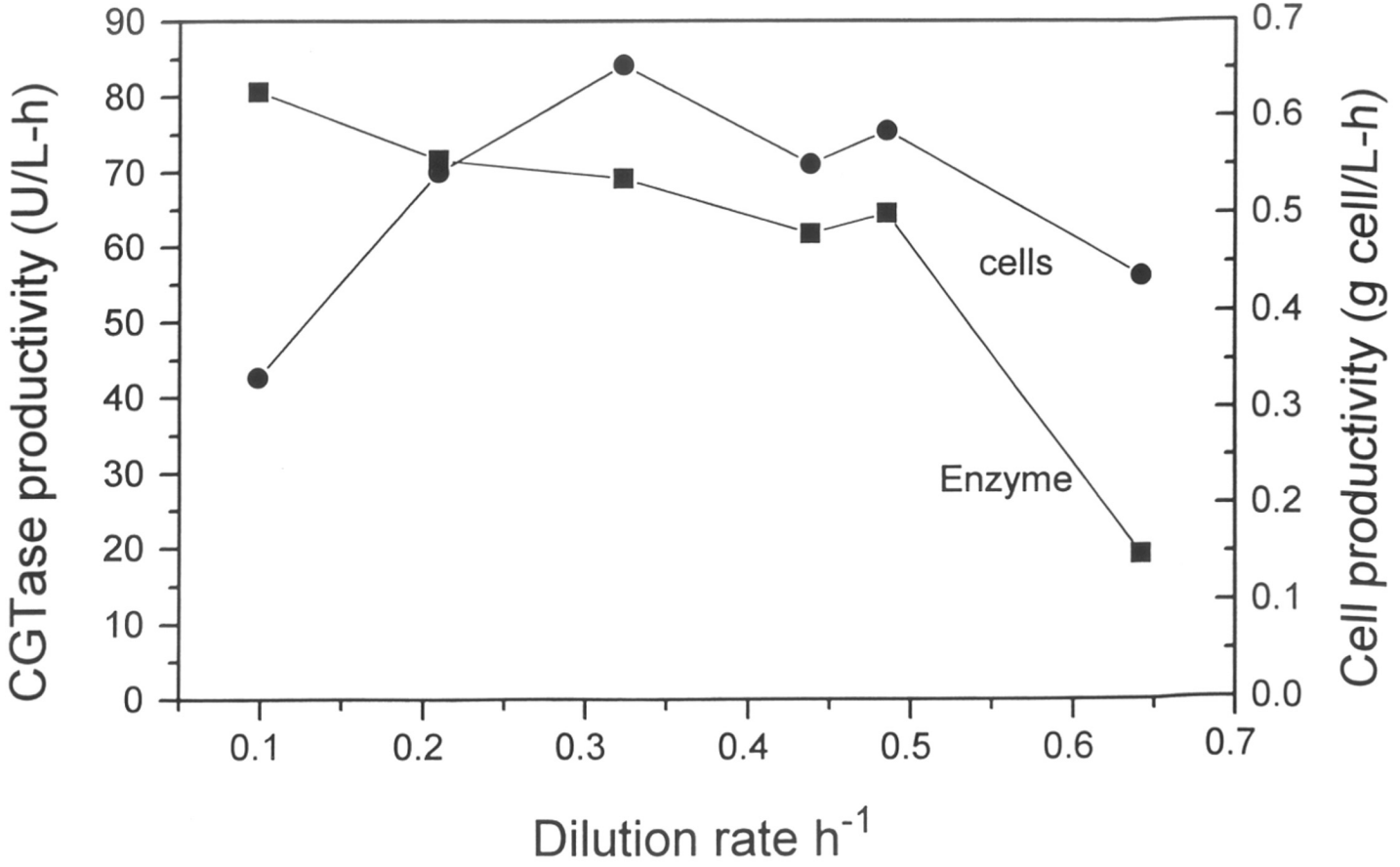

Figure 4.22: CGTase and cell mass volumetric productivities obtained in continuous culture.

glucose based medium as compared to batch culture. The maximum volumetric productivity obtained was 5.4 kU/L-h. In the present studies we found low productivities and low enzyme yields in continuous culture as compared with batch.

Most continuous culture studies are done under chemostat conditions using a single nutrient limitation. Such studies normally employ minimal (defined) media such that the concentration of the growth limiting nutrient can easily be estimated. In the present study a complex medium was used to grow *B. firmus* under non-chemostat conditions. Non-chemostat continuous culture is an equally invaluable tool to study synthesis of inducible enzymes (Wang *et al.*, 1979) under different dilution rates. The present study enabled us to observe the effect of starch and peptone concentration on growth and enzyme production.

4.3.9 Effect of cell density on CGTase production

During the time course studies it was observed that CGTase secretion showed a strong correlation to cell growth. To confirm this observation *B. firmus* cells actively producing CGTase were harvested by centrifugation, washed with fresh medium and later suspended in the same amount of fresh medium and incubated further. The results are shown in Figure 4.23. It could be observed that the cell growth started immediately on resuspension in fresh growth medium without any lag phase. Though the specific enzyme activity with respect to cell mass remained constant in both the phases of growth ($\approx$ 40 U/g cells), the enzyme productivity in the later phase was 0.056 U/mL-h as compared to 0.02 U/mL-h in the first phase, an increase of 2.76 folds. These results clearly indicate that the CGTase production in *B. firmus* is related to total cell mass. Further experiments on growth of the bacterium with cell recycle confirmed the observation.

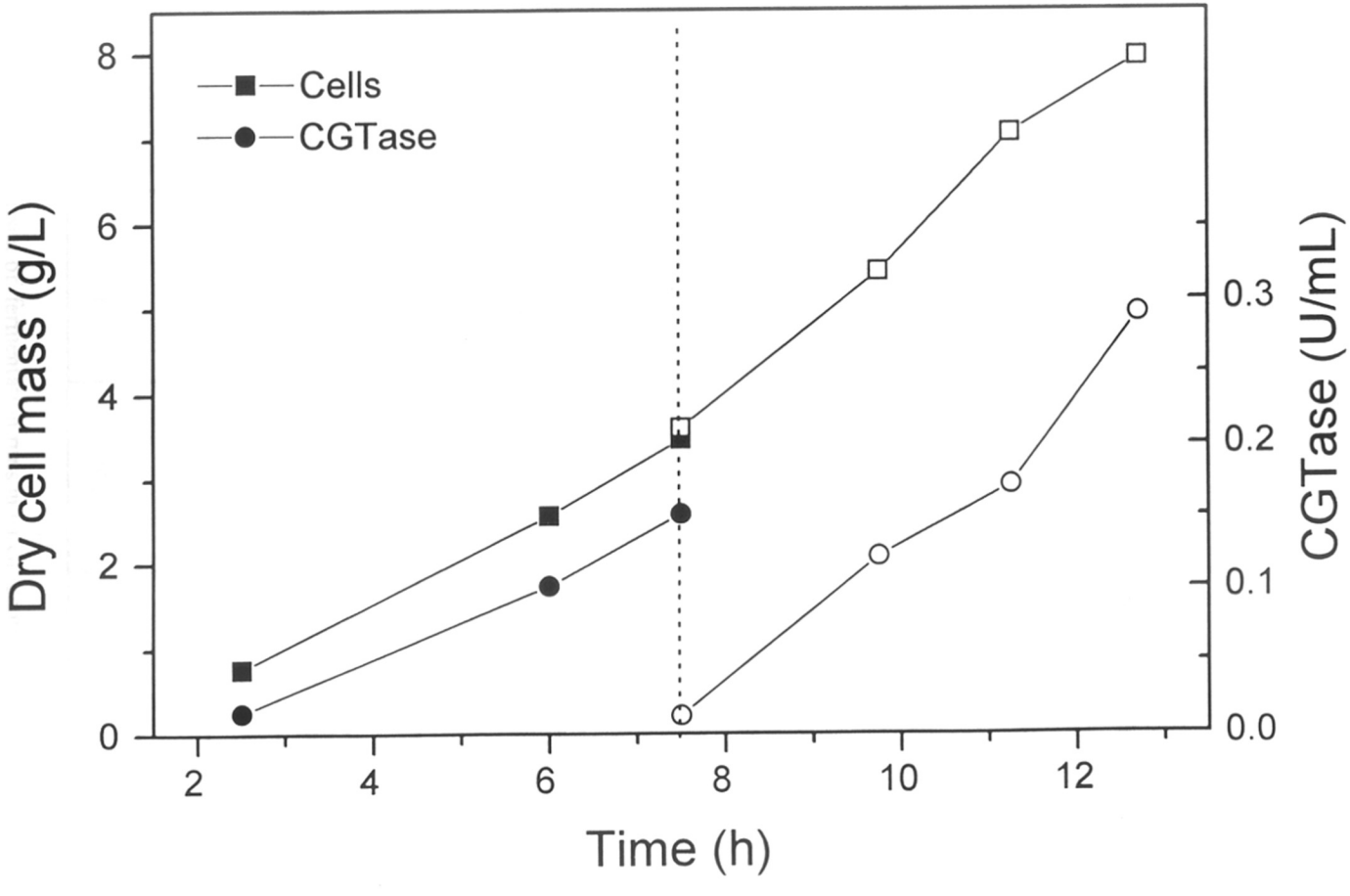

Figure 4.23: Dependence of CGTase production on cell mass. Open symbols represent profiles after second inoculation.

4.3.10 Production in cell recycle bioreactor

B. firmus was cultivated in a 1 L Gallenkamp fermenter with 700 mL working volume (Figure 4.24). After a batch period of 11 h a microfiltration membrane module was coupled to the fermenter and the run continued. Fresh medium was added to the fermenter at a flow rate of 1 mL/min and equivalent volume of permeate was collected through the microfiltration module. After 24 h the feed rate (and the permeate rate) was increased to 2 mL/min. The cell mass retained in the fermenter and enzyme activity in the permeate were estimated. The results are shown in Figure 4.25. During the 56 h of operation of the cell recycle reactor the dry cell mass increased 5.9 fold from an initial 5 g/L to a final 29.8 g/L. As compared to the initial activity of 0.9 U/mL in batch culture at the start of recycle operation, 1.6 U/mL and 2.25 U/mL CGTase was produced at the cell densities of 10.9 g/L and 29.8 g/L respectively. After an initial dip from 9.85 to 9.1 in the first 8 h of the recycle mode, the pH of broth in the fermenter remained almost constant at 9.1. Table 4.4 shows a comparison of CGTase production in cell recycle reactor with batch and continuous culture.

Mode	Time or Residence time (h)	Cell mass (g/L)	CGTase (U/mL)	Volumetric productivity (U/L-h)
Batch	19.5	6.4	2.9	148.7
Continuous	10.2	3.5	0.93	91.14
	5	2.6	0.34	71.4
Cell recycle	11.6	10.94	1.6	137.6
	5.9	29.8	2.25	382.5

Table 4.4: Comparison of fermentation runs in batch, continuous and cell recycle modes.

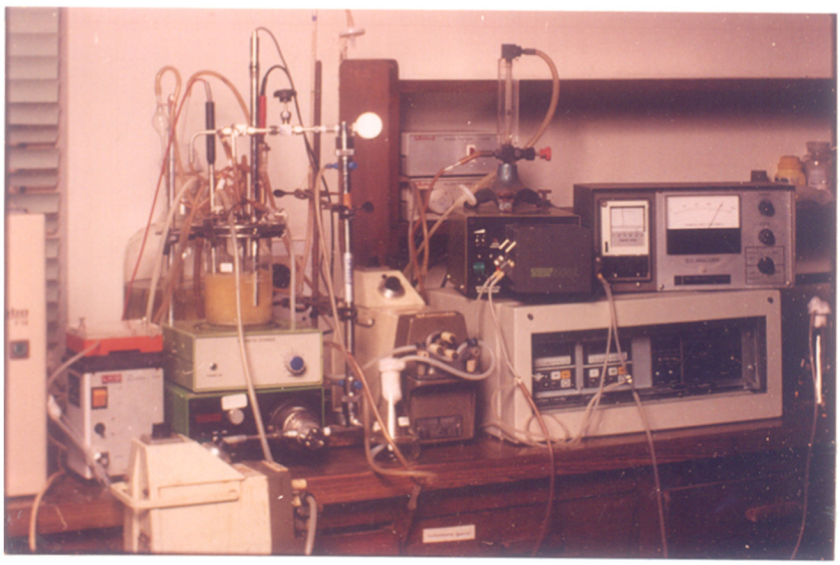

Figure 4.24: Membrane recycle bioreactor used in the present study.

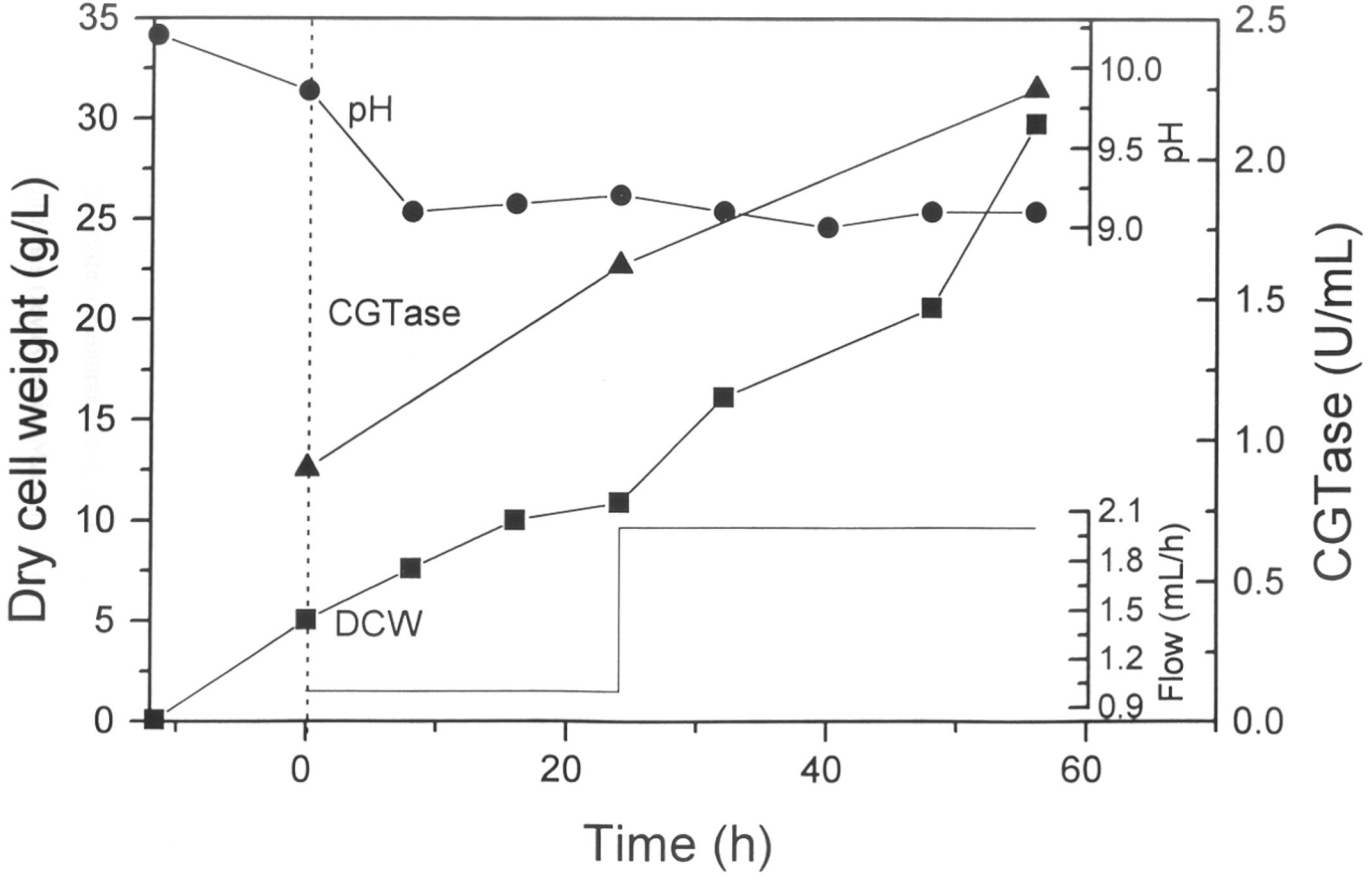

Figure 4.25: CGTase production in cell-recycle reactor. Dotted line indicates switch over to recycle phase.

It is quite clear that a membrane recycle reactor gives the best volumetric productivity for CGTase, although the highest enzyme activity is obtained in batch culture.

There are no reports available on production of CGTase in a membrane bioreactor. Jamuna *et al.* (1993) reported production of CGTase in a fluidized bed reactor with *B. cereus* cells immobilized in alginate beads. These workers found upto 13-fold increase in productivity of CGTase as compared to free cells. Though, the above results cannot be strictly compared with a membrane recycle bioreactor studies, the fact that an increased biomass concentration in the reactor results in higher productivity, supports our findings. There are a few reports available on production of other extracellular or intracellular enzymes in cell recycle mode. Holst *et al.* (1985) reported higher productivity (3.5 times of batch) of the intracellular enzyme superoxide dismutase produced by *Streptococcus lactis* whose growth was inhibited by the acidic products in batch culture. Nipkow *et al.* (1989) obtained 11-12 fold exo-β-amylase as well as cell mass in an anaerobic fermenter coupled to a ceramic microfiltration membrane. A recent work of Morcel and Biedermann (1994) on α-amylase production by *B. amyloliquifacins* in a cell recyle reactor compares well with the work in the present study. The authors obtained increased volumetric productivity in the cell recycle mode when compared to batch, but the enzyme yields were lower.

We envisage the following reasons for low yields of CGTase in spite of the high cell density obtained.

i) Incomplete permeation of CGTase through the membrane

ii) Attrition of cell mass during the cell recycle

iii) Sub-optimal concentration of the inducing agent, starch, in the reactor

In a microfiltration system membrane fouling and formation of a dynamic layer often leads to retention of proteins (reviewed by Marshall *et al.*, 1993). Due to this phenomenon,

recovery of enzymes from bacterial fermentation broths has been reported to be affected (Nagata *et al.*, 1989; Nipkow *et al.*, 1989). In a study of production of exo-β-amylase using ceramic membranes coupled to the fermenter, Nipkow *et al.* (1989) found 20-75% enzyme retention by the membrane in a maltose based medium that further increased to 50-95% when maltodextrins were used in the growth medium. Similarly, Morcel and Biedermann (1994) observed 25% and 35% retention of α-amylase in a polypropylene based membrane module while using maltose or starch based medium, respectively. It is quite evident that retention of enzyme by the membrane can lead to decreased overall productivity in a cell-recycle bioreactor. In the present study enzyme activity in the cell bleed was not determined. The Carbosep membrane module used in this work was often used to clarify fermentation broth where no significant losses in enzyme recovery were found. However prolonged usage of this membrane can lead to fouling, the effect of which probably accounts for decreased enzyme recovery.

Another reason for low enzyme yields could be attributed to death/disintegration of bacterial cells in the recycle reactor. In a cross-flow filtration system high cross flow rates are desirable to minimize membrane fouling. However, high cross-flow rates can lead to disintegration of bacterial cells due to high shear (> 1 kN/cm) in the recycle loop (Shimuzu *et al.*, 1992). Moreover, the poor performance of a membrane recycle bioreactor under high cell concentrations has also been linked to nutrient or mass transfer limitations (Qureshi and Cheryan, 1989). These factors could eventually lead to cell death in the bioreactor. However, using laser flow cytometry, Hoffman *et al.* (1987) found no change in the physiological state of yeast cells with or without cell recycling. In another report it was observed that shear stress in a microfiltration module actually improved the fermentation performance of *Clostridium acetobutylicum* (Afschar *et al.*, 1986). In our studies, no physical damage to the bacterial cells could be observed under microscopic examination. Thus cell attrition may not be a cause of low enzyme yields. However,

detailed studies on correlation between shear rate and release of cellular constituents need to be performed to draw final conclusions.

Concentration of starch in the growth medium plays a very important role in the production of CGTase. As observed in batch and continuous culture studies maximum CGTase is produced when the residual starch concentration is optimal. CGTase production in a cell recycle bioreactor is governed by similar observations on residual starch. As starch is also consumed by cells as a carbon source, for a particular reactor configuration, with a certain amount of cell density, there is a specific concentration of starch, or its degradation products, required for maximal CGTase production. This observation is substantiated by shake flask experiments carried out in our laboratory recently (unpublished results). These experiments were performed to find out the optimal concentrations of the nutrients, starch and yeast extract for maximal CGTase production. The results shown in Figure 4.26 are self explanatory. At the starch concentration of 10 g/L and yeast extract concentration of 5 g/L the DCW and CGTase activity were 2.2 g/L and 0.16 U/mL respectively. As the yeast extract concentration was increased to 25 g/L, CGTase activity doubled while the cell mass increased to 3.4 times. However at the starch concentration of 5 g/L, after similar increase in yeast extract, though the cell mass doubled, the CGTase activity increased 8.6 fold. These results clearly emphasize the importance of starch concentration in the growth medium. In a cell recycle bioreactor the concentration of cells continuously increases with time. Therefore proper optimization of feed nutrients (including starch concentration) is necessary as it certainly affects the enzyme yields. In the present study low enzyme yields due to above-mentioned reasons cannot be ruled out.

The use of a membrane bioreactor for production of microbial enzymes is a recent technique. Not only does this system provide an opportunity for high volumetric productivities and high enzyme yields in the fermentation process but it also offers an

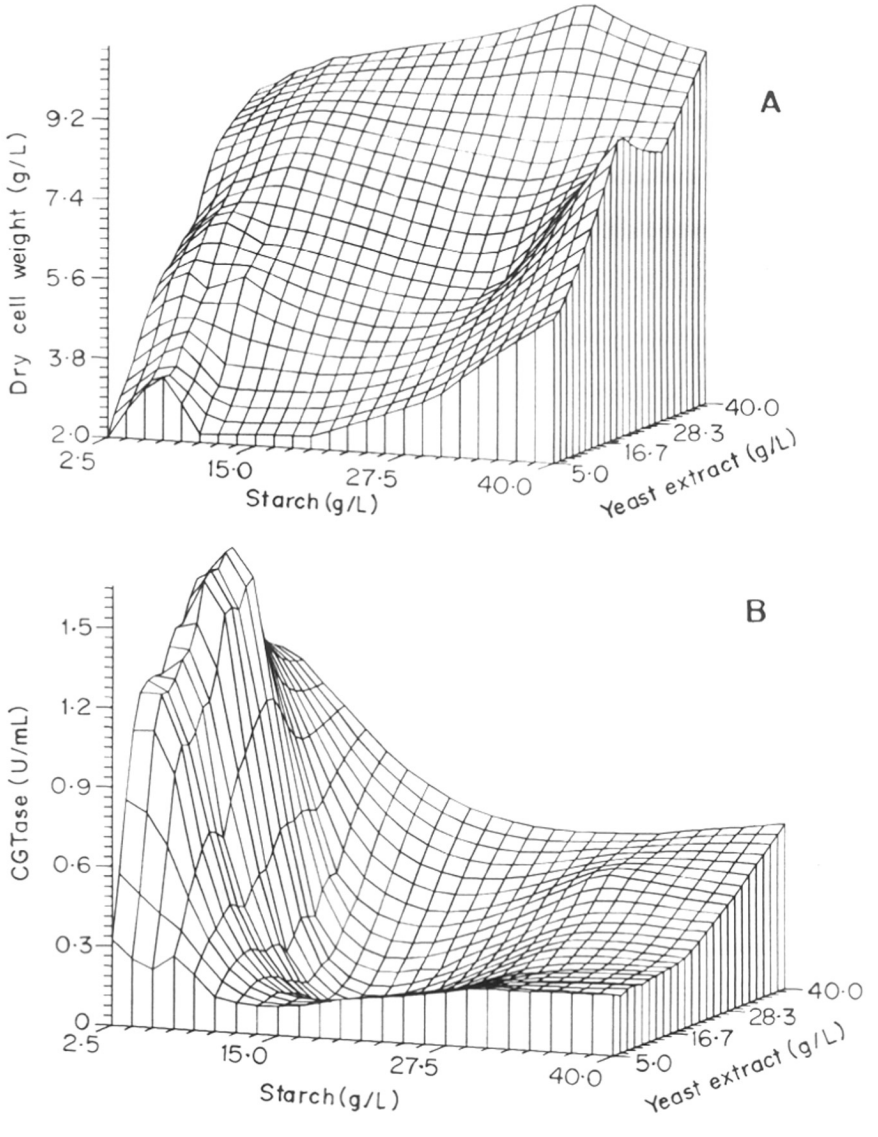

Figure 4.26 : Effect of starch concentration in the growth medium on cell mass and CGTase activity. A: Cell mass. B: CGTase

integrated downstream processing for enzyme production. As pointed out by Sicard and Saniez (1987), for industrial use, purification of CGTase is unnecessary and simple microfiltration and ultrafiltration of fermentation broth is sufficient for CD production. Thus, if proper optimization studies are done, CGTase production in a cell recycle bioreactor can offer a cost-effective method for production of this commercially important enzyme.

4.4 CGTase purification

The CGTase from *B. firmus* was purified using a two step procedure comprising of an affinity separation using a modified starch column followed by anion exchange chromatography on diethyl amino ethyl (DEAE) Sephrose column. The results of purification steps are summarized in Table 4.5. The purified enzyme preparation had a specific activity of 35.6 U/mg protein (the protein concentration was estimated by measuring absorbance at 280 nm). The overall recovery of enzyme was approximately 70%.

Step	Total volome (mL)	Total activity (U)	Total protein (mg)	Specific activity ($U.mg^{-1}$)	Folds purification	Yield (%)
Cell free supernate	950	1301.5	3752.5	0.35	1	100
Starch affinity	32	917.3	28	32.8	94.4	70.5
DEAE Sepharose	12	908	25.5	35.6	102.7	69.8

Table 4.5: Purification of CGTase.

4.4.1 Starch adsorption

The enzyme was bound to freeze-thawed modified corn starch at 2-5 °C and eluted at 45 °C. The crude broth obtained after removing the suspended cells was directly loaded onto the starch column. However, to avoid long loading times the volume of broth was often decreased by ultrafiltration through a hollow fibre module of molecular weight cut off 20,000 Da (Nitto Denko, Japan, Model NTU 3250C). This step gave a 8-10 fold concentration with > 99% retention of enzyme activity. The enzyme had strong affinity for the modified starch. One gram (wet weight) of starch could bind upto 80 U enzyme. The enzyme could not be eluted by a shift in pH (from 6 to 10), increase in ionic strength (upto 2 M NaCl) or elution with substrate (0.5% w/v soluble starch). The enzyme was eluted by raising the temperature of column to 45 °C. At this temperature bound CGTase acted on starch and hydrolysed it. In this process the enzyme was desorbed and was recovered by buffer washing. High binding capacity permitted use of small amounts of starch for complete recovery of enzyme from broth. In order to avoid breakthrough of enzyme during loading a starch column of long bed height had to be used. It was observed that yields of enzyme obtained through the column never exceeded 20-25% and considerable amounts of enzyme remained bound to the starch as seen by the gradual disintegration of starch on prolonged exposure to elevated temperature. To circumvent this problem a simple modification was made in the elution procedure. The flow during washing and elution of the column was reversed. The enzyme was now eluted in this way with increased yield of 70–80%.

The extent of adsorption of enzyme was found to be dependent on the source and type of starch. The enzyme could bind to corn, potato and tapioca raw starches. Corn starch had the maximum binding capacity and it was therefore used to make modified starch. *B. firmus* CGTase was unable to hydrolyse raw corn starch even though it could bind to it. Thus, it would be the ideal affinity matrix for purification of CGTase. However, its

binding capacity was much less than that of freeze-thaw modified starch. This could be explained by much larger surface area of the modified starch. Freeze-thawed starch was therefore preferred for affinity purification of CGTase. Makela *et al.* (1988) also observed that 'any modification of starch was effective only when it increased the adsorption surface'. Adsorption onto starch has been one of the most common steps in the purification of CGTase (Table 2.2). This process has been used with various modifications for better adsorption and yields, e.g. raw starch in combination with ammonium sulphate, ethanol or filter aids was used to facilitate adsorption. The elution of CGTase was carried out in the presence of maltose, ammonium sulphate, ethanol or β-CD (Table 2.2), with enzyme recovery varying from 42 to 100%. In the present work 70-80% of enzyme could be recovered. As shown in Figure 4.27, most of enzyme was eluted at 45 °C. Further increase in the elution temperature (upto 60 °C) did not prove effective because very low enzyme concentration were observed in subsequent fractions which had to be discarded.

4.4.2 Ion exchange chromatography

The affinity purified preparation contained minor impurities such as linear and cyclic maltooligosaccharides produced by starch hydrolysis. These could be easily removed by DEAE Sepharose chromatography. The enzyme was loaded at pH 7.0 and the column was washed with 0.1 M NaCl in buffer. The pure enzyme was eluted with 0.2M NaCl in buffer.

4.5 Characterization of CGTase

4.5.1 Molecular weight

The purified preparation showed a single band on SDS-PAGE (Figure 4.28). Using standard molecular weight markers the enzyme molecular weight was calculated as 78,000

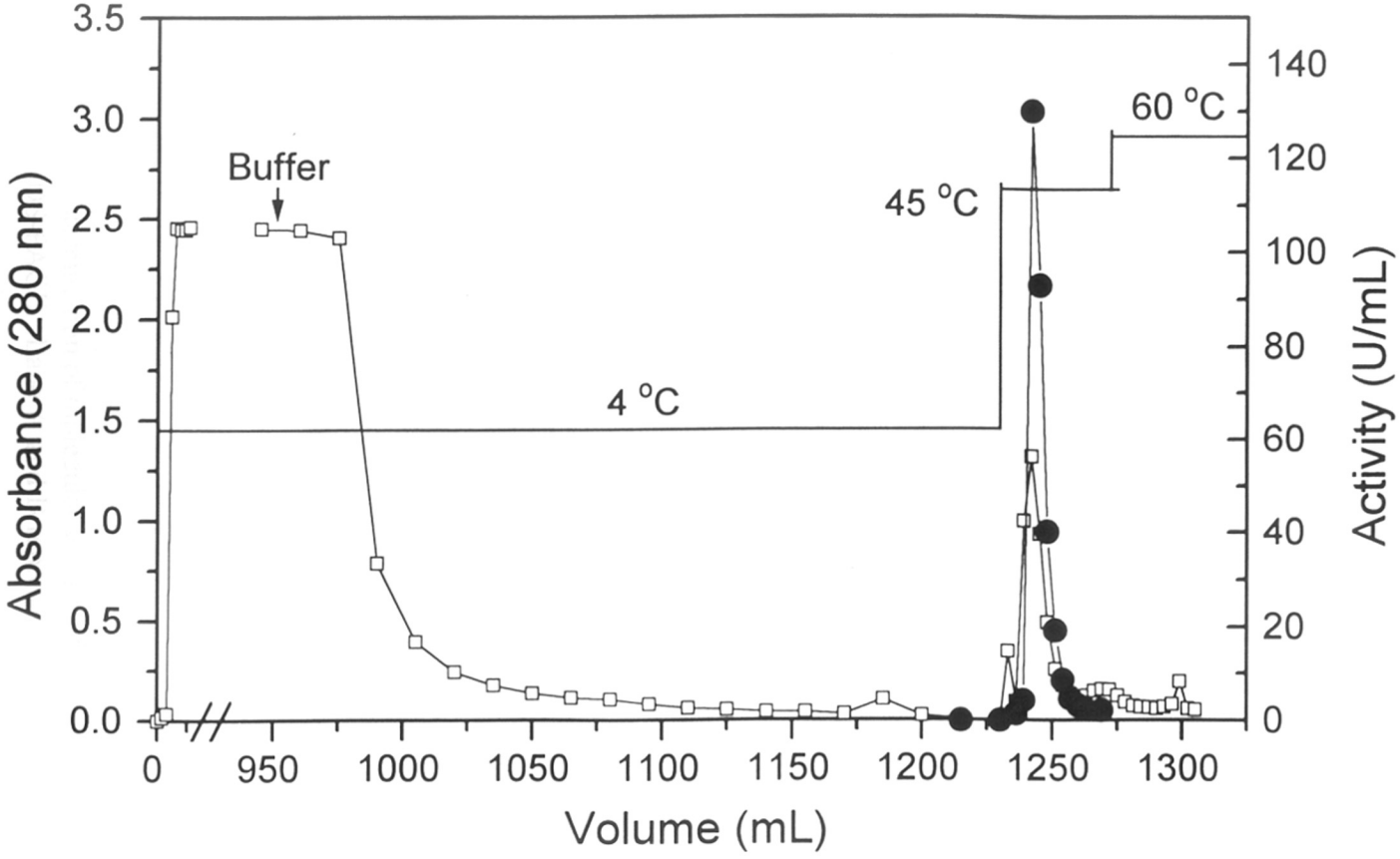

Figure 4.27: Elution profile of CGTase on Starch column .

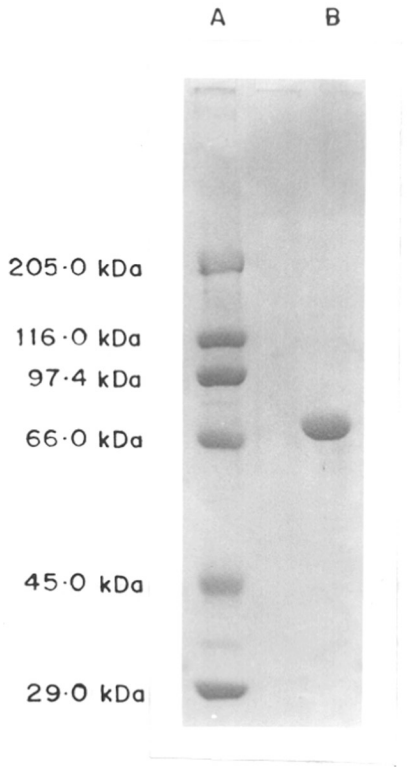

Figure 4.28: Determination of molecular weight of the purified preparation of CGTase by SDS-PAGE. Lane A and B represent standard molecular weight markers and CGTase, respectively.

Daltons. CGTases are monomeric or dimeric proteins with molecular weight ranging from 68,000 to 170,000 (Table 2.3). *Bacillus sp.* 290-3 which also belongs to the *B. firmus/B. lentus* group has CGTase of molecular weight 75,000 (Englbrecht *et al.*, 1990). Other CGTases with similar molecular weights are those from *B. circulans* E 192 (Bovetto *et al.*, 1992a), *B. obhensis* (Sin *et al.*, 1991), *Bacillus sp.* A2-5a (Kometani *et al.*, 1994) and *B. stearothermophilus* (Joong-Hoon *et al.*, 1990).

4.5.2 Effect of pH on activity and stability

The CGTase from *B. firmus* appears to have a wide pH optimum (Figure 4.29). Though the maximum activity was observed at pH 6 and 7.5, the enzyme expressed more than 95% activity between pH 6–8 as determined by HPLC after a 20 min enzyme assay. The activities at pH 9.5 and 10.5 were, respectively, 62.9 and 25.9% of the maximum, thus indicating the alkalotolerant nature of CGTase. The enzyme was not active below pH 4.5 or above pH 10.5. When assayed by PHP method optimum activity was found around pH 6.0. However the results obtained by HPLC must be considered more accurate for reasons mentioned earlier.

The CGTase was stable over a pH range of 7–10 (> 90% activity) when kept at 10 °C for 24 h in different pH buffers (Figure 4.30). It was most stable at pH 9.0 in diethanolamine buffer. At pH 11.0 the CGTase still retained more than 80% of its initial activity. The enzyme lost all its activity below pH 4.0 and above pH 12.0. At higher temperatures the enzyme was unstable in the alkaline pH range. At 60 °C the enzyme was most stable at pH 7.0. The exact reason for this behaviour is not known. However, the narrow stability range at high temperatures may be due to the inherent temperature instability of this CGTase.

Characteristically CGTases have a pH optimum of around 6.0 and they are stable between pH 6-8 (Table 2.3). *B. firmus* CGTase also showed similar behaviour. Other

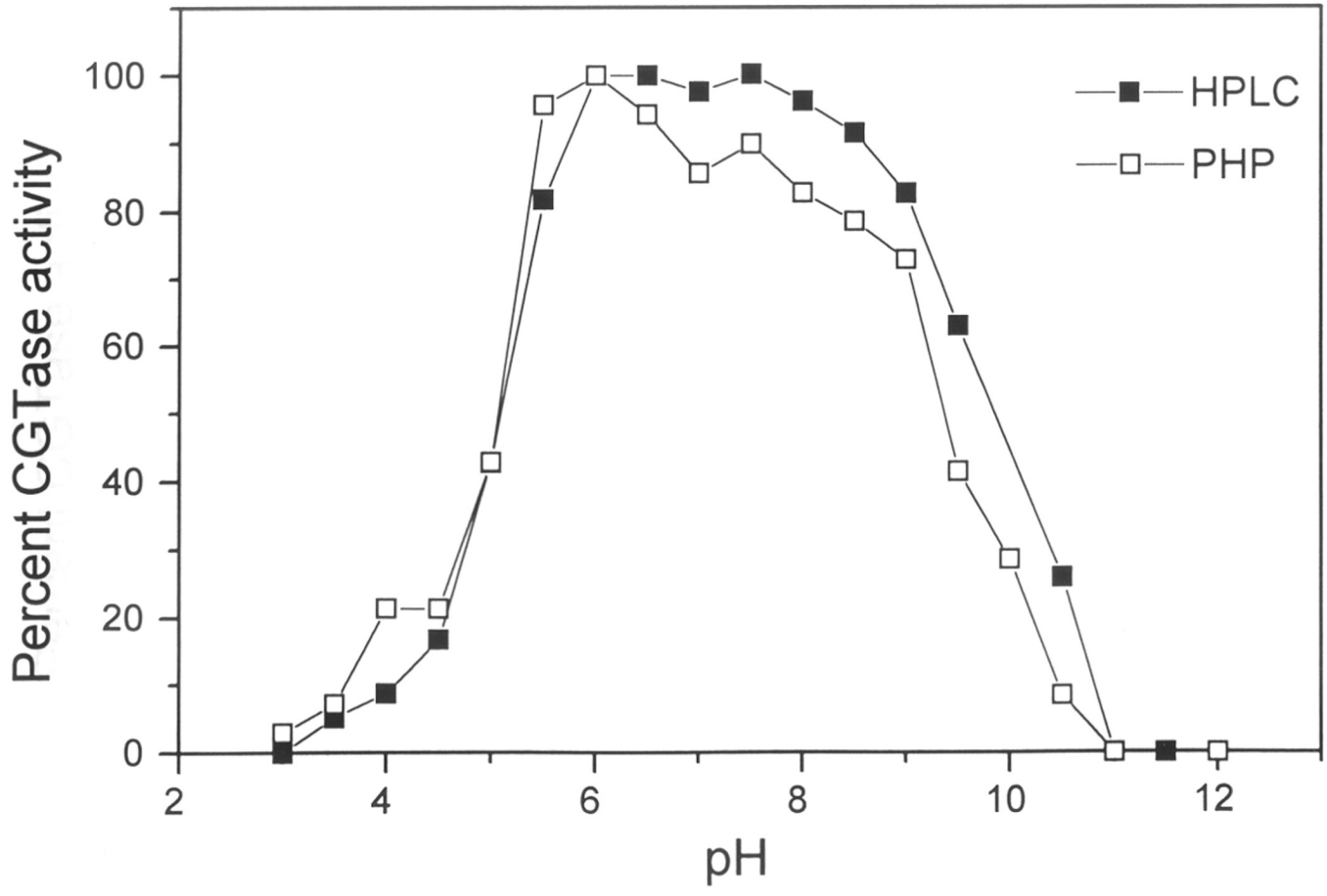

Figure 4.29: Effect of pH on CGTase activity.

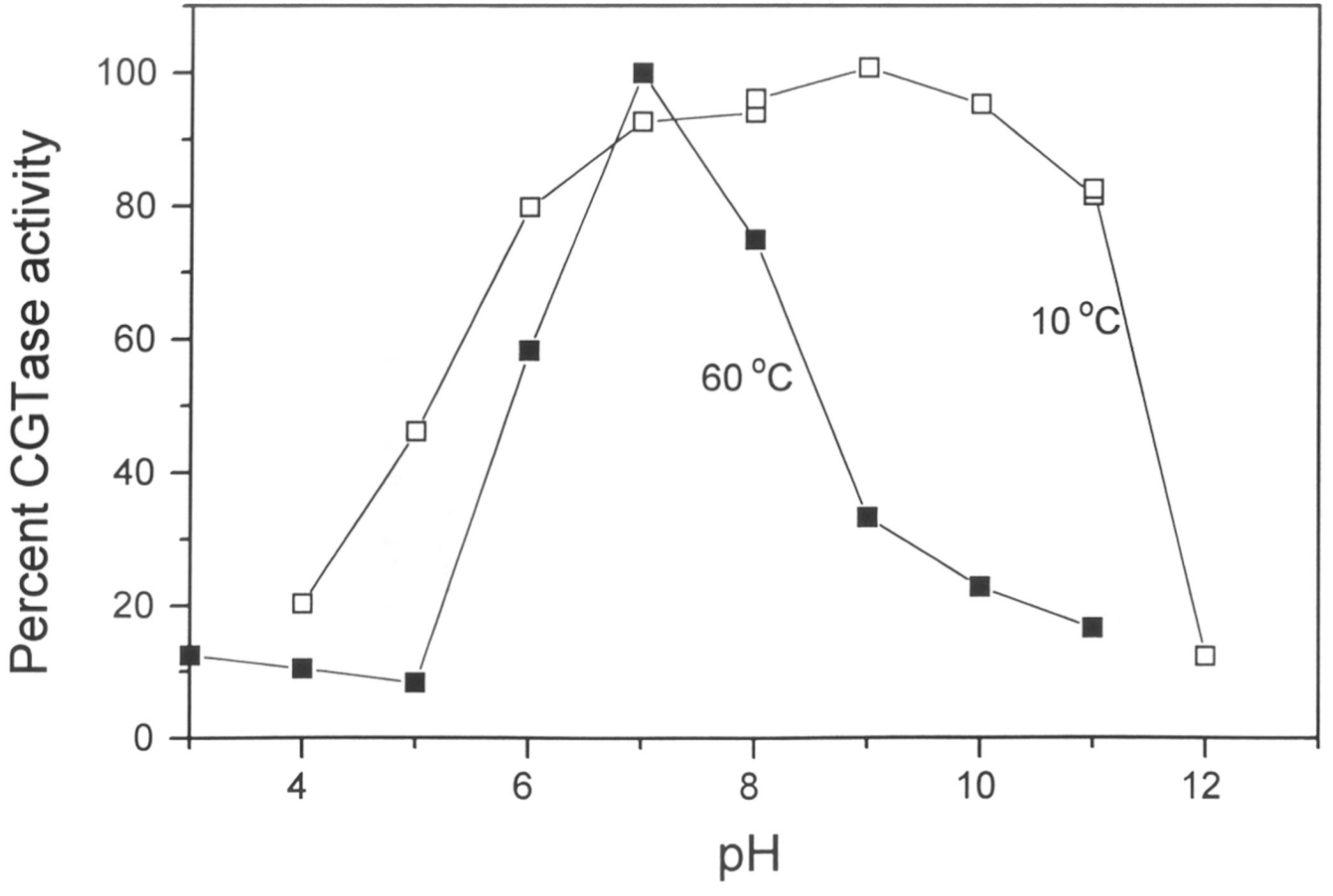

Figure 4.30: Stability of CGTase at different pH (at 10 and 60 °C)

CGTases with pH profiles similar to this CGTase are those from *B. lentus* (Sabioni and Park, 1992), *B. obhensis* (Yagi *et al.*, 1986); *Bacillus sp.* 290-3 (Englbrecht *et al.*, 1990) and *B. stearothermophilus* TC-60 (Kitahata and Okada, 1982a).

4.5.3 Effect of temperature on activity and stability

The optimum temperature for CGTase activity of *B. firmus* using soluble starch was 65 °C (Figure 4.31). At 75 °C, only 48% activity was observed whereas the enzyme was inactivated above 80 °C.

The *B. firmus* CGTase was not thermostable. The enzyme rapidly lost its activity at temperatures above 30 °C on incubation for 20 min (Figure 4.32). Though presence of starch provided protection against temperature denaturation (as observed during starch affinity purification step), calcium ions had no effect on thermostability of this enzyme.

Temperature optima of most CGTases are in range 50-70 °C (Table 2.3). The enzyme from thermophiles such as *Thermoanaerobacter* (Norman and Jorgensen, 1992) and *Thermoanaerobacterium thermosulphurigenes* (Wind *et al.*, 1995) are stable even at 80 °C. Like amylases the stability of CGTases is enhanced in the presence of substrate, product or Ca^{+2} ions (Bovetto *et al.*, 1992a). The CGTase of *B. firmus* is different than other CGTases as Ca^{+2} does not provide any thermostability. *Bacillus sp.* 290-3 CGTase is another CGTase whose activity is unaffected by presence of Ca^{+2} ions (Englbrecht *et al.*, 1990).

It was observed that pure CGTase (from *B. firmus*) could be stored for long time between pH 7–9 at low temperatures. In one of the experiments, the CGTase retained more than 75% of its initial activity on storage in diethanolamine buffer at 0-4 °C for two years.

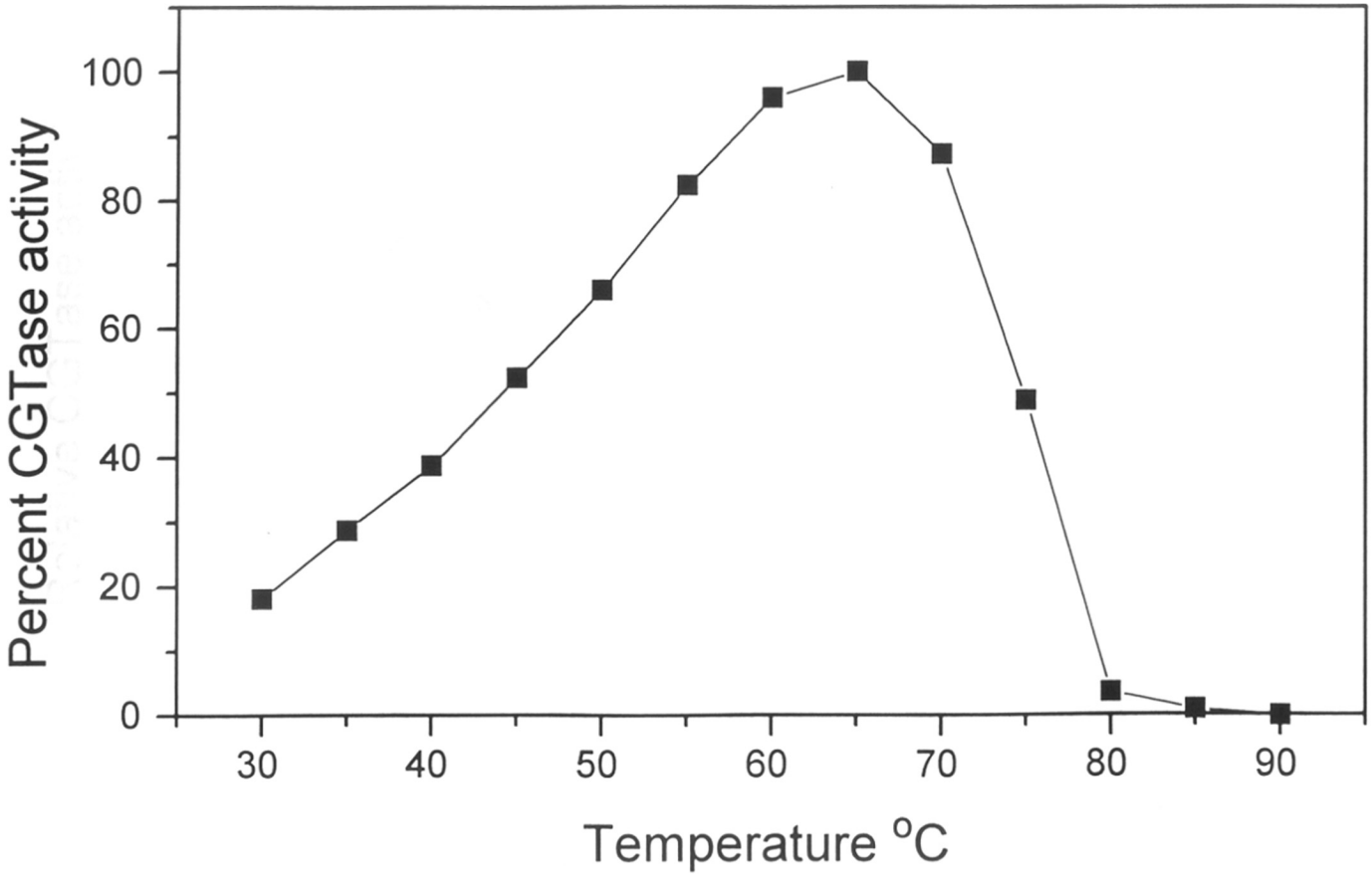

Figure 4.31: Effect of temperature on CGTase activity.

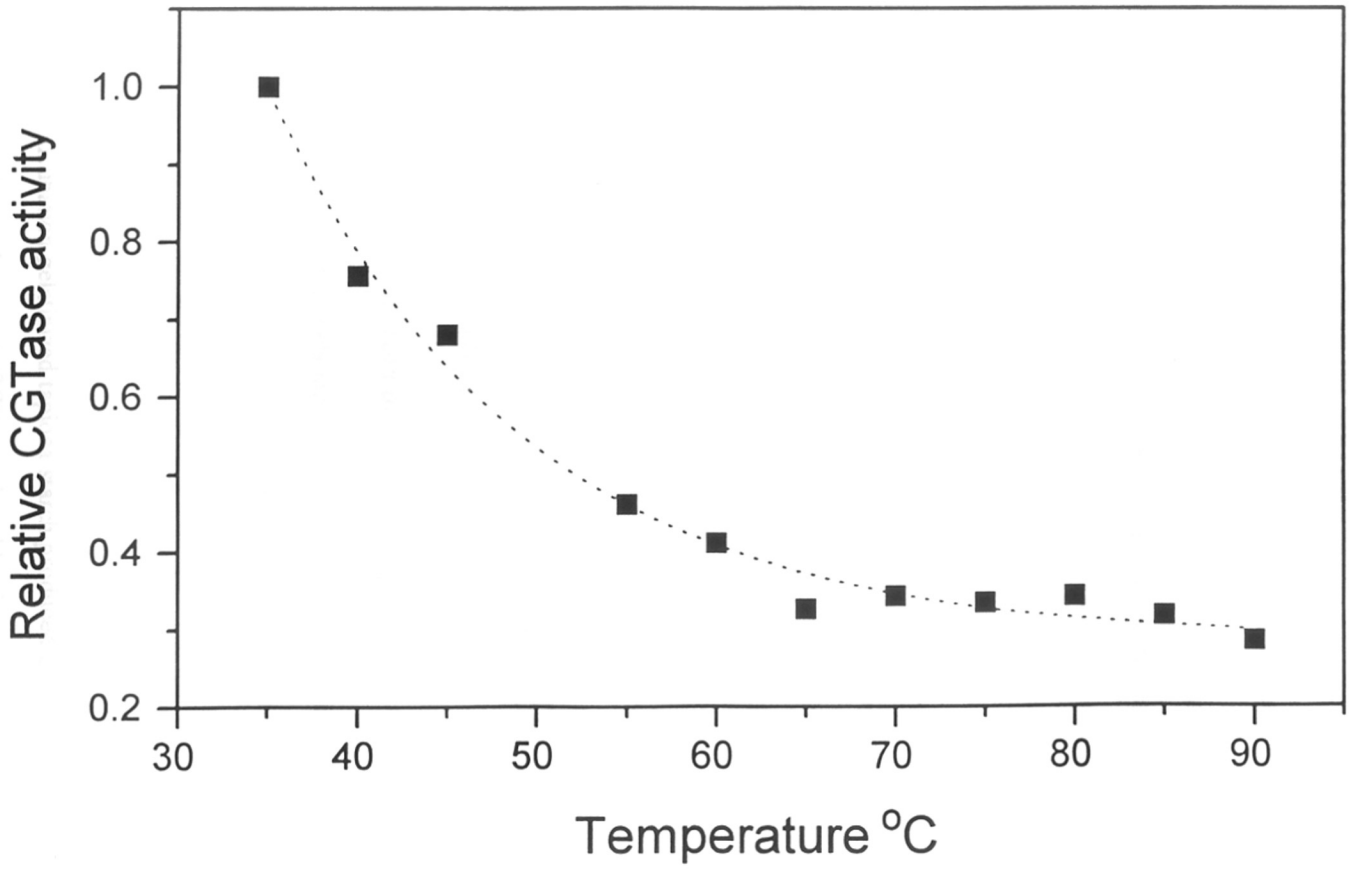

Figure 4.32: Temperature stability of CGTase.

4.6 Action of CGTase on starch

4.6.1 Viscosity of starch solutions treated with CGTase

The viscosity of a 1% solution of gelatinized tapioca starch was measured on addition of different amounts of *B. firmus* CGTase. As shown in figure 4.33 there was a rapid decrease in viscosity of starch solutions. The rate of decrease in viscosity was proportional to added CGTase concentration. With 0.176 U/mL enzyme the viscosity of the starch solution decreased to less than half within one minute of CGTase addition. After 10 min of digestion the final viscosity of the starch solution was about 0.5 cps which was less than 20% of the initial viscosity. It was also observed that the viscosity of control increased from 2.29 cps to 3.04 cps during 10 min of incubation at 60 °C. This increase may be due to concentration of starch solution as a result of evaporation.

Decrease in viscosity of starch solutions is mainly due to rapid breakdown of long chains of amylose/amylopectin via the disproportionation activity of CGTase. When compared with the rate of cyclization activity (Figure 4.34) it is evident that disproportionation reaction is very fast. Thus, in formation of CDs the cyclization and not the disproportionation, is possibly rate limiting.

4.6.2 Action on starch from different sources

The conversion of native, gelatinized and hydrolysed (soluble) starches from different sources, into CDs, was investigated. *Bacillus firmus* CGTase readily acted on raw starch slurry to produce CDs (Figure 4.34). As compared to hydrolyzed potato starch (commercially available as soluble starch DE 8) 26% more CDs were produced from tapioca starch powder. On gelatinization higher yields of CDs were obtained. After 5 hours of incubation gelatinized tapioca starch produced 22% more CDs with respect to raw powder. The total amount of CDs produced by the enzyme from various starches was

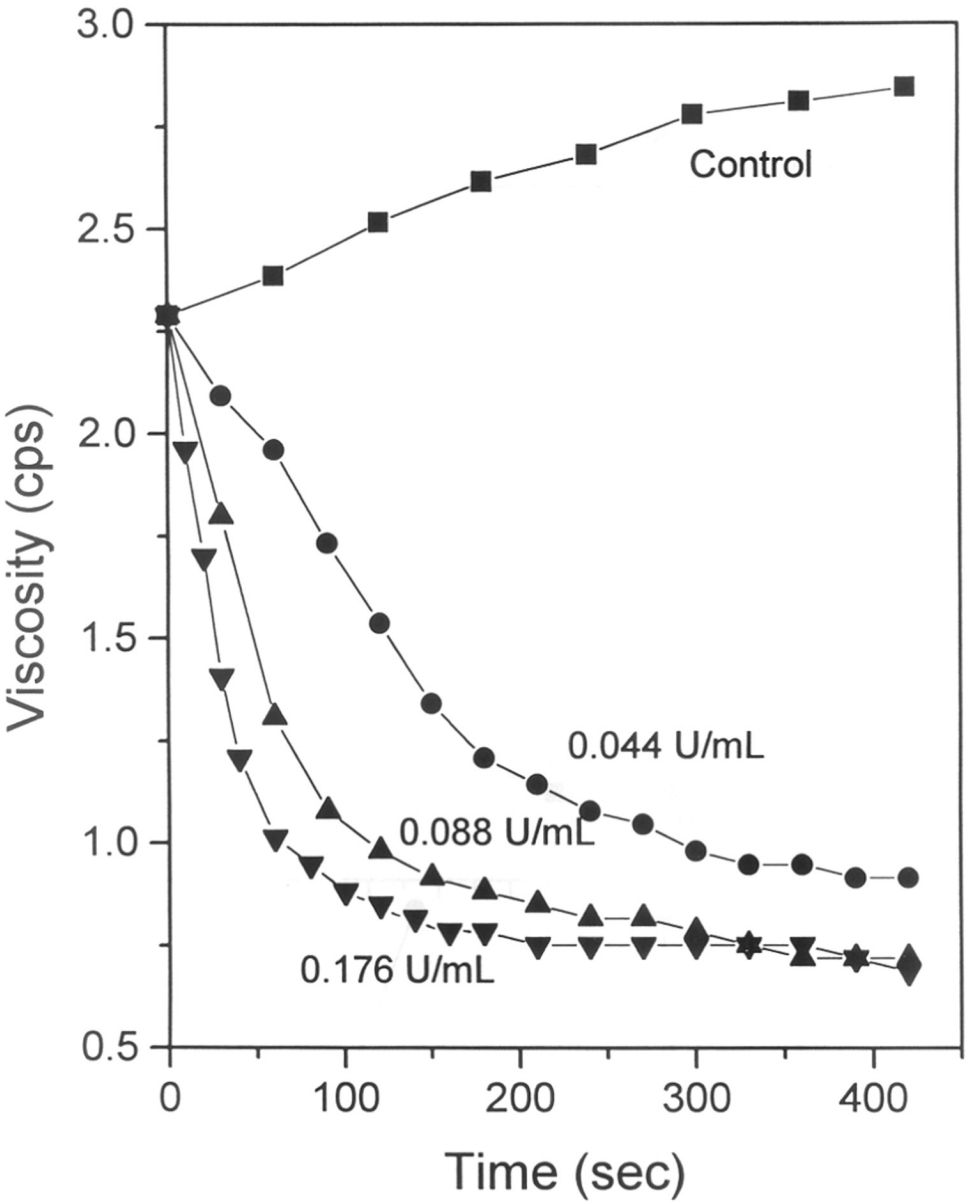

Figure 4.33: Change in viscosity of native starch by action of CGTase.

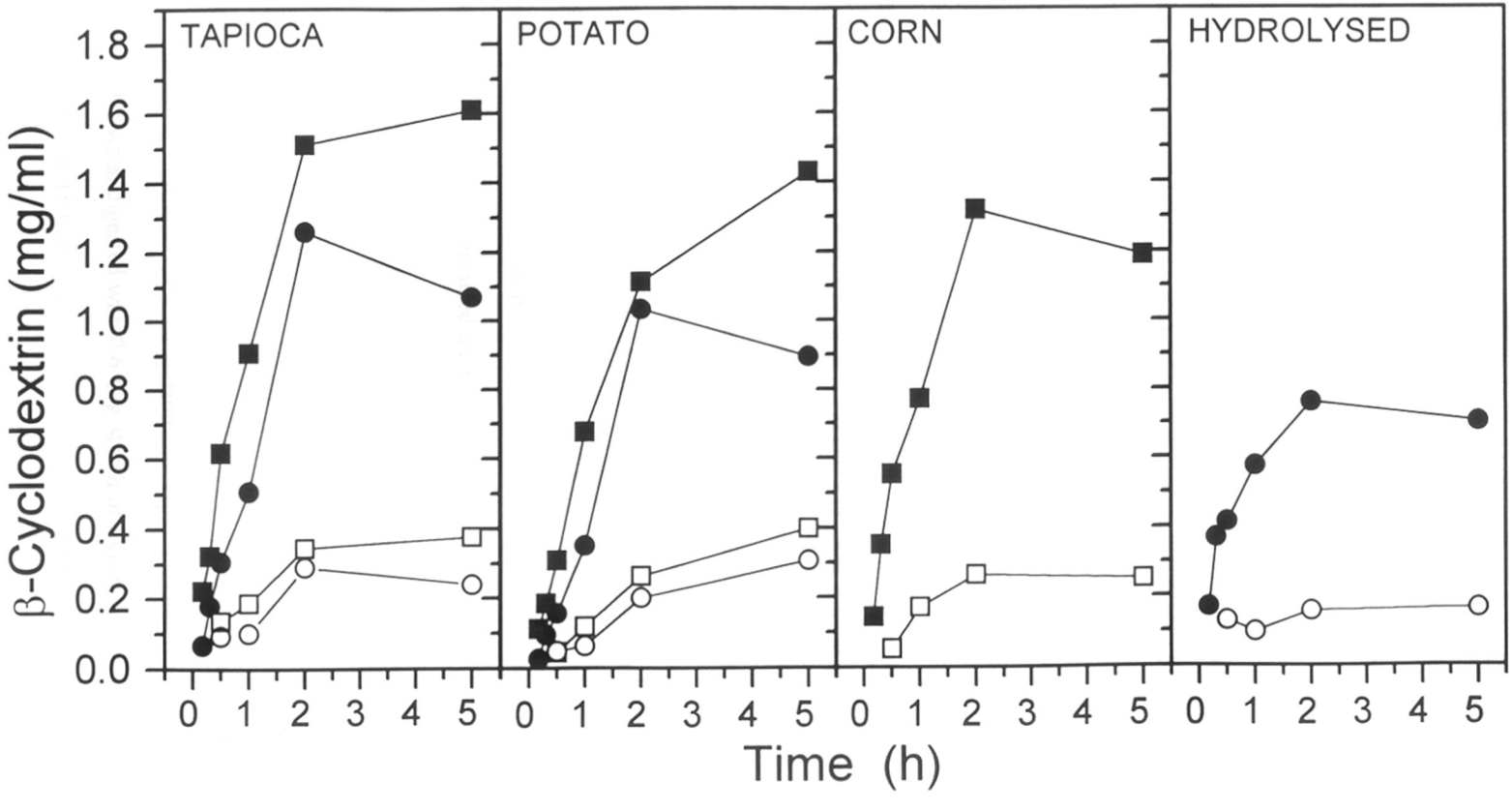

Figure 4.34: Cyclodextrin production from raw (—□—) and gelatinized starch (—○—). Closed symbols represent β-CD and open γ-CD

in the following decreasing order: Tapioca > Potato > Corn. The CGTase could not attack raw corn starch powder although gelatinized corn starch was degraded. The precise reason for this behavior is not known but it can be attributed to the unusual structure of the corn starch granule. Corn starch contains large amounts of lipids, proteins and ash as compared to tuber starches like potato and tapioca (Galliard and Bowler, 1987). When gelatinized, the structure of the starch granule is broken and it may become amenable to enzyme attack.

There are no reports on raw starch degrading CGTase in literature. Lee and Park (1992) reported high yields of CDs from extruded corn starch powder. However raw starch in its native form gave less than 10 % yields when compared with enzyme hydrolyzed (DE 10) or extruded starch. Raw starch degrading amylase from *Bacillus* sp. B1018 which has strong homology with CGTase has been documented to have some CGTase activity (Itkor *et al.*, 1989; Itkor *et al.*, 1990). The CGTase from *B. firmus* however, is completely free of amylase activity (Figure 4.35). Action of CGTase from *B. circulans* E 192 on raw maize starch revealed an unusual behaviour (Villette *et al.*, 1992). There was a slow hydrolysis of the starch granules (glucose and maltose being main products) without the formation of CDs. The hydrolysis was enhanced in presence of acceptors like salicin and glucose. No such hydrolysis was detected with *B. firmus* CGTase and the corn starch remained unutilized.

4.6.3 Cyclodextrin production

Tapioca starch was used for further studies as it was found to be the best substrate. Various concentrations of gelatinized tapioca starch were incubated with excess of CGTase (based on complete conversion of starch to CDs) and the reaction products were analysed on HPLC. About 49% of gelatinized tapioca starch (at 10, 50 g/L) was converted to CDs when digested with 0.6 U/g-starch of CGTase. However the conversion decreased to half with 150 g/L substrate (Figure 4.36). By the end of eight hours more

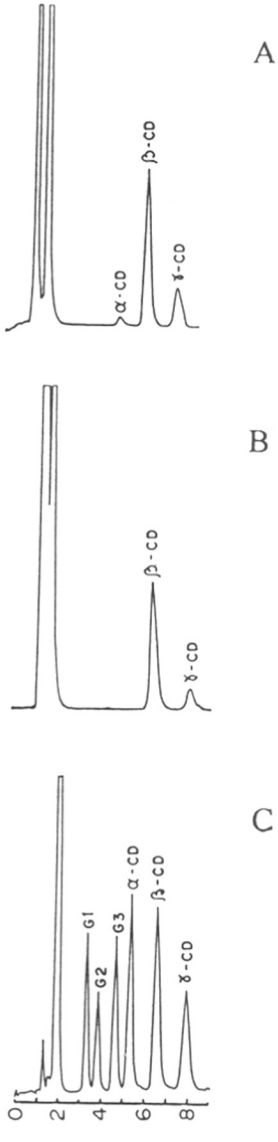

Figure 4.35: Formation of CDs from Tapioca starch slurry (50 g/L) treated with CGTase as determined with HPLC. C: standard B: after 6 h A: at the end of 24 h.

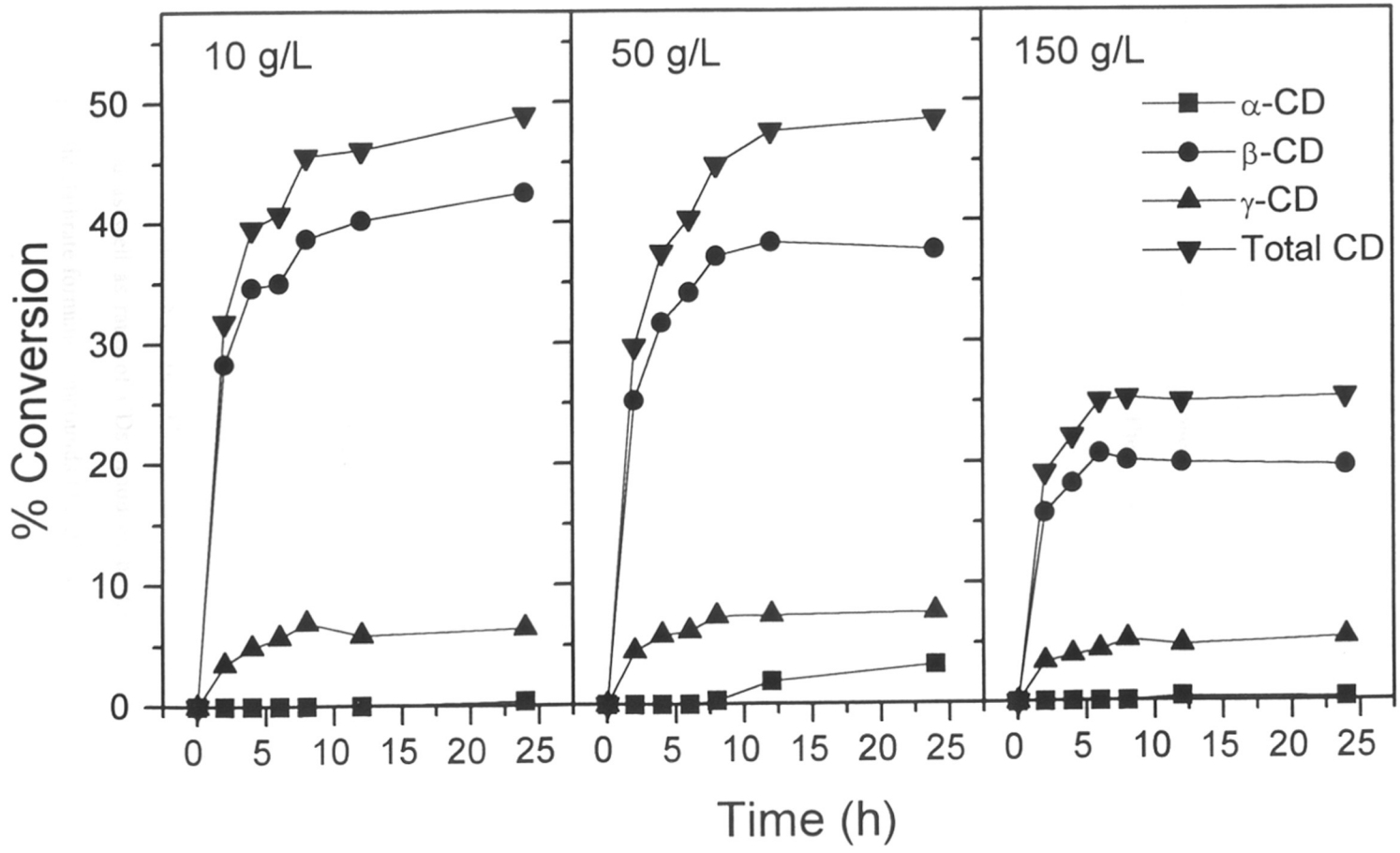

Figure 4.36: Conversion of Tapioca starch to cyclodextrins.

than 90% reaction was over with β and γ-CD as the only reaction products. No linear maltooligosaccharides were detected (Figure 4.35). However, on further incubation small amount of α-CD was also produced. After 24 hours of incubation the ratio of α : β : γ CDs produced from 50 g/L starch was 0.08 : 1.0 : 0.2. The ratio of cyclodextrins varied with the incubation time and the starch concentration. Other workers have recorded similar observations (Sato and Yagi, 1991). These results indicate that production of CDs in a specific ratio is not a unique property of the enzyme but depends on the type of starch, enzyme to substrate ratio, time of reaction and the nature of enzyme itself. Therefore proper optimization of these variables is required for efficient production of CDs.

There are reports of CGTases that exclusively produce γ-CD from starch. Kato and Horikoshi (1986) reported *Bacillus* sp. 313 CGTase that formed γ-CD with 5% yield. The CGTase from *Bacillus* sp. AL-6 produced 8% γ-CD from starch in the initial phase of reaction (Fujita *et al.*, 1990). Another γ-CGTase producing bacterium *Bacillus* sp. 290-3 that belongs to *B. firmus/lentus* group has been isolated (Englbrecht *et al.*, 1990). The CGTase from this bacterium produces equal amounts of β and γ-CDs during the early stage of reaction. However the yields of CD obtained from this CGTase have not been reported. Recently, Mori *et al.* (1994a) isolated a strain of *Brevibacterium* sp. producing large amounts of γ-CD. After 24 h of reaction this CGTase yields upto 20% γ-CD from 1 % starch solution. Though a process to manufacture CDs using this CGTase has been patented by a Japanese company, 'Amano-Pharma' (Mori *et al.*, 1994b), it is not known whether this enzyme is used for commercial production.

Due to low yields, γ-CD is presently manufactured commercially as a side-product of other high CD yielding CGTases (Horikoshi, 1979; Yagi *et al.*, 1986; Sin *et al.*, 1994). Moreover the yield as well as ratio of CDs produced by CGTase can be modified by addition of specific clathrate forming compounds like ethanol (Shiraishi *et al.*, 1989, Fujita

et al., 1990), glycyrrhizic acid (Sato and Yagi, 1991), C_{12} cyclic compounds (Rendleman, 1992) etc. Therefore the choice of suitable CGTases in industry is based on high yields of CDs. *B. firmus* CGTase produces γ-CD comparable to industrially used strains with overall yields of β and γ-CD being 40% and 8% respectively. Economical production of CDs requires a minimum number of unit operations with high yields. The CGTase produced by *B. firmus* reported here produces CDs from raw starch, hence it has potential for industrial exploitation.

4.6.4 Structure of CGTase treated starch granules

A suspension of raw tapioca starch was treated with *B. firmus* CGTase and the starch granules were examined under SEM after gold deposition. Native starch granules have uneven globular structure with an approximate size of 5-15 μ (Figure 4.37A). On treatment with CGTase the granules split open (Figure 4.38 A & B) and later disintegrated on further incubation. In control experiment small amount of swelling (10-15 %) of tapioca granules was observed (Figure 4.37B). Potato starch granules also gave similar results.

Effect of CGTase on microscopic changes of intact starch granules have not been studied. Lee and Park (1992) investigated CGTase action on extruded corn starch using SEM. On extrusion the starch granules expanded around 3-5 times. When subjected to CGTase, these granules were gradually fragmented and finally dissappeared. These workers suggested that extruded swollen starch generated a large amount of accessible surface area for CGTase action that increased CGTase yields. Extensive work has been done on amylase mediated hydrolysis of starch (reviewed by Galliard and Bowler, 1987). Amylases from different sources attack starches in different manner. Alpha amylase hydrolyses wheat starch granules by eroding them all over the surface (Evers and McDermott, 1970). However, it preferentially attacks the starch inside the longitudinal grooves that run along the starch granule. Glucoamylase from *Aspergillus niger* also

behaves in a similar manner (Evers *et al.*, 1971). Cereal α-amylases act by penetration at local points of the granule surface thereby forming 'pits' followed by an 'inside-to-outside' digestion within the granule (Sargent, 1980). Thus it appears that the enzyme attack on starch granules is localized (and initiated) on the physical defects present in these granules. If the granules are deliberately broken mechanically, enzymatic hydrolysis is faster. Action of *B. firmus* CGTase also appears to be of similar nature. At the first instance local hydrolysis takes place which opens up the granule and later the fragments are further hydrolysed to produce cyclic and/or linear oligosaccharides.

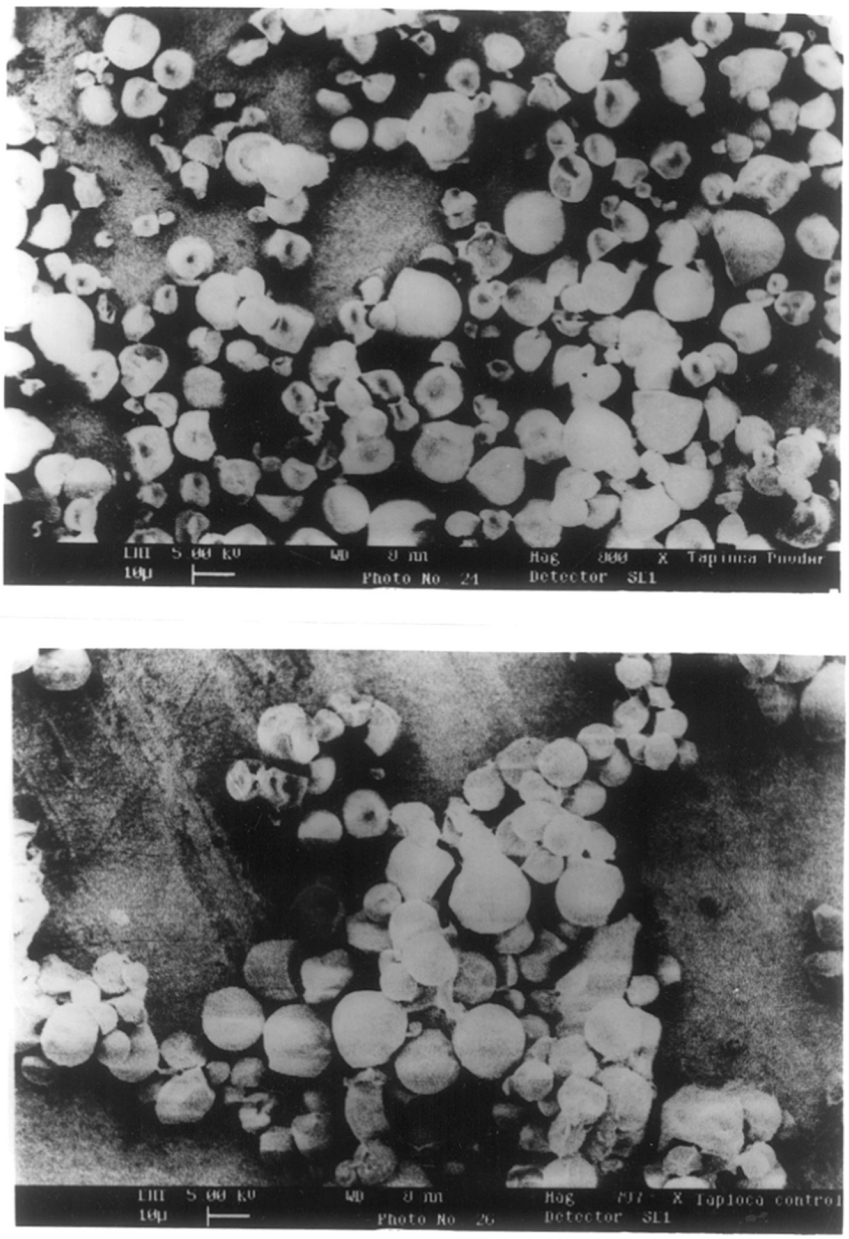

Figure 4.37: Ultrastructure of tapioca starch granules as seen by scanning electron microscopy. A: Native starch granules. B: Untreated starch granules in buffer – control experiment.

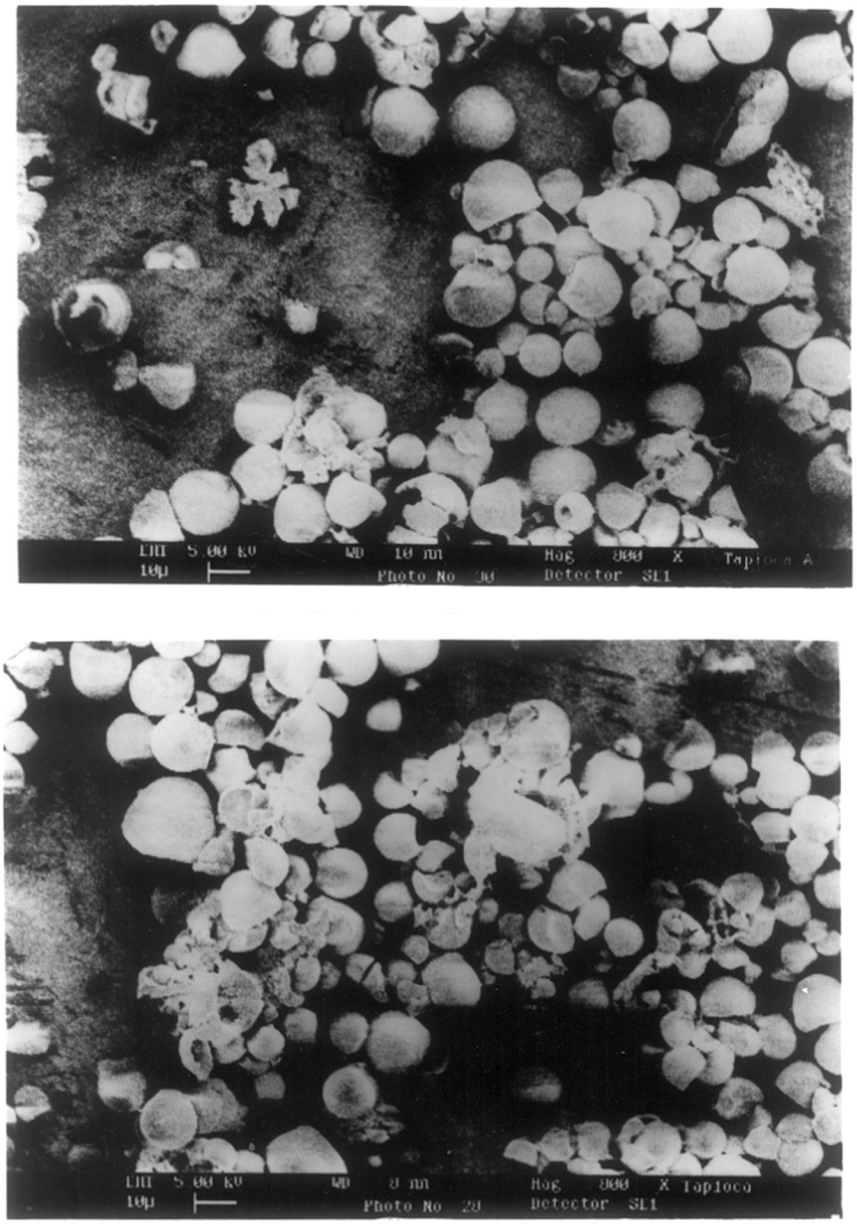

Figure 4.38: Ultrastructure of tapioca starch granules treated with CGTase as seen by scanning electron microscopy.

5. Conclusions

An alkalotolerant bacterium, identified as *Bacillus firmus*, that produced large amounts of the enzyme, cyclodextrin glycosyl transferase (CGTase), was isolated from soil. This enzyme degrades raw starch to produce cyclodextrins (CDs) and is free of any amylase activity. Based on specificity and accuracy of estimation a modified phenolphthalein based spectrophotometric assay and an HPLC-based assay were selected for routine work.

B. firmus is a mesophilic organism that grows between temperatures 28-40 ^{o}C. It grows well between pH 7-10.5. No growth takes place below pH 7 - a characteristic used to identify the *Bacillus* to the species level. Cell growth and CGTase activity was similar in media buffered with 1% Na_2CO_3 or that with pH controlled at 9.0. CGTase production was induced by starch and repressed by simple sugars like glucose. Some enzyme (about 10%) was produced constitutively.

Detailed cell growth studies were done in shake flasks as well as fermenters. In a complex medium containing starch, peptone and yeast extract, higher cell mass as well as CGTase activity were obtained in fermenter runs. Lower cell mass in shake flasks could be attributed to inherent oxygen limitations. The pattern of CGTase production differed in shake-flask and fermenter. While in shake flask the CGTase production was growth-associated, in fermenter the enzyme was produced in late log phase. This difference in enzyme production was linked to lower specific growth rate in shake flask. In fermenter, at lower agitation, the cell growth was severely limited by oxygen availability. The maximum specific growth rate dropped from 0.44 h^{-1} at 600 rpm to 0.34 h^{-1} at 400 rpm. Concomitantly specific CGTase productivity also decreased by 33% at 400 rpm. Under favourable conditions maximum CGTase activity obtained was $\approx$ 3 U/mL in batch culture. Both the total and reducing sugars decreased with time during the cell growth. It was postulated that the utilization of starch by *B. firmus* probably occured by uptake of mono or disaccharides produced by CGTase-mediated inter- and intra-molecular

transglycosylation. The formation of these sugars was promoted by their utilization by the bacterium.

Vent gas analysis presented problems due to dissolution of CO_2 in the alkaline medium. Stoichiometric analysis revealed that 55% of the carbon utilized was incorporated in the biomass. The overall equation representing the aerobic growth of *B. firmus* under the conditions of study was:

$$10.05\ CH_2O + 10\ CH_{1.96}O_{0.75}N_{0.25} + 7.04\ O_2 =$$

$$6.31\ CH_{1.78}O_{0.99}N_{0.16} + 8.69\ CH_{1.68}O_{0.96}N_{0.16} + 5.05\ CO_2 + 6.93\ H_2O + \Delta G$$

Continuous culture studies revealed inhibition of CGTase synthesis by unutilized starch in the medium. Lower dilution rates resulted in higher enzyme activity as well as cell growth. In further experiments, it was found that CGTase production was dependent on the cell density in the reactor. Thus in shake flask experiments with cell supplementation as well as in membrane recycle bioreactor studies, higher cell mass gave more CGTase activity. High CGTase volumetric productivity was obtained in the cell recycle bioreactor (CRB) as compared to that in batch and continuous cultures. Relatively low enzyme yields, obtained in the CRB, were thought to be due to incomplete permeation of enzyme through the microfiltration membrane or to the sub-optimal starch concentrations in the reactor. The importance of starch concentration for high CGTase yields was confirmed in a separate shake-flask study.

CGTase was purified in a two-step procedure consisting of an initial starch affinity binding step followed by ion-exchange chromatography. The enzyme was purified about 100-fold with 70% recovery. The capacity of starch to adsorb CGTase was found to be dependent on the type of starch and the surface area available for adsorption. The purified enzyme had a specific activity of 35 U/mg protein.

CGTase had a molecular weight of 78,000 Da as determined by SDS-PAGE. It had a wide pH optimum of 6-8 and was stable over a pH range of 7-10, retaining > 90% activity. At a higher temperature (60 °C) it was most stable at pH 7.0. The enzyme showed maximum activity at 65 °C. Stability studies (20 min incubations) revealed that the enzyme was unstable at temperatures above 30 °C.

The CGTase rapidly decreased the viscosity of gelatinized starch. Within 10 min the viscosity of starch fell to less than 20% of the original. This indicated that the cyclization, and not the disproportionation, activity of the CGTase was rate-limiting. The production of CDs was found to be dependent on the source and nature of starch, its initial concentration and the reaction time. For the production of CDs, tapioca starch was the best substrate for this CGTase. With a concentration of 10 and 50 g/L gelatinized starch, 49% conversion to CDs was obtained. On increasing the starch concentration to 150 g/L the conversion decreased to 25%. Scanning electron microscopy revealed the mode of splitting open of starch granules on treatment with CGTase.

The CGTase produced large amounts of γ-CD comparable to industrially used enzymes reported in literature. It was concluded that this novel raw starch degrading enzyme from an alkalotolerant bacterium has great potential for the production of β- and γ-cyclodextrins.

6. Epilogue

Though the research on cyclodextrins was initiated more than 100 years back (Villiers, 1891), most of the progress has been made during the eighties. The work of Pringheim, Freudenberg, Cramer, French and Bender (M) generated copious knowledge by the sixties. However, the price of CDs fell down only in mid-eighties when a number of bacteria producing CGTase were isolated and the production of CDs was realized on a large scale (Bender, 1992). The prices of β-CD crashed from $ 2000/kg down to about $ 40/kg (Figure 6.1, data courtesy Dr. J. Szejtli, personal communication). The other two CDs viz. α and γ-CDs are also now available, though rather expensive, at $ 50/kg and $ 300/kg respectively ($ 200/kg and $ 1200/kg in 1990). In the second phase of research, the CGTase was cloned and produced in large amounts (Schmid, 1989). The demand and the market for CDs has truly been developed by conscious research and development. For the profitable marketing and end use of CDs the price of CDs must go further down to about $ 5/kg (Szejtli, 1992). For this to happen, the consumption of CDs must increase and secondly efficient processes for manufacture of CDs must be developed. In the process for economical CD production, particularly α and γ-CDs, their separation from the reaction mixture is the main bottleneck. This is due to the fact that these CDs are highly soluble in aqueous medium and separating them from linear oligosaccharides is tedious. Therefore an effort is being made by workers all over the world to isolate novel CGTases that produce a large amount of a particular CD with minimum non-cyclic products.

The CGTase from *B. firmus* as mentioned in the present report degrades raw starch to produce CDs thus eliminating the need for pre-hydrolysis of starch by external addition of amylases. Moreover, no maltooligosaccharides have been detected in the reaction products of this CGTase. These characters are highly desirable for the eventual use of CGTase in CD production. Though two CGTase producing bacteria have been isolated recently which produce large amounts of γ-CD (Fujita *et al.*, 1990 and Mori *et al.*, 1994a),

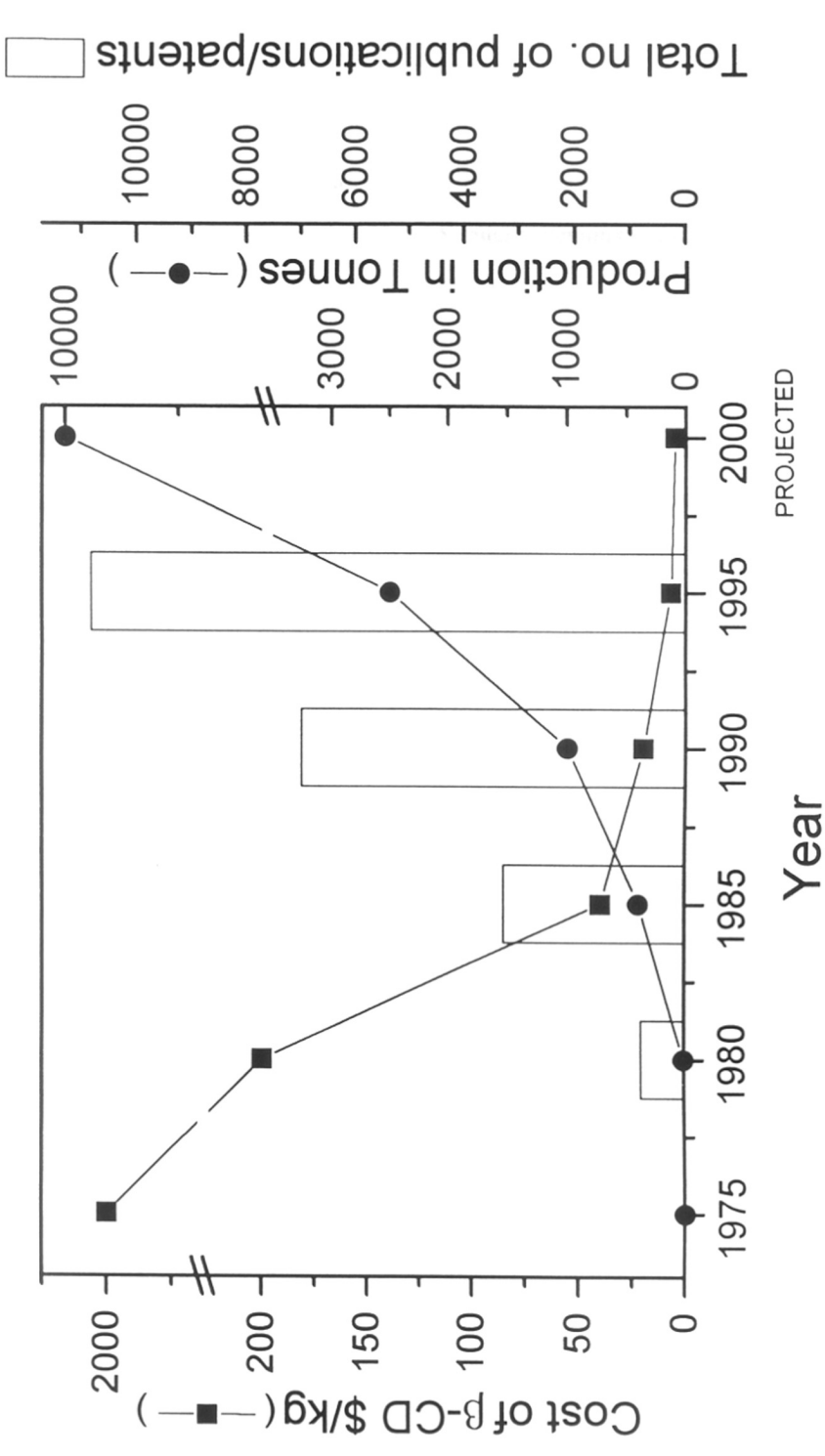

Figure 6.1: Scenario for cyclodextrin research, development and market.

starch digests of these enzymes still contain other CDs. Another approach for obtaining better CGTases has been to 'engineer' them (Tao, 1991) to produce the desired products. Limited success has been achieved in this direction. Replacement of a single amino acid in CGTase of *B. obhensis* by site-directed mutagenesis has been reported to double the production of γ-CD (Sin *et al.*, 1994). Similarly Penninga *et al.* (1995) have been able to derive the mutants of a *B. circulans* whose CGTase gave altered CD ratios of starch digests and caused a shift in reaction products from linear to cyclic ones. Protein engineering seems to hold a promise in future to give tailor-made CGTases with enhanced stability, altered substrate specificity and novel catalytic capabilities. Efforts are also being made in our laboratory to clone and modify the CGTase of *B. firmus*.

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8. APPENDIX

APPENDIX I

List of cultures tested of CGTase production:

National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune	
Bacillus circulans	NCIM 2107
	NCIM 2160
	NCIM 2161
Bacillus macerans	NCIM 2131
	NCIM 2186
Plant Biotechnology Institute, Canada	
Bacillus macerans	B-61
	B-68
	B-96
Bacillus circulans	B-30
	B-132
ETH-Zentrum, Zurich	
Bacillus macerans	B-4036
Division of Biological Sciences, NRC, Canada	
Bacillus macerans	NRC-5563
	NRC-9073
	NRC-9074
	NRC-9075
Bacillus circulans	NRC-9022
	NRC-9023
	NRC-9090
Department of Environmental Biology, Guelph, Ontario	
Bacillus circulans	360
	513

All Union Collection of Microorganisms, USSR

Bacillus macerans	B-506
	B-697
	B-698
Bacillus circulans	B-692
	B-729
	B-1242

APPENDIX II

Composition of cocktail buffer :

Citric acid	1.92 g
NaH_2PO_4	1.77 g
Tris	1.21 g
Glycine	0.75 g
Distilled water to	500 ml

Adjust pH with 0.2N NaOH and the buffer is ready.

Citrate-phosphate buffer:

Citric acid (0.2 M) and $NaHPO_4.2H_2O$ (0.4 M). Add in following proportions and make up the volume to 100 mL with distilled water.

pH	Citric acid (mL)	$NaHPO_4.2H_2O$ (mL)
3	39.8	10.2
4	30.7	19.3
5	24.3	25.7
6	17.9	32.1
7	6.5	43.6
8	titrate	49

Diethanolamine-HCl buffer:

Add HCl to 50 mL of 0.4 M diethanolamine and titrate till desired pH is obtained (pH range 8-11). Make volume to 100 mL with distilled water.

Sodium phosphate (dibasic)-sodium hydroxide buffer:

Add NaOH to 50 mL of 0.5 M sodium phosphate and titrate till desired pH is obtained (pH range 11-12). Make volume to 100 mL with distilled water.