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The effects of reaction conditions on the production of γ-cyclodextrin from tapioca starch by using a novel recombinant engineered CGTase

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Abstract

A novel mutant enzyme namely H43T CGTase can produce up to 39% γ-cyclodextrin (γ-CD) compared to the native enzyme which produces only 10% γ-CD. The effect of the reaction conditions on γ-CD production was studied using this mutant CGTase. The effects of substrate–buffer combination, starch pretreatment and concentration, pH, additives and finally the use of a debranching enzyme improved the γ-CD ratio further. The tapioca–acetate pair gave the highest conversion (16% conversion) among four types of starch and four buffer system combinations. Gelatinized starch was preferred compared to raw tapioca starch in producing a high percentage of γ-CD and conversion rate. Higher pH especially pH 8–9 led to a higher proportion of γ-CD, and was relatively more apparent when the concentration of starch was increased. Forty-six percent γ-CD was produced using 2.5% gelatinized tapioca starch at pH 8. Pullulanase enzyme was found to be useful in reducing the viscosity of tapioca starch paste thus increasing the efficiency of utilization of starch by CGTase by at least 20- to 30-fold. Up to 48% γ-CD can be produced when 4% pullulanase-pretreated tapioca starch was reacted with the CGTase mutant. It was also found that the supplementation of the reaction mixture with glucose, toluene, or cyclododecanone improved the γ-CD yield by 42.2, 46.4, 43.4, and 43.4%, respectively. All the parameters involved have been shown to affect the product specificity of the mutant H43T CGTase transglycosylation mechanism.

Keywords: *Bacillus* sp. G1; Cyclodextrin glucanotransferase; Gamma-cyclodextrin; Pullulanase; Site-directed mutagenesis; Tapioca starch

1. Introduction

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an industrially important enzyme that produces cyclodextrins (CDs) from starch or starch-like substrates. α-, β- and γ-CD contain closed ring structures with six, seven and eight glucose units, respectively, joined by α-1,4-glucosidic bonds. The doughnut-shaped CDs have an interior portion that easily form inclusion complexes with many organic substances or drugs which can change the physicochemical properties of the latter molecule, such as its solubility and stability. In practical terms, due to its relatively larger cavity volume γ-CD can form inclusion complexes with larger compounds in cases where the volume of the interior cavity of α-CD and β-CD is not sufficiently large to do so. Another advantage of γ-CD is its high solubility (23.2%, w/v, at 25 °C) in water, which is almost double and 10 times that of

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α-CD and β-CD, respectively [1]. The major disadvantage of γ-CD production by CGTase is that α-, β- and γ-CDs are produced as a mixture where the amount of γ-CD is generally small and the ratio of CDs greatly depends on the bacterial source of the enzyme [2], and the reaction time and conditions [3]. CGTase catalyzes four reactions (cyclization, coupling, disproportional and hydrolysis) which occur simultaneously through different kinetic [4] and thermodynamic mechanisms [5]. The net contribution of these reactions determines the overall yield and product specificity in a prolonged reaction [4].

The source of the CGTase greatly influences the production of cyclodextrin since the amino acid sequence and the folding of the protein structure determine the outcome of the kinetic equilibrium. Enzyme engineering of CGTase at central active site cleft [6], subsite-3 and subsite-7 [4] were reported to be important for enriching the production of certain types of CD. Alternatively, modifying the enzymatic reaction conditions could also help in enriching the yield of the target type of CD; such parameters include starch source [7,8], starch concentration [9], buffer type [3,10], reaction pH [10], presence of additives [11–13] and presence of precipitants [14]. Some of the studied parameters are reported to have a beneficial effect on CD production while contradictory results have been reported in other publications. Thus, it can be concluded that reaction condition studies are still essential for each case of interest.

This is the first report on the effects of reaction parameters on the ratio of γ-CD to the total amount of CDs produced by the utilization of a mutant CGTase (mutant H43T). A recombinant form of the enzyme derived from the predominant β-CGTase G1 [8] was protein tailored at subsite-3 of the protein structure. The mutant exhibited the ability to produce a higher percentage of γ-CD. This study shows that combining protein engineering and reaction manipulation, enriches the production of γ-CD significantly, and is relatively more effective than altering one single mode alone.

2. Materials and method

2.1. Bacterial strain, DNA manipulation, and purification of mutant CGTase H43T

The alkalophilic bacteria identified as *Bacillus* sp. G1 was isolated from soil and the nucleotide sequence of the CGTase gene was submitted to the National Center for Biotechnology Information (NCBI) database (accession number AY770576). The Megaprimer-PCR method [15] was used to construct mutant CGTase H43T. Internal mutagenized primer 5′-CCACCACAATACTTGGTAAGATCTATACAG-3′ was used to generate the "megaprimer" which was subsequently used as the forwarding primer to complete the whole gene amplification. The PCR product was digested with restriction enzyme *Xba*1 and *EcoR*1 (Promega) and ligated to plasmid pUC19 that was digested with the same enzymes. Mutagenesis was confirmed by DNA sequencing. The mutant protein was expressed by *E. coli* JM109 in 1 l LB/amp media, and the crude enzyme was harvested after overnight incubation at 37 °C, 200 rpm. The method used to purify the mutant CGTase H43T is similar to the procedure used to purify the wild type CGTase [8]. The γ-CD forming activity of the purified mutant CGTase was determined by the bromocresol green (BCG) method [16]. One unit of γ-CD forming activity is defined as the amount of enzyme that produces 1 μmol of γ-CD per minute. In all the following reactions (Sections 2.2–2.6), approximately 0.012 U of diluted purified enzyme was used per ml of reaction mixture. Under normal conditions, this corresponded to 1.2 U of enzyme per gram of starch. The enzymatic reaction was carried out for 18 h based on the initial screening process which showed that the enzymatic reaction had reached steady state after 18 h (data not shown). Two or more runs were conducted for all the enzymatic reactions.

2.2. Screening of substrates and buffers on γ-CD production

Corn and potato starch were purchased from Merck, soluble starch from Goodrich Chemical Enterprise (GCE), amylose and glycogen were from Sigma. The tapioca starch used was of food grade and sourced from a local manufacturer. Substrates were boiled for 10 min in each 20 mM buffer system (phosphate, acetate, MES, citric), cooled to room temperature before adding diluted CGTase. Purified mutant CGTase H43T was reacted with 1 ml of 1% (w/v) substrate in selected buffer systems at pH 6 and 60 °C for 18 h. Enzymatic reactions were later stopped by boiling for 10 min. A concentration of 1% substrate was chosen for the basis of comparison because certain starch solutions (typically tapioca starch) at high concentrations are very viscous, difficult to handle and result in lower yield as reported in a study using the wild type CGTase G1 [8].

2.3. Effects of raw and gelatinized starch on γ-CD production

Raw starch slurry was freshly prepared before use by suspending 1–4% (w/v) tapioca starch in 20 mM acetate buffer of pH 6. Gelatinized starch was prepared by heat treatment in a boiling water bath for 10 min. After cooling to room temperature, mutant CGTase H43T was added. Reactions were carried out as described earlier.

2.4. Effects of pH on γ-CD production

Mutant CGTase H43T was reacted with 1 and 2.5% gelatinized tapioca starch in 20 mM buffers ranging from pH 6 to 10. The buffers used were acetate buffer (pH 6), phosphate buffer (pH 7–8) and glycine–NaOH buffer (pH 9–10).

2.5. Effects of starch pretreatment using a debranching enzyme on γ-CD production

Debranching of amylopeptin in 1, 2.5 and 4% (w/v) gelatinized tapioca starch–acetate buffer were carried out using pullulanase (Promozyme 400 l, Sigma). Concentrations of 0.1 and 0.3% (v/v) pullulanase were added to the starch and incubated for 30 min at 50 °C. Mutant CGTase H43T was

added after the debranching activity had been stopped by boiling.

2.6. Effects of additives, precipitants and solvent on γ-CD production

Mutant CGTase was reacted with 1 ml of 1% gelatinized tapioca starch–acetate buffer supplemented with additives (5–20% $CaCl_2$, 10–50 mM glucose), solvent (10–30% ethyl alcohol) and precipitants (5–40% toluene, 2.5–8% limonene, 0.0057–0.115 mM cyclododecanone). These chemicals were only added once at the beginning of the reaction.

2.7. Analysis of cyclodextrins by HPLC

The ratio of cyclodextrins produced was analyzed using a Waters HPLC system with separation carried out using an Econosphere NH2 (5 μm, 250 mm × 4.6 mm) column. The peaks were eluted with 70:30 acetonitrile–water at a rate of 1 ml/min. An RI detector was used to detect the reaction products. The standards used for calibration were glucose, maltose, maltotriose, maltotetraose and CDs and were of high purity grade purchased from Sigma and Supelco.

2.8. Determination of enzyme kinetic parameters

The enzyme kinetic values for the purified enzyme were determined by incubating 0.2 ml of samples with soluble starch in 1 ml of 0.1 M phosphate buffer (pH 6.0). The data was produced according to the γ-CD cyclization assay. K_m and V_{max} values were then determined from a Hanes–Woof plot. The turnover number (k_{cat}) for CGTase G1 was calculated by dividing V_{max} by the molar concentration of CGTase ($k_{cat} = V_{max}/(E)_0$).

3. Results and discussion

The study conducted by Sian et al. [8] showed that a purified CGTase from *Bacillus* sp. G1 produced about 10% γ-CD and 90% β-CD when reacted with 1% tapioca starch in phosphate buffer (pH 6). With tapioca starch as the substrate, no α-CD was formed using CGTase produced by *Bacillus* sp. G1. In this study, an engineered enzyme from the predominant β-CD producer CGTase from *Bacillus* sp. G1 was constructed by mutation at subsite-3 of the recombinant enzyme. A comparison of the amino acid sequence of CGTase G1, mutant H43T and other CGTases is shown in Fig. 1. The varying patterns of residue at subsite-3 can be clearly distinguished between the different groups of CGTase. For α-CD and α-/β-CD producers, lysine (K) or arginine (R) is mainly found at subsite-3 whereas for the β-CD producer either in the absence or presence of minor quantities of α-CD, histidine (H) is found at this site while γ-CD producers have threonine (T) at corresponding location (Table 1). Therefore, a site-directed mutagenesis based on rational design was conducted in this work. Threonine has a shorter and less complex side chain than histidine. The mutation at this position in recombinant CGTase of *Bacillus* sp. G1 created extra space which affects the cyclization activity to allow the formation of a larger cyclodextrin molecule in the extra space at subsite-3. This finding is in agreement with the results reported by van der Veen et al. [17] which suggested that a relatively short side chain at the same position is accompanied by a clear preference for the production of the larger size cyclodextrin. In addition to its importance for product specificity, the residue at subsite-3 is also involved in the binding of starch and cyclodextrin during the cyclization process. Shortening the side chain after mutation to threonine may cause the changes in position of the sugars which bind during the cyclization reaction [18].

Table 1
Comparison of amino acid sequence at subsite-3 of various CGTase

CGTase source	Sequence at subsite-3 region	Main product	Accession number
Bacillus macerans	HS-NL**K**LYF	α	P04830
T. thermosulfurigenes EM1	HT-SL**K**KYF	β/α	P26827
Bacillus licheniformis	CS-NL**K**LYC	α/β	P14014
Bacillus circulans No. 8	CS-NL**K**LYC	β	CAA48404
Alkalophilic *B.* sp. 17.1	CT-NL**R**LYC	β	P30921
Bacillus circulans 251	CT-NL**R**LYC	β	P43379
Alkalophilic *B.* sp. 1011	CT-NL**R**LYC	β	P05618
Alkalophilic *B.* sp. 1.1	CI-DL**H**KYC	β (No α)	P31746
Alkalophilic *B.* sp. G1[a]	CI-DL**H**KYC	β (No α)	AAV38118
Mutant CGTase H43T$^+$	CI-DL**T**KYC	β/γ	This study
Bacillus firmus/lentus 290-3	CL-DL**T**KYC	γ/β	CAA01436
Bacillus clarkii 7364	CS-DL**T**KYC	γ	BAB91217
Alkalophilic *B.* sp. G-825-6	CL-DL**T**KYC	γ	BAE87038

[a] The native CGTase used to construct mutant H43T CGTase$^+$.

The novel mutant enzyme namely H43T CGTase can produce up to 39% γ-CD and 61% β-CD when reacted with 1% tapioca starch. The increment in γ-CD production was approximately four times that obtained using the native CGTase and was higher than that of other subsite-3 mutants described in other publications. Table 2 shows the spectrum of CD production by various CGTase mutants reported in previous studies. Moreover, another advantage of mutant CGTase H43T is that it does not produce α-CD which facilitates isolation of the other types of CD. The wild type CGTase from *Bacillus* sp. G1 produces approximately 0.85 mg/ml total CDs when 1% tapioca starch in phosphate buffer is used as the substrate. Under the same reaction conditions and enzyme concentration, mutant CGTase H43T was only able to generate 0.55 mg CDs/ml. The shorter side chain of threonine compared to histidine at residue 43 caused the interaction between the enzyme and starch–CD complex to become weaker as the distance between them is greater and this lead to a reduction in the total CD produced. Kinetic study revealed that the K_m and k_{cat} values for the wild type CGTase G1 was 2.9 mg/ml and 2349 s^{-1}, respectively. However, the values for mutant CGTase H43T were lower, 1.9 mg/ml and 2103 s^{-1} for K_m and k_{cat}, respectively. Uitdehaag et al. [18] reported that mutation at Arg47 (corresponding to residue 43 in CGTase G1 numbering) can cause a change in binding affinity (K_m) and cyclization turn-over number.

Although mutant CGTase H43T was able to produce 39% γ-CD and 61% β-CD, the percentage production of γ-CD is still

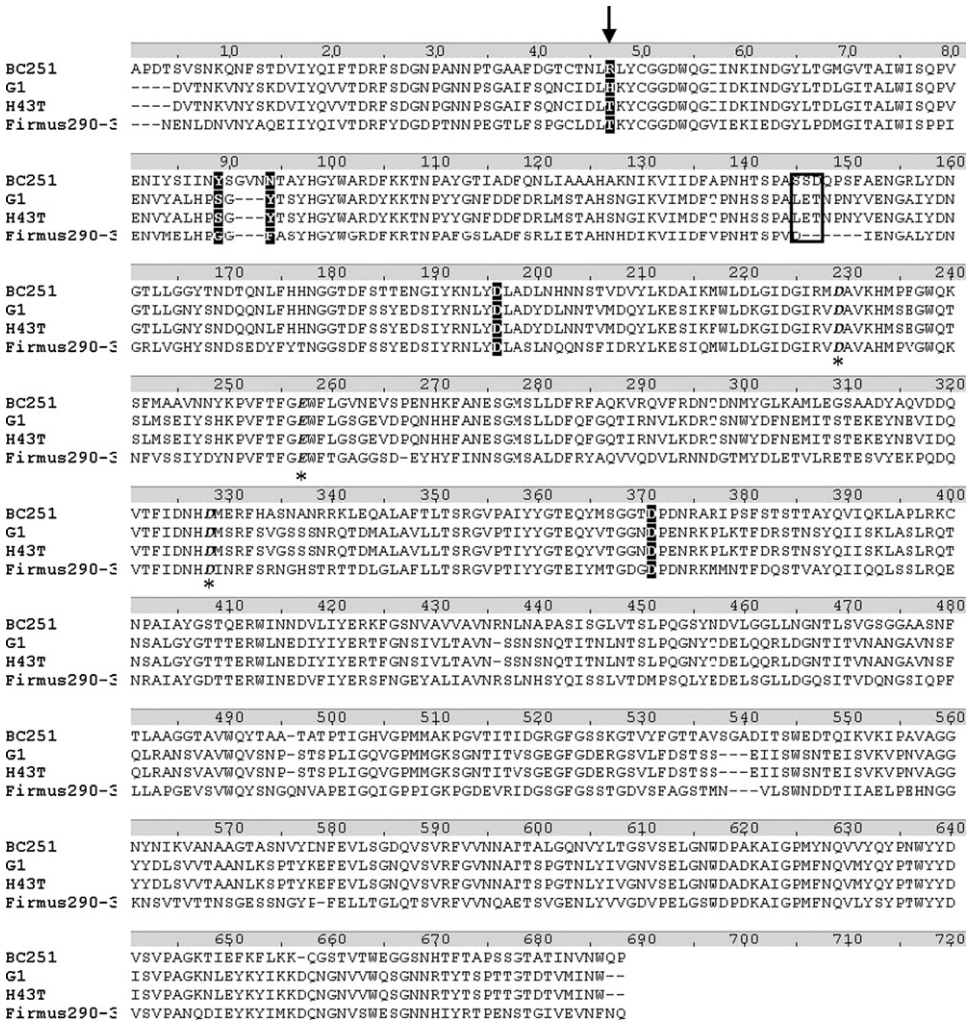

Fig. 1. Comparison of amino acid sequence of CGTase *Bacillus circulan* 251 (BC251), CGTase *Bacillus* sp. G1 (G1), CGTase mutant H43T (H43T) and CGTase *Bacillus firmus/lentus* 290-3 (Firmus 290-3). The catalytic residues for CGTase are indicated by *. The location of subsite-7 is shown in the square box. All the amino acids at subsite-3 are highlighted and the residue mutated in this study is indicated by the arrow.

lower compared to the wild type γ-CGTases, typically CGTase from *Bacillus firmus/lentus* 290-3 [9] which can produce almost the same quantity of β-CD and γ-CD. Therefore, the effect of reaction conditions on the performance of mutant CGTase H43T was studied with the aim of improving the percentage of γ-CD formed.

3.1. Effects of substrates and buffers on γ-CD production

The effects of various substrates (tapioca, potato, soluble, corn starch, amylose, glycogen) in different buffers were studied to identify the best substrate–buffer combination for γ-cyclodextrin production, using the engineered CGTase. As

Table 2
Ratio of cyclodextrins produced by parent and mutants CGTase from various sources

CGTase strain	Mutation site	CD spectrum (α-CD:β-CD:γ-CD)		Reference
		Before mutation	After mutation	
Bacillus sp. G1	H43T	0:90:10	0:61:39	This study
Bacillus firmus var. *alkalophilus*	H59Q and Δ(154–160)	0:83:17[a]	No changes	[31]
Thermococcus sp. B1001	Y267W	85:8:7[a]	70:17:13[a]	[32]
Bacillus circulans No. 8	Y195W	11:68:21[a]	6:39:55[a]	[33]
	Δ(145–151)D		6:54:40[a]	
Bacillus circulans 251	Y195W	13:64:23	18:63:19	[34]
Bacillus ohbensis	Y188W	0:85:15[a]	0:57:43[a]	[6]

[a] Numbers were estimated from graph.

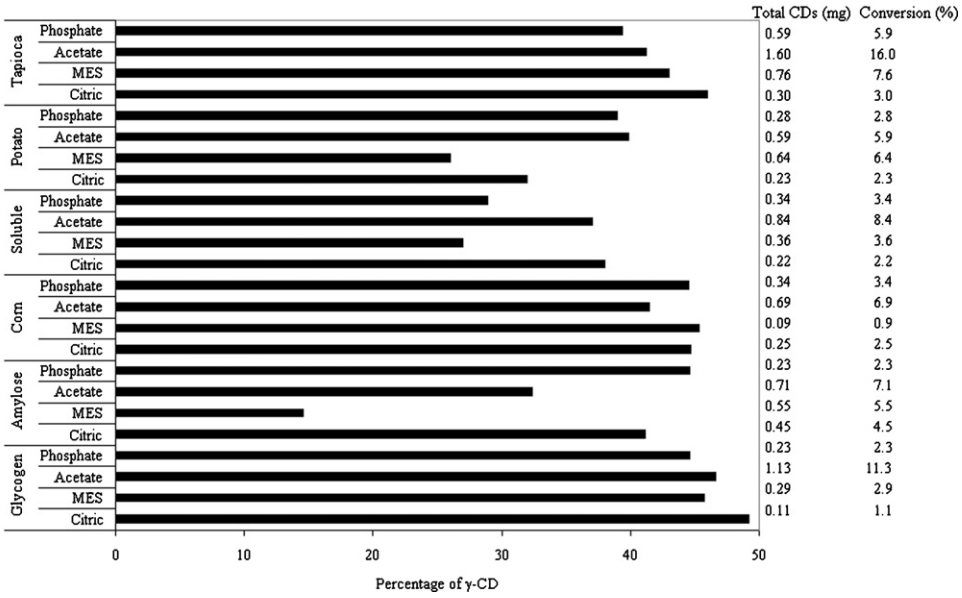

Fig. 2. Effect of substrate selectivity and buffer systems at pH 6; 1% gelatinized substrates were used throughout.

shown in Fig. 2, the use of tapioca starch in a different buffer system leads to a significant improvement in the γ-CD ratio: acetate buffer (41% γ-CD), MES (43% γ-CD) and the highest percentage of γ-CD was found using citric buffer (46% γ-CD). Despite enhancing the formation of γ-CD from 39% in phosphate buffer to 46% γ-CD when citric buffer was used, conversion to total CDs, however, was relatively low as only half the amount of total CDs could be produced in comparison to the tapioca–phosphate system as shown in Fig. 2.

When the γ-CD ratio and conversion capability factor were compromised, the tapioca–acetate system gave the numerically highest conversion rate (16%) while maintaining a high ratio of γ-CD (41%) which is almost three times the amount of cyclodextrins (16% conversion) produced under normal conditions. In fact, by using the tapioca–acetate system, conversion to CDs was the highest compared to all the substrate–buffer combinations studied in this work (Fig. 2).

It was observed that there were fewer short oligosaccharides formed when tapioca starch was used (Fig. 3). Conversion to CDs was almost four times less for potato starch compared to tapioca starch, whereas the formation of linear oligosaccharides was relatively higher when potato starch was utilized (Fig. 3). HPLC analysis showed that potato starch used in this experiment was slightly richer in free short linear maltooligosaccharides. The presence of short oligosaccharides can interfere with the efficiency of the cyclization reaction as these molecules can promote more disproportional and coupling activities than cyclization activity, the latter being responsible for the formation of CD. Disproportional reactions will only result in various lengths of linear oligosaccharides and those that are less than eight glucose units in length are unsuitable as substrates for the formation of cyclodextrin since the cyclization reaction follows the equation $G_n \leftrightarrow G_{n-x} + cG_x$, (where $n \geq 8$, $9 \geq x \geq 6$), G_n are α,1-4-glucopyranosyl chains of length n, and cG_x are cyclodextrins of ring size x [19]. Triggering of disproportional activity would compete with cyclization reactions, and may lead to a reduction in the synthesis of CDs. Overactivation of the coupling reaction will cause the CD ring to be opened up to linear oligosaccharides and hence will decrease cyclodextrin yield [20]. Furthermore, small oligosaccharides such as glucose and maltose that are formed from by-reactions are inhibitors of CGTase leading to a reduction in CDs formed [21].

3.2. Effects of raw and gelatinized tapioca starch on γ-CD production

Since the tapioca–acetate buffer combination gave good conversion (16%) and a high percentage of γ-CD (41%), it was chosen as the basis for comparison in all the following experiments. A higher percentage of γ-CD was produced from

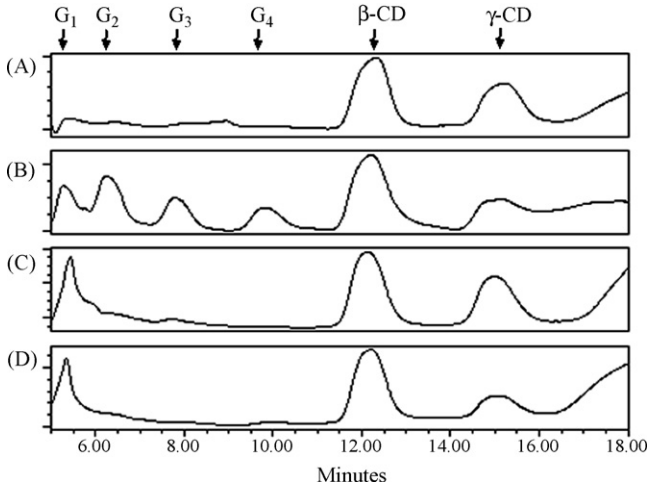

Fig. 3. Typical HPLC chromatograms for the different sources of starch. (A) tapioca starch; (B) potato starch; (C) corn starch; (D) soluble starch; G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose.

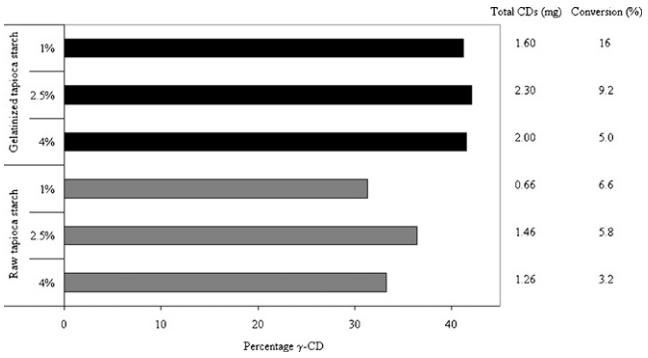

Fig. 4. Effects of raw and gelatinized tapioca starch in sodium acetate buffer (pH 6).

gelatinized starch compared to the native form (Fig. 4). The total amount of CDs produced was also higher when gelatinized tapioca starch was used compared to raw starch.

Raw starch has a compact crystalline structure which is not easily degraded by common starch degrading enzymes [22] due to the weak interaction of CGTase with raw tapioca starch granules. The native crystalline structure of starch is disrupted by heating in the presence of water, a phenomenon known as gelatinization. Gelatinized starch swells irreversibly to many times its original size and creates a large surface to volume area for enzymatic reaction [22,23]. Another reason for the superior γ-CD production in the presence of gelatinized tapioca starch is that during gelatinization, heat leaches out amylose [24] providing more contact sites between the substrate and enzyme.

Increasing the concentration of gelatinized starch will significantly reduce the conversion rate of starch to CDs as shown in Fig. 4. Tapioca starch appears to be 'cloudy' when gelatinized and becomes more viscous as the concentration increases. This is a common problem in the starch industry especially when high starch concentrations are used. Normally high temperature is required to liquefy the starch. An alternative approach frequently used in industry to liquefy starch slurry is to use amylase, however, it was found that low molecular weight oligosaccharides produced by the hydrolytic reaction inhibits CD production [20].

3.3. Effects of pH on γ-CD production

A relatively higher percentage of γ-CD was achieved under alkaline conditions with a maximum percentage of γ-CD (46%) observed at pH 8 and pH 9 and the ratio was noticeably higher when the starch concentration (2.5% starch) was elevated (Fig. 5). Although the percentage of γ-CD was enhanced at a higher pH, the amount of total CDs produced however, was less than that produced at pH 6 which appears to be the optimum pH for the mutant CGTase enzyme activity (data not shown). It has been reported that the ability of CGTase to produce γ-CD can be enhanced by using alkaline conditions and improved to a more obvious level using higher starch concentrations [10,21]. This could be due to the different binding modes of the enzyme and substrate at different pHs [10].

3.4. Effects of debranching enzyme on γ-CD production

The content of amylose in tapioca starch was reported to be 16.27 ± 0.32% [25]. Compared to other starches such as corn and potato, the content of amylopectin (the branched portion) in tapioca starch is higher than that of amylose [24,25]. CGTase efficiently degrades starch to CD by its action on the amylose portion of the molecule and not on the amylopectin moiety. The effect of the debranching enzyme pullulanase to improve the ratio of γ-CD produced using this mutant H43T CGTase was studied.

Fig. 6 shows that the amount of γ-CD produced was greater when tapioca starch was treated with pullulanase. When 2.5% tapioca starch was treated with 0.1 and 0.3% pullulanase, there was a reduction in the total CDs produced while the percentage of γ-CD increased. This was also observed for 1% tapioca starch. In both cases (1 and 2.5% tapioca starch), although the ratio of γ-CD produced was improved when pullulanase was used,

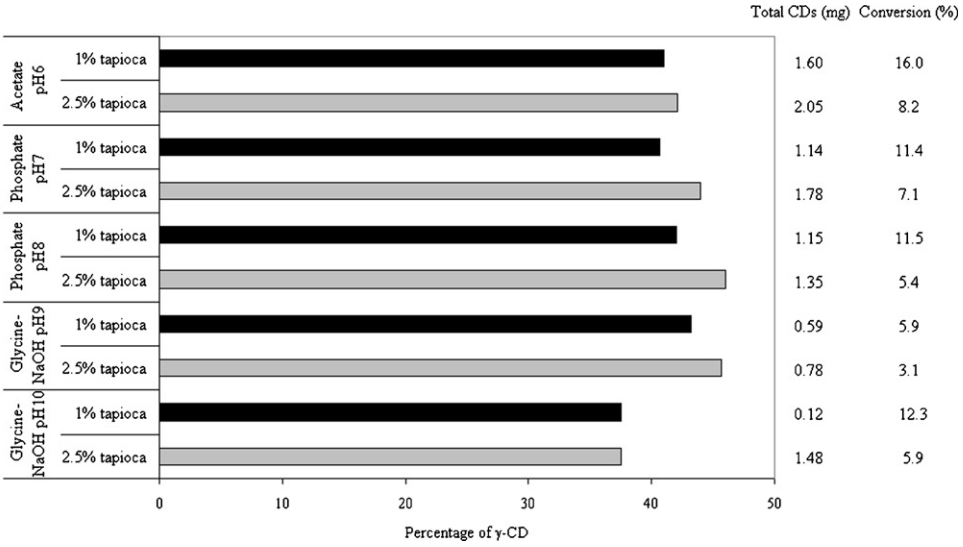

Fig. 5. Effects of different concentrations of gelatinized tapioca starch at pH 6–10.

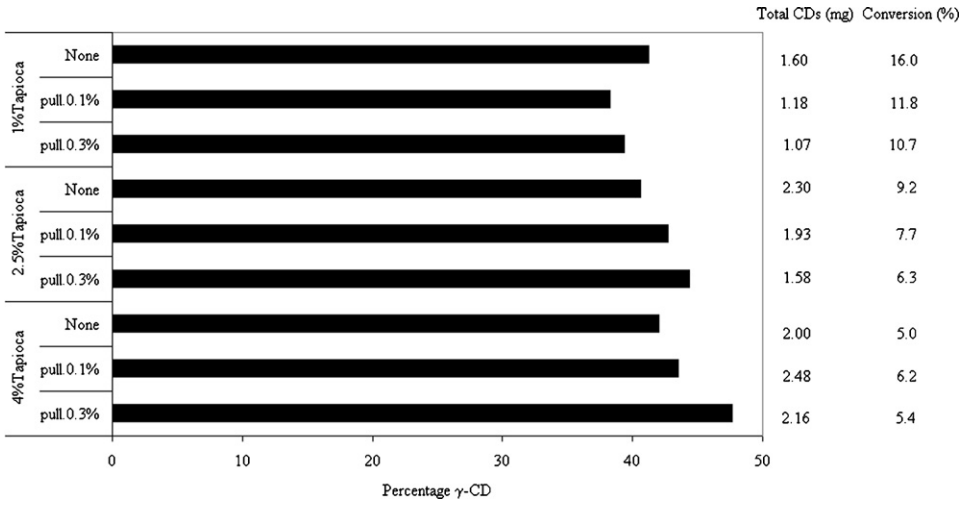

Fig. 6. Effects of starch pretreatment with pullulanase. Gelatinized tapioca starch was digested with 0.1 or 0.3% (v/v) pullulanase; pull., pullulanase.

the total conversion of starch to CDs decreased. Production of cyclodextrin using CGTase is influenced by substrate quantity (concentration), substrate quality (amylose–amylopectin ratio and viscosity), and the presence of inhibitors (glucose produced from pullulanase action on starch) where these parameters are related to each other in this context. Equilibrium between these factors determines the final ratio of γ-CD and total CD production. Our initial analysis on starch treated with pullulanase using HPLC showed that a high level of glucose was produced (data not shown). Glucose has been reported to be an inhibitor of CGTase since both glucose and starch bind at the same position in CGTase molecule, i.e. at the maltose binding site (MBS) which affects the activity of the enzyme [19,26].

An increment in the total CDs produced was observed when pullulanase was used in 4% tapioca starch as shown in Fig. 6. The overall conversion to CDs for the control was 5% while 6.2 and 5.4% conversions were observed with 0.1 and 0.3% pullulanase pretreatment of the starch, respectively. Interestingly the percentage of γ-CD produced was increased from 42 to 48% when 0.3% pullulanase was used. Pullulanase reduced the viscosity of the starch slurry and produced abundant quantities of free linear α-1-4-glucopyranosyl as substrate for CD production. In the presence of a rich substrate concentration, more CDs are produced and this effect is greater than the negative effect caused by glucose inhibition. In low starch concentrations (e.g. in the case of 1 and 2.5%) as shown in Fig. 6, the use of the debranching enzyme only leads to excess of glucose which inhibits CGTase activity and this negative effect of pullulanase overrides its positive effects in reducing viscosity and increasing the amylose content. From the results obtained with different starch concentrations and the varying degree of debranching, it can be concluded that the amount of pullulanase used is critical since it controls the variable of substrate concentration, viscosity and the inhibition effect. Only in an appropriate concentration where the pros outperform the cons would pullulanase be useful in γ-CD production.

In studies conducted by Pishtiyski and Zhekova [7], Szejtli [27] and Saha and Zeikus [28], pullulanase was also used together with CGTase in cyclodextrin production, however, no enhancement of the γ-CD ratio was observed by these authors. Pishtiyski and Zhekova [7] reported that preliminary treatment of starch with α-amylase and pullulanase was inefficient and unnecessary because it did not lead to an increase in the yield of CDs. In addition, no significant diversity in CD profile was observed. The results from this study, however, contradict the results of previous researchers [7].

3.5. Effects of additives, precipitants and solvent on γ-CD production

The effects of supplements on γ-CD production using 1% gelatinized tapioca starch in acetate buffer (pH 6) are shown in Table 3. The addition of $CaCl_2$ is known to increase the thermostability of CGTase. Calcium ion binds at two locations in the protein structure as shown by X-ray crystallography [29]. Addition of $CaCl_2$ to the reaction mixture did not lead to a significant difference in γ-CD ratio or total conversion (Table 3).

Table 3 also summarizes the effect of ethyl alcohol on the production of γ-CD using the H43T mutant CGTase. Reactions at 60 °C (which is the optimum temperature) using mutant CGTase H43T and the addition of 10, 20 or 30% ethyl alcohol showed no improvement in γ-CD specificity, but reduced the conversion rate progressively as the concentration of alcohol increased. In this study, the activity of mutant H43T is inversely proportional to the percentage of ethyl alcohol added. This could be due to inactivation of enzyme since reactions were carried out using the optimum temperature of 60 °C. Mori et al. [12] reported that the presence of 20% ethyl alcohol in the reaction mixture at 40 °C greatly enhanced the proportion of γ-CD. In another example, the yield of γ-CD increased with ethyl alcohol concentration up to 30% [11]. In both successful examples, reactions were carried out at 40 °C since the enzyme is inactivated at temperature higher than 50 °C in the presence of ethyl alcohol [12]. All the reported studies were conducted at reaction temperatures lower than the actual optimum temperature of the CGTases used. Therefore, a further study of the effect of ethyl alcohol was carried out at

Table 3
Relative comparison of the effects of selected additives, precipitants and solvent on cyclodextrin production and γ-CD ratio

Additives and concentration	γ-CD (%)	β-CD (%)	Total CDs (mg)	Conversion (%)
No additives	41.0	59.0	1.60	16.0
$CaCl_2$ (mM)				
5	40.6	59.4	1.61	17.0
10	40.6	59.4	1.61	16.1
20	40.4	59.6	1.61	16.1
Ethyl alcohol (60 °C) (%)				
10	39.4	60.6	0.99	9.9
20	40.2	59.8	0.42	4.2
30	23.3	76.7	0.23	2.3
Ethyl alcohol (40 °C) (%)				
0	35.0	65.0	0.93	9.3
10	26.7	73.3	0.93	9.3
20	30.3	69.7	0.67	6.7
30	20.5	79.5	0.61	6.1
Glucose (mM)				
10	40.3	59.7	1.47	14.7
30	42.0	58.0	1.40	14.0
50	42.2	57.8	1.33	13.3
Toluene (%)				
5	41.9	58.1	1.52	15.2
25	41.2	58.8	1.48	14.8
40	46.4	53.6	1.45	14.5
Limonene (%)				
2.5	37.5	62.5	1.56	15.6
5	37.7	62.3	1.73	17.3
8	37.4	62.6	1.63	16.3
2-Butanone (%)				
4	40.0	60.0	0.77	7.7
2-Butanone + 0.0057 mM cyclododecanone	41.9	58.1	0.78	7.8
2-Butanone + 0.028 mM cyclododecanone	41.9	58.1	0.78	7.8
2-Butanone + 0.115 mM cyclododecanone	43.4	56.6	0.77	7.7

40 °C which is lower than the optimum temperature for H43T CGTase. The results show that at 40 °C, the percentage of γ-CD dropped to 35% and that only a quarter of total CDs generated at 60 °C were produced. The activity and product specificity of mutant H43T is greatly influenced by reaction temperature. This study shows that ethyl alcohol increases β-CD yield rather than γ-CD as shown in Table 3.

Table 3 indicates that at 10 and 50 mM glucose, inhibition of mutant CGTase H43T was found to be 8 and 17%, respectively. Looking at the individual amounts of β-CD and γ-CD produced by mutant H43T in the presence of glucose (data not shown), it appears that as the concentration of glucose increases the quantity of β-CD produced clearly falls significantly compared with that of γ-CD whose percentage yield was enhanced. In this study it has been shown that glucose might have a greater inhibitory effect on the formation of β-CD than of γ-CD.

γ-CD forms inclusion complexes with certain solvents and chemicals. If the precipitants are applied at the beginning or middle of the reaction, a portion of the γ-CD will be precipitated causing an imbalance in the kinetics equilibrium, and driving the reaction towards producing more γ-CD. In this study, some of the commonly used precipitants such as toluene, limonene, and cyclododecanone were added to a 1% tapioca–acetate buffer system to improve γ-CD production using the mutant H43T CGTase. All additives were added only once at the beginning of each reaction.

Toluene at a concentration of 40% (v/v) was added to the reaction mixture and the results show that it increases the production of γ-CD up to 46.4%. The γ-CD ratio was relatively higher than the control as shown in Table 3. The decline in the total amount of CDs produced was less than 10%, probably due to some enzymatic inactivation caused by the solvent. The high volatility of toluene makes it a useful precipitant because the toluene–γ-CD complex is not tightly bound and can easily be disrupted by simple heat treatment. However, the use of toluene is not recommended when the CDs produced are to be used in food and drugs.

In a patent file, Ammeraal [30] described the use of limonene for purifying β-CD from a reaction mixture. It was reported that limonene preferentially precipitated β-CD and in a second step precipitated γ-CD from a mixture of CDs. It was also reported that the overall yield of β-CD was increased in the presence of limonene. Unfortunately, utilizing low concentration (2.5–8%) of limonene together with mutant H43T did not result in an increase in the production of γ-CD (Table 3).

2-Butanone used to dissolve cyclododecanone is itself inert to product specificity, nevertheless it inactivates mutant CGTase H43T. Only half the amount of total CDs were formed in the presence of 2-butanone compared to the control as shown in Table 3. Approximately 43.4% γ-CD was produced when the reaction was supplemented with 0.115 mM cyclododecanone. Nevertheless, Rendleman [14] managed to change the product specificity from an α-CD:β-CD:γ-CD ratio of 11.2:15.6:2.8 to 3.7:37.6:41.8 in the presence of cyclododecanone by adding 10 incremental aliquots of fresh CGTase from *Bacillus macerans* to the reaction mixture. The percentage of γ-CD was increased to 72.2% using 35 increments of CGTase.

4. Conclusion

Mutant H43T CGTase produced 39% γ-CD while the parent enzyme produced only 10% in the tapioca–phosphate (pH 6) system. Manipulating reaction parameters increased the percentage of γ-CD produced. These parameters include acetate buffer (41% γ-CD), pH 8–9 (46% γ-CD), 0.3% pullulanase pretreatment of starch (48% γ-CD), 50 mM glucose (42.2% γ-CD), 40% toluene (46.4% γ-CD), and 0.115 mM cyclododecanone (43.4% γ-CD). The improvement was achieved as a result of the effects of all the parameters used on the structure of the mutant H43T. This has led to product specificity and changes in total CD production of the mutant CGTase H43T via an intermolecular transglycosylation. Our findings with regard to this mutant enzyme are compatible with those of other researchers [7–14] who also showed that the use of different starches, pH, solvents, etc., together with native CGTase can affect the transglycosyla-

tion reaction, which leads to the different product specificities. We have demonstrated that the enhancement of γ-CD production can be achieved via the combination of protein engineered CGTase and manipulation of the reaction conditions. Using both these methods in combination provides better results than the use of either one alone. It is therefore concluded that mutant CGTase H43T serves as a potential enzyme for use in the manufacture of γ-CD.

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