Cyclodextrin glucanotransferases convert starch into cyclic α-1,4-glucans, called cyclodextrins (CDs). Cyclodextrins were identified in 1891 and structurally characterized in the preceding years. The main products of CGTases are α-, β-, and γ-CDs, composed of 6, 7, or 8 glucose residues, although, much larger cyclic glucans are produced in the early phases of the reaction (Tereda et al., 1997; Zheng et al., 2002; Qi et al., 2007). In nature, certain bacteria and archaea presumably excrete CGTases to monopolize on the starch substrate, converting it into CDs, which cannot be utilized by competing microorganisms (Pajatsch et al., 1999; Hashimoto et al., 2001).

**Starch as a Substrate for CGTase Production**

Starch is one of the abundant polymers found on earth, and serves as the main source for most living organisms. It is a major component of crops and an important raw material for food industrial processes (Swinkels, 1985). Starch serve as a kind of energy storage for plant and is mostly found in seeds and roots in the form of granules. Starch is a complex carbohydrate, \((C_6H_{10}O_5)_x\), consisting of two types of glucan polymers; amylose and amylopectin, where \(x\) ranges from 50 to thousands. These glucose units are linked together by α-(1,4) or α-(1,6) glycosidic bonds to form one huge molecule starch (Hasnah et al., 2000).

In general, common starches comprise of 15%-25% of amylose and 75%-85% of amylopectin (Kennedy et al., 1987). These two fractions occur in different proportions in starches from different sources and contribute to their different physical and chemical properties. In a case of special breed, or waxy starches no amylose or a very low amount of amylose is present (Tegge et al., 1984). At the other extreme, amylopectin potato that has been genetically modified contains 90-98% of amylopectin (Szejtli, 1998).
Naturally, starch can be degraded or hydrolyzed to smaller molecules by acids or enzymes excreted by microorganisms that utilize starch as carbon source. Starch degradation is a complex process; necessitates a set of enzyme, commonly named as starch hydrolazes. One of the enzyme groups involved in starch degradation is called \( \alpha \)-amyrase family or known as glycosyl hydrolaze family (Park et al., 2000). Glucoamylase, isoamylase, \( \beta \)-amylase, pullulanase, \( \alpha \)-glucosidase, cyclodextrin glucanotranferase and fungal \( \alpha \)-amylase belong to this group. These enzymes are further divided into endo-acting and exo-acting enzymes. Endo-acting enzyme hydrolyzes bonds randomly at the inner part of starch or \( \alpha-(1,6) \) bonds and produces linear and branched oligosaccharides with various sizes. \( \alpha \)-amyrase belongs to the endo-acting group and is widely distributed in most living organisms. Whereas, glucoamylase, isoamylase, \( \beta \)-amylase, pullulanase, \( \alpha \)-glucosidase, and cyclodextrin glucanotranferase (CGTase) are the exo-acting enzymes. Exo-acting enzymes cleave at \( \alpha-(1,4) \) bonds at the non-reducing end in starch molecule and produce only low molecular weight products mainly glucose and maltose.

**CGTase Production**

CGTase is produced by *Thermoanerobacterium* (Norman and Jorgensen, 1992) and some species of *Bacillus*, such as *Bacillus megaterium* (Kitahata et al., 1974), *Bacillus macerans* (Stavn and Granum, 1991; Steighardt and Klein, 1993), *Bacillus stearothermophilus* (Ahn et al., 1990), *Bacillus klebsiella* (Gawande and Patkar, 1999), *Bacillus firmus* (Goel and Nene, 1995) and *Bacillus lentus* (Sabioni and Park, 1992). The CGTases from most microorganisms are extracellular enzymes (Preist, 1977). CGTases from different organisms were given in table 2.1.
Table 2.1. Comparison of CGTases from various microorganisms

<table>
<thead>
<tr>
<th>Origin</th>
<th>Weight (Da)</th>
<th>Optimum Temp (°C)</th>
<th>Optimum pH</th>
<th>Major Product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paenibacillus</em> sp. F8</td>
<td>-</td>
<td>50</td>
<td>7.5</td>
<td>β</td>
<td>Yagi et al., 1980</td>
</tr>
<tr>
<td><em>Brevibacterium</em> sp. no 9605</td>
<td>-</td>
<td>45</td>
<td>8.0-9.0</td>
<td>γ</td>
<td>Mori et al., 1994a; Mori et al., 1994b</td>
</tr>
<tr>
<td><em>Thermoanaerobacter</em> sp. ATCC 53627</td>
<td>75,291</td>
<td>90-95</td>
<td>5.8</td>
<td>B</td>
<td>Norman and Jorgensen, 1992; Starnes et al., 1996; Starnes et al., 1992</td>
</tr>
<tr>
<td><em>Thermoanaerobacterium</em> thermosulfurigenes EMI</td>
<td>75,100</td>
<td>80-85</td>
<td>4.5-7.0</td>
<td>β/α</td>
<td>Wind et al., 1995</td>
</tr>
<tr>
<td><em>Micrococcus varians</em> M 849</td>
<td>-</td>
<td>55-65</td>
<td>5.0-8.0</td>
<td>α/β</td>
<td>Yagi et al., 1980</td>
</tr>
<tr>
<td><em>Bacillus circulans</em> E 192</td>
<td>-</td>
<td>60</td>
<td>5.5-5.8</td>
<td>β</td>
<td>Bovetto et al., 1992</td>
</tr>
<tr>
<td><em>Bacillus macerans</em> IFO 3490</td>
<td>74,008</td>
<td>55</td>
<td>5.2-5.7</td>
<td>α</td>
<td>Kitahata and Okada, 1982a; Kitahata and Okada, 1982b; Fujiwara et al., 1992</td>
</tr>
<tr>
<td>Alkalophilic <em>Bacillus</em> sp. 38-2 Neutral CGTase</td>
<td>-</td>
<td>50</td>
<td>7.0</td>
<td>β</td>
<td>Nakamura and Horikoshi, 1976a; Horikoshi, 1988</td>
</tr>
<tr>
<td>Alkalophilic <em>Bacillus</em> sp. 38-2 Acidic CGTase</td>
<td>-</td>
<td>45</td>
<td>4.5-4.7</td>
<td>β</td>
<td>Nakamura and Horikoshi, 1976b; Horikoshi, 1988</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em> M5A1</td>
<td>69,029</td>
<td>-</td>
<td>6.0-7.5</td>
<td>α/β</td>
<td>Bender, 1977; Bender, 1983; Bender et al., 1986</td>
</tr>
</tbody>
</table>

CGTase Isolation

Cyclodextrin glucanotransferases are members of the largest family of glycoside hydrolases acting on starch and related α-glucans (Stam et al., 2006). The first 3D structure of this enzyme (Klein and Schulz, 1991) revealed that CGTases are five domain proteins with the active site located at the bottom of a (β/α) 8-barrel in the A domain. Substrates bind across the enzyme’s surface in a long groove formed by the domains A and B that can accommodate at least 7 glucose residues at the donor subsites and 3 at the acceptor subsites as revealed by kinetic studies and crystal
structures of substrate/inhibitor/product-CGTase complexes (Bender, 1990; Kanai et al., 2001; Leemhuis et al., 2003a; Wind et al., 1998; Schmidt et al., 1998; Uitdehaag et al., 1999).

The C-terminal region of CGTases is formed by C, D, and E-domains. The function of domain D is unknown, domain C has been implied in substrate binding ((Penninga et al., 1996) and domain E is a raw starchbinding domain (Dalmia et al., 1995; Chang et al., 1998). The E-domain is classified as a family 20 carbohydrate binding module (CBM20) (Cantarel et al., 2009; Machovic and Janecek, 2006).

Cyclodextrin glucanotransferases can also transfer the glycosyl-intermediate to a second α-glucan yielding a linear product (disproportionation) or to water (hydrolysis). In addition, CGTase can degrade CDs by opening the CD ring and transferring the linearalized CD to a sugar acceptor to yield a linear oligosaccharide (coupling). The large amount of structural information together with site-directed mutagenesis data have been used to elucidate the mechanistic functions of the residues at the catalytic center of CGTases (Nakamura et al., 1993; Leemhuis et al., 2003b; Klein et al., 1992; Haga et al., 2003).

Nikolina et al., 2009 produced cyclodextrin gluconotransferase using cells of obliged alkaliphiles Bacillus pseudocaliphilus 20 RF and Bacillus pseudocaliphilus 8SB from Bulgaria habitats by three different techniques; on two types of poly sulfone membranes; entrapped in agar gel beds containing magnetite and by nano particles of silanized magnetite covalently bound on the cell surface.

Gawande and Patkar in 2001 produced a novel raw starch degrading cyclodextrin glycosyl transferase from Klebsiella pneumoniae AS-22 and purified by ultra filtration, affinity and gel filtration chromatography. The specific cyclization activity of the pure enzyme preparation was 523 units/ mg of protein. The molecular
weight of the pure protein was estimated to be 75kDa with SDS-PAGE and gel filtration. The isoelectric point of the pure enzyme was 7.3.

A novel thermophilic anaerobic, rod shaped, non spore forming, gram positive bacterium was isolated from an oil field in Turkey by Ayse Avci and Sedat Donmez in 2009 that produces cyclodextrin glycosyl transferase from starch. According to the some morphological, biochemical and 16S rRNA analysis, the strain belongs to the genus *Thermoanaerobacter*. The strain has CGTase activity optimum at 80°C and pH 7.0-8.0.

Rita and Rajni in 2002 isolated a new cyclodextrin glycosyltransferase from an alkaliphilic *Bacillus agaradhaerens* strain LS-3C, from an Ethiopian soda lake, and was purified up to 43-fold by starch adsorption with a yield of 50%. The enzyme was a monomer with an estimated molecular weight of 110kDa, representing the largest *Bacillus* CGTase reported so far. The CGTase was stable over a very wide pH range, 5.0-11.4, at 25°C and was most active at pH 9.0.

Kim *et al.*, 1998 produced cyclodextrin glycosyl transferase from *Paenibacillus* sp F8. The molecular weight was estimated to be 72kDa by SDS-PAGE. The initial production ratio of α-CD, β-CD, γ-CD and δ-CD from soluble starch was 0.09:1.0:0.25:0.14.

A new CGTase was obtained from *Bacillus firmus*, strain 7B, isolated from oat soil culture, using a high alkaline pH medium containing 1% Na₂CO₃. The enzyme was characterized in soluble form for pH 5-11, temperature from 30-85°C, using a 1% maltodextrin substrate solution and appropriate buffers. It produced mainly β-CD and the cell free supernatant had a precipitating activity measured by trichloroethylene method that is a 100-fold greater than that of the enzyme *Bacillus firmus*, strain 37 previously studied by their group (Cristiane *et al.*, 2007).
Sonia Jemli et al., 2007 isolated a bacterial strain designed US 132 from a Tunisian soil and its production of a potent cyclodextrin glycosyltransferase activity was studied. They identified the strain as *Paenibacillus pabuli* by sequencing of the 16S rDNA and the 16S-23S internal transcribed spacer (ITS). This enzyme exhibited a maximal activity at 65°C, in presence of 10 mM Calcium and was most active at pH range 5.5-9 with an optimum at 6.5 using 10% (w/v) of potato starch. This CGTase produced a high level of cyclodextrins reaching 42 g/l with a β-cyclodextrin ratio of 63%.

**Chemical Modification**

Before recombinant DNA technologies were developed, proteins engineers relied on chemical modification of amino acids such as lysine, cysteine, etc., to improve enzyme function and to gain insights into functionally important residues. When combined with mass spectrometry, one can determine exactly which amino acid residues are modified, as shown for *Bacillus circulans* DF 9R CGTase (Costa et al., 2009). Chemical modifications of amino acids can have various effects on the reaction specificity as demonstrated for *Thermoanaerobacter* CGTase, where succinylation and acetylation enhanced the transglycosylation (Alcalde et al., 2001) and hydrolytic activities of the enzyme, respectively (Alcalde et al., 1999). Chemical modification is also the first step in the synthesis of cross-linked enzyme crystals, which are insoluble particles that retain catalytic activity under harsh conditions such as extreme pH, high temperature, and high solvent concentrations as demonstrated for *Bacillus macerans* CGTase (Kim et al., 2003).
Cross Linking

Cross-linking can also be performed in the presence of substrate/product molecules, known as molecular imprinting, where one tries to fixate a productive conformation of an enzyme. Molecular imprinting of *Paenibacillus* sp. A11 and *Bacillus macerans* CGTase with $\gamma$-CD yielded CGTase crystals that converted over 10% of starch into CDs larger than $\gamma$-CD (Kaulpiboon *et al.*, 2007) while the corresponding wild-type does not produce these large CDs.

Immobilization

Closely related to the chemical modification method is the immobilization of enzymes onto particles to facilitate the recovery of the expensive biocatalyst from product streams for reuse of the expensive biocatalyst. In addition, immobilization usually stabilizes the biocatalyst under industrial settings. A small number of reports have described the immobilization of CGTases covalently to supports, such as Eupergit C (Martin *et al.*, 2003a) and glyoxyl-agarose (Ferrarotti *et al.*, 2006), or by entrapment in sodium alginate beads (Arya and Srivastava, 2006). Covalent immobilization is, generally, more favorable as the biocatalyst is not leaking away, but unfortunately, this typically reduces the activity of CGTase to below 10% due to the inaccessibility of a large portion of the immobilized enzyme for the polymeric substrate. One report describes an alternative strategy to facilitate the recovery of biocatalyst, namely, entrapping bacterial cells that display CGTases on the surface in polyvinyl-cryogel beads (Martin *et al.*, 2003b). Chemical modification processes can, therefore, provide a means of improving the application range of CGTases in industry.

Paulo *et al.*, 2006 presented the immobilization of the *Thermoanaerobacter* cylcomaltodextrin glucanotransferase enzyme into cross-linked 6% agarose beds activated by high density of linear aldehyde groups (glyoxal-agarose) that allow the
establishment of multi-attachment enzyme-support bonds. The immobilization conditions were 25°C, pH 10 and 5h of contact time. The immobilization yield was almost 100% and the activity recovery was 32%.

Anna et al., 2005 studied the continuous cyclodextrin glucanotransferase production by *Bacillus circulans* ATCC 21783 in four systems: free cells in a stirred reactor; free cells in a bubble column reactor; agar-entrapped and membrane-immobilized cells in a bubble column reactor. The performance of the process with free cells led to a higher enzyme level, specific CGTase activity and enzyme productivity compared to systems using immobilized cells. However the residual enzyme activity was 96 to 97% for immobilized cells and 78-86 % for free cells. Immobilization of CGTase reported in the literature was given in table 2.2.
Optimization by statistical methods

Optimization of medium composition for CGTase production has been reported by many scientists (Tonkova, 1998; Bender, 1986, Lee et al., 1992; Hedges, 1992; Gawande et al., 1998) The prerequisite to achievement of high product yield would be the design of an efficient CGTase production medium, which would render the process more economical. The conventional practice of a single factor optimization which is done by maintaining other factors involved at an unoptimal level does not allow interactions between factors involved to be studied. This method is also time consuming and requires a large number of experiments to be carried out to determine the optimal level of each factors (Box and Hunter, 1978; Davies, 1993). The use of Central Composite Design (CCD) allowed determination of levels of various parameters to be carried out with the interrelation between each parameters evolved simultaneously (Montgomery, 1991; Shiow-Ling and Wen-Chang, 1997). This method is successfully applied in the optimization of medium composition Khuri and Cornell, 1987). The utility of statistical experimental design for media optimization has also been demonstrated by Zhu et al., 1996, Park and Reardon, 1996 and Goel and Nene 1995.

Media optimization for feed stream using statistical experimental design for the production of novel CGTase by fed batch fermentation had not been reported before. On the other hand, the optimizing of media formulation in batch fermentation was readily available. Optimization of either biomass growth, enzymes, certain extracellular proteins and bioactive metabolitesa in batch fermentation has been cited by many authors such as Lee and Chen, 1997, Dey et al., 2001, Liu et al., 2001, Khairizal et al., 2004, Chen et al., 2002, Li et al., 2002 and Roshanida et al., 2004.
Few reports on the effect of carbon/nitrogen ratio on CGTase production by batch fermentation were available (Adriana et al., 2002).

Optimization of nutrient feeding was developed by Wan Salwanis et al., 2007 to overcome the limitation in batch fermentation and to increase the CGTase production from *Bacillus* species TS1-1 in fed batch fermentation. The results were analysed using three dimensional response surface plot, and the optimized values of carbon and nitrogen concentration of 3.30 % (w/v) and 0.13 % (w/v) were obtained respectively. According to them CGTase activity increased up to 80.12 units/ml which is 13.94 % higher as compared to batch fermentation (70.32 units/ml).

Cyclodextrin glucanotransferase was produced when the *Bacillus* sp TS1-1 was grown in a medium containing sago starch, yeast extract, phosphorous and mineral salt sources, using a shake flask mode at 37°C for 24 h. Response Surface Methodology was applied to optimize the medium constituents with respect to CGTase production and activity. A 2^4 full factorial design was carried out to identify the significant effect of medium components towards CGTase production. The variables involved in this initial screening study were sago starch, yeast extract, K_2HPO_4 and MgSO_4.7H_2O. Statistical analyses of results have shown that only sago starch and yeast extract have a significant effect on CGTase production (Khairizal et al., 2004).

Pui-Woon Yap et al., 2010 investigated the optimization technique of the cultivation medium in enhancing the CGTase production by a locally isolated alkalophilic *Bacillus* sp. Optimization was conducted by using change-a-factor-at-a-time method. From the study, sago starch was found capable in improving the CGTase production with the yield of 18452U/gm at 0.1%w/v of starch.
Purification

Different separation techniques were evaluated for CGTase purification from the culture supernatant. Among these was affinity chromatography and β-CD Sepharose 6B gel which has often been used for the purification CGTase (Akimuru et al., 1991; Larsen et al., 1998).

Volkova et al., 2001 used butyl – toyopearl column followed by DEAE-Sephacel and purified the CGTase with 13-fold concentration. Laxman et al., 2008 investigated the production and purification of starch digesting cyclodextrin transferase from alklophilic Bacillus firmus. Fermentation was carried out in 141 bioreactor at 28°C using a medium containing dextrin, yeast extract, peptone, \((\text{NH}_4)\text{H}_2\text{PO}_4\) and \(\text{MgSO}_4.7\text{H}_2\text{O}\). The enzyme obtained had homogeneity and the molecular weight was 76 kDa confirmed by SDS-PAGE.

Dae-Hyuk Kweon et al., 2005 demonstrated the poly-lysine tag can be employed for the immobilization of enzyme on a solid support without deterioration of its enzymatic characteristics. CGTase derived from Bacillus macerans was fused to consecutive 10 lysine residues (CGTK10ase) and electrostatically immobilized on a cation exchanger. Analyses on the binding characteristics, effects of pH and temperature on enzyme stability and operational stability indicate that the poly-lysine tag is also effective for non-covalent immobilization of CGTase.

Ho Kek et al., 2005 successively purified a cyclodextrin glucanotransferase by ammonium sulphate purification and affinity chromatography on α-CD (epoxy)-Sepharose 6B column. The specific activity of the CGTase was increased approximately 2200-fold, from 8.43 U/mg protein to 18,866 U/mg protein. SDS-PAGE showed that the purified CGTase was homogeneous and the molecular weight of the purified CGTase was about 75 kDa.
Chapter II  Review of Literature

Sonia Jemli et al., 2007 purified β-CGTase from a newly isolated Paenibacillus pabuli US 132 strain which was purified to homogeneity by hydrophobic interaction chromatography and starch adsorption.

Ilma Hiroko Higuti et al., 2003 isolated CGTase producing strain from different soil samples and they identified as Bacillus firmus. It was partially purified by starch adsorption in a yield of 51%. Maximum enzyme activity occurred at pH 5.5 and 8.5.

Anna Vassileva et al., 2007 purified CGTase from Bacillus circulans ATCC 21783 by ultrafiltration and a consecutive starch adsorption. Total enzyme yield of 75.5% and purification factor of 13.7 were achieved. And they found that CGTase was most active at 65°C, the enzyme was thermo stable up to 70°C, and 64% of the original activity retained at 70°C after 30min heat treatment.

CD production

Cyclodextrins are produced in thousands of tons from starch annually by several manufactures, and demands are still rising. The starch is first liquefied, usually via an energy consuming jet-cooking step (Buchholz and Seibel, 2008). Unfortunately, the total conversion of starch into CDs is closer to 50% than 100%. One of the reasons for this lack of efficiency is that CGTases have difficulty in bypassing the α-1,6-branches in amylopectin yielding CGTase limit dextrin (van der Maarel et al., 2002). The addition of isoamylase or pullulanase debranching enzymes increases the accessibility of the amylopectin fraction of starch, thus, increasing the CD yield (Rendleman, 1997).

Cyclodextrin yields are also limited due to enzyme product inhibition (Gaston et al., 2009) and breakdown of CDs by CGTases into linear oligosaccharides in the coupling reaction. The effects of both product inhibition and CD degradation are
minimized by keeping the CD concentrations in the reactor low, which is generally achieved by adding complexing agents leading to the precipitation of the CDs. Moreover, the type of complexing agent used strongly influences the ratio of α-, β-, and γ-CD produced (Blackwood and Bucke, 2000; Biwer et al., 2002; Zhekova et al., 2009). The breakdown of CDs is reduced further by restricting the accumulation of short oligosaccharides through the use of CGTases with low hydrolytic activity. Indeed, it has been shown that at high concentrations of saccharides, CGTases do not produce CDs from starch (Martin et al., 2001).

The other major issue in CD production is that CGTases produce a mixture of CDs. A selective purification step is, thus, required to obtain pure α-CD, β-CD, or γ-CD, through the use of complexing agents during CD synthesis and the variation in solubility of the different CDs to allow selective precipitation (Matioli et al., 2000; Lee and Kim, 1992; Son et al., 2008). The source of CGTase is the key factor in the type of CDs produced, along with reaction parameters such as the type of starch used, the buffer composition, reaction temperature and pH (Qi et al., 2005; Kamaruddin et al., 2005; Alves-Prado et al., 2008).

Tae et al., 1995 produced cyclodextrins using moderately heat treated corn starch. This method has many merits. They used 65°C heating temperature, heating time 1 h, concentration of substrate 7.5%, amount of enzyme loaded 48 units/gm of substrate. Using these conditions they got 50% cyclodextrin content, 25 % substrate conversion yield, 5.22 mg of cyclodextrin as enzyme productivity.

Ilma Hiroko Higuti et al., 2004 studied the effect of source and concentration of starch on the yield of cyclodextrin and CGTase. CGTase was more active at pH5.5. Best results were obtained for β-CD with the use of corn starch.
Applications

Cyclodextrins have numerous applications in the pharmaceutical, cosmetics, and food and textile industry, etc., as reviewed (Martin del Valle, 2009; Li et al., 2007), because of their capacity to encapsulate hydrophobic molecules within their hydrophobic cavity. Encapsulation is used to solubilize hydrophobic molecules in water (CDs have a hydrophilic outside), which is particularly advantageous as many drug molecules are poorly soluble in water (Loftson and Duchene, 2007), or to protect guest molecules from light, heat, or oxidizing conditions (Astray et al., 2009). Cyclodextrins are also used to lower the volatility of odour molecules in perfumes and room refreshers for controlled release of the odour. In the chemical industry, CDs are used in the separation of enantiomers to extract toxic chemicals from waste streams and in soil bioremediation (Fava and Ciccotosto, 2002). Various other applications of CDs include the suppression of undesirable (bitter) tastes and the extraction of compounds such as cholesterol from foods (Szente and Széjti, 2004; Széjti and Szente, 2005).