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Production of a Thermoactive B-cyclodextrin Glycosyltransferase with a High Starch Hydrolytic Activity from an Alkalitolerant *Bacillus licheniformis* Sk 13.002 Strain

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ABSTRACT

The effects of different carbon, nitrogen and metal ion sources on the production of a β-cyclodextrin glycosyltransferase from a new alkalitolerant *Bacillus licheniformis* SK 13.002 strain were studied and effects of pH and temperature on the cyclization and hydrolysis activities assessed. Soluble starch was the best (0.131±0.003 U mL^{-1}) carbon source while peptone combined with yeast extract (0.105±0.002 U mL^{-1}) was an essential organic nitrogen source for enzyme production. $MgSO_4$ (0.130±0.003 U mL^{-1}) and $FeCl_2$ (0.127±0.001 U mL^{-1}) showed similar effect for CGTase production. Effect of $FeCl_2$ on CGTase fermentation production has not been reported before. Glucose (0.0192±0.002 U mL^{-1}) and maltose (0.0354±0.001 U mL^{-1}) repressed enzyme production while $CuSO_4$, $ZnSO_4$ and $ZnCl_2$ completely inhibited CGTase synthesis. The CGTase could significantly hydrolyze starch into short linear saccharides, with the hydrolysis activity exceeding cyclization activity four times. The enzyme showed an optimal cyclization activity (0.102±0.004 U mL^{-1}) at pH 7.0 while optimal hydrolysis activity (0.461±0.003 U mL^{-1}) was at pH 6.0. These activities were both optimal at 65°C. At 70 and 75°C, the relative cyclization activities were 87 and 50%, respectively, while those for hydrolysis were 98 and 93%, respectively. Therefore, *B. licheniformis* SK 13.002 CGTase has a potential for industrial application in processes where thermal activity is required. The hydrolytic activity of this CGTase is thought to be due to partial retention of ancestral enzyme function from evolution over time. However, this side reaction is undesirable since it produces short saccharides that are responsible for the breakdown of the cyclodextrins formed, thus limiting their yield.

Key words: Cyclodextrin, cyclization, enzyme intermediate, *Bacillus licheniformis,* saccharides

INTRODUCTION

Cyclodextrin glycosyltransferase (CGTase) is an important industrial enzyme used to produce cyclic α-(1,4)-linked oligosaccharides called cyclodextrins (CDS) from starch and can also be used to produce other oligosaccharides with novel properties (Weijers *et al.*, 2008). CGTase catalyzes mainly transglycosylation reactions (cyclization, coupling, disproportionation) but can also exhibit, to a lesser extent, α-amylase-like activity, hydrolyzing starch into short linear saccharides. The main products of CGTases are cyclic α-, β- and γ-CDS, composed of 6, 7, or 8 glucose residues, respectively. CDS have numerous applications in the pharmaceutical, cosmetics, textile, food, as

well as bioremediation and separation processes, as reviewed (Biwer *et al.*, 2002; Fava and Ciccotosto, 2002; Li *et al.*, 2007; Martin Del Valle, 2004).

CGTases are extracellular inducible enzymes produced predominantly by *Bacillus* species. However, production by a variety of other bacterial species has also been reported. These enzymes are members of the largest superfamily of α-amylase enzymes that act on starch and related α-glucans, called glycoside hydrolyase family 13 (GH13). All family members share a conserved active site architecture along with four short conserved sequence regions embedded in a TIM $(\beta/\alpha)_8$ structural fold, indicating evolutionary diversification from a common enzyme ancestor (Janecek *et al.*, 2003). All members either hydrolyse and/or transglycosylate α-glucosidic linkages to produce α-anomeric mono- and oligo-saccharides. It is therefore the type of acceptor substrate utilized which determines enzyme reaction specificity, a water molecule in the case of α-amylases and a hydroxyl group of a sugar substrate for the glycosyltransferases. Continuous evolution has resulted in enzyme intermediates partially retaining the initial ancestral function while catalyzing new functions (Aharoni *et al.*, 2005). In some cases this has resulted in misidentification of CGTase enzymes (Wind *et al.*, 1995). While, most CGTases generally exhibit low starch hydrolytic rates, *Thermoanaerobacterium thermosulfurigenes* EM1 and *Thermoanaerobacter* sp. ATCC 53627 CGTases display unusually high hydrolytic activity, but still low compared with α-amylases. These CGTases also tend to be highly thermostable (Kelly *et al.*, 2009a, b).

In the present study, the effects of different carbon, nitrogen and metal ion sources on the production of a β-CGTase from a new alkalitolerant *Bacillus licheniformis* SK 13.002 strain, capable of efficiently hydrolysing starch into short linear saccharides in addition to production of CDS, were studied. Effects of pH and temperature on the cyclization and hydrolysis activities of the enzyme were also determined.

MATERIALS AND METHODS

All the materials used for the experiment were procured in year 2009 and the study was conducted between December 2009 and May 2010.

Materials: Soluble starch, cyclodextrin standards, dextrin from maize starch and all other analytical grade chemicals were procured from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Maltodextrin, corn starch, cassava starch, potato starch and wheat starch were purchased from different manufacturing companies in China.

Bacterial strain and culture conditions for CGTase production: Strain SK 13.002, identified by biochemical and 16S rDNA gene sequencing as *Bacillus licheniformis*, was isolated from a soil sample by our laboratory. The gene sequences for this strain have been deposited to the NCBI GenBank database under accession number GU570959.

The fermentation medium used for CGTase production contained per liter: 10 g soluble starch, 10 g soy peptone, 5 g yeast extract, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$ and 7 g Na_2CO_3, added separately after autoclaving. A 3% (v/v) strain inoculum was transferred into a 250 mL conical flask containing the fermentation media and incubated at 37°C for 48 h with continuous orbital shaking at 200 rpm. The cells were harvested by centrifugation (10,000 rpm, 15 min, 4°C) and the supernatant used as the source of the crude enzyme and for activity assays. Estimation of cell growth as optical density was done at 600 nm. The experiments were done in triplicates.

Effect of initial pH on CGTase production: The effect of initial pH on CGTase production was studied by varying Na_2CO_3 concentrations in fermentation media at (w/v) of 0, 0.1, 0.4, 0.7 and 1.0%.

These concentrations corresponded to initial pHs of 6.56, 8.44, 9.52, 9.92 and 10.11, respectively.

Effects of media composition on CGTase production: Soluble starch (1% w/v), soy peptone (1% w/v) and yeast extract (0.5% w/v) in the basal fermentation media were substituted by various carbon sources and nitrogen sources respectively, while the other ingredients were kept constant. The effect of metal ions on the production of CGTase was studied by replacing $MgSO_4 \cdot 7H_2O$ with other metal ion sources by 0.02% (w/v).

Time course production of CGTase: Fermentation using optimal media components was carried out for 96 h using *Bacillus licheniformis* SK 13.002 strain to produce the CGTase enzyme. Samples were collected at regular intervals to measure the cyclization activity and concentration of glucose in the media.

CGTase activity assays

Cyclization activity: Cyclization activity (used as a standard for CGTase production) was measured, according to a modified method by Savergave *et al.* (2008), as a function of the β-CD production rate, using soluble starch (hydrolyzed by boiling for 3 min) as substrate at 1% (w/v) in 50 mM Tris-HCl buffer, pH 7.0, at 65°C for 15 min. The β-CD produced in the assay was determined based on colour fading at 550 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of β-CD per minute under the assay conditions.

Hydrolysis activity: The starch hydrolyzing activity was determined by increase in the concentration of reducing sugars during incubation of enzyme with 1% (w/v) hydrolyzed soluble starch in 50 mM sodium acetate buffer, pH 6.0, at 65°C for 15 min. The concentrations of reducing sugars were determined by DNS method (Miller, 1959) using glucose as standard. One unit (U) of hydrolysis activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as glucose per minute under the assay conditions.

CGTase activities as a function of temperature and pH: The enzyme cyclization and hydrolysis activities at different temperatures and pHs were determined as in above sections. The only difference being that the temperature and pHs were set to different values of pH 3.5-10 and temperature 25-85°C. Buffers used to set the pH values were: Sodium acetate for pH 3.5-6.0; Tris-HCl for pH 7.0-8.0 and Glycine-NaOH for pH 9.0-10.0.

Statistical analysis: The results were subjected to statistical Analysis of Variance (ANOVA) using SPSS Inc. PASW 18 software. The significant differences between means were determined by Duncan Multiple Range Test at $p<0.05$ and $p<0.01$ levels.

RESULTS AND DISCUSSION

Bacterial strain growth and effect of initial pH on CGTase production: The strain grew well in a medium without Na_2CO_3 which had a pH around neutral but produced very small amount

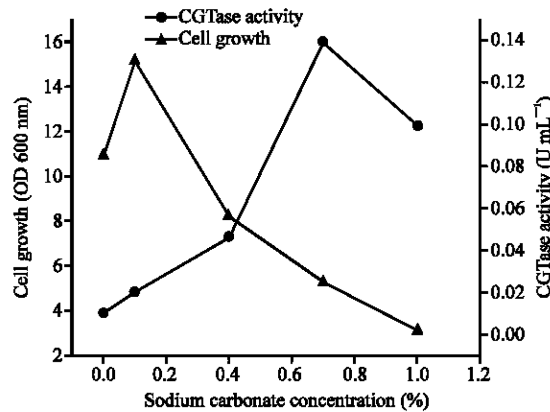

Fig. 1: Effect of initial pH and Na_2CO_3 concentration on cell growth and CGTase production

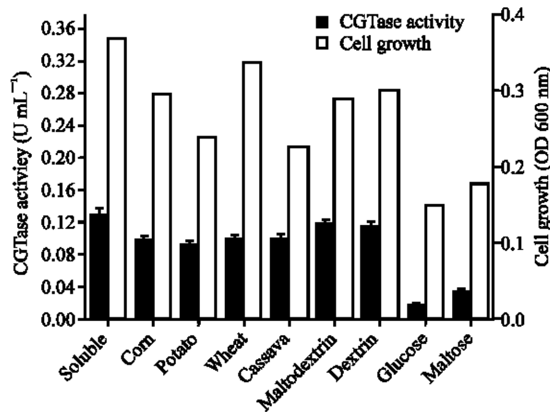

Fig. 2: Effect of different carbon sources on cell growth and CGTase production by *B. licheniformis* SK 13.002 strain. (Statistical significant differences were: $p<0.01$ for soluble starch, maltodextrin and dextrin; $p<0.01$ for corn, potato, wheat and cassava starches; $p<0.01$ for glucose and $p<0.01$ for maltose)

of enzyme as shown in Fig. 1. However, it was also capable of growing in alkaline media with a 0.7% (w/v) Na_2CO_3, which corresponded to pH 9.92 and produced the highest amount of CGTase enzyme activity of 0.139±0.002 U mL^{-1}. This result indicated that *Bacillus licheniformis* SK13.002 strain is alkalitolerant and that a suitable concentration of Na_2CO_3 for adjusting pH is essential for production of its CGTase. It also produced predominantly **β**-CD than other CDS from starch hence it was classified as a **β**-CGTase producer.

Effect of carbon sources on CGTase production: The different carbon sources used revealed that *Bacillus licheniformis* SK 13.002 strain could produce CGTase from all tested starches and the highest activity (0.131±0.003 U mL^{-1}) was achieved from soluble starch, followed by maltodextrin (0.119±0.004 U mL^{-1}) and dextrin (0.116±0.001 U mL^{-1}) from maize starch as shown in Fig. 2. Cassava, wheat potato and corn starches produced almost the same amounts of CGTase with an average of 0.098±0.003 U mL^{-1} CGTase activity. All these carbon sources may have contained an inducer for CGTase production and the source seemed not to be so important for

enzyme production. However, when statistical analysis of variance was done to check significant differences for CGTase production by the different carbon sources, it showed that soluble starch, maltodextrin and dextrin from maize starch were not significantly different at $p<0.01$ level while corn, potato, wheat and cassava starches were also found not to be any significantly different between each other at the same confidence level. The differences in activity obtained may be due to differences in their physical and chemical properties which had no marked impact on CGTase production.

Even though glucose and maltose supported cell growth, CGTase production was very negligible, with 0.0192±0.002 and 0.0354±0.001 U mL^{-1} produced, respectively. Glucose and maltose exerted a catabolite repressive effect on CGTase production, which is a generally observed phenomenon for the synthesis of extracellular bacterial enzymes (Tonkova, 1998). Similar data trend was also observed for CGTase production by *B. lentus* (Sabioni and Park, 1992) and alkaliphilic *Bacillus* sp. ATCC 21783 (Nakamura and Horikoshi, 1976) where mono- and disaccharides showed repression on enzyme production. However, *Bacillus stearothermophilus* (Stefanova *et al*., 1999) and *Bacillus amyloliquefaciens* (Abdel-Naby *et al*., 2000) showed preference to glucose as a carbon source for CGTase production. CGTases in general have a higher affinity to disaccharides compared with monosaccharides which suggests that the acceptor-binding site can recognize at least two glucopyranose moieties (Weijers *et al*., 2008).

Different carbon sources have been reported for maximum production of CGTase for different microbial strains. It has been suggested by Ibrahim *et al*. (2005) that the production of CGTase is a specific reaction process between the microorganism and the carbon source, hence *Bacillus licheniformis* SK 13.002 appears to prefer starch more than simple sugars.

Effect of nitrogen sources on CGTase production: The influence of organic and inorganic nitrogen sources on CGTase production indicated that the strain only produced the enzyme when organic nitrogen was present in the medium. Soy peptone combined with yeast extract was found to be the best nitrogenous compound for the production of CGTase enzyme with activity of 0.105±0.002 U mL^{-1} (Fig. 3). However, soy peptone alone was able to provide the best cellular growth for the strain but could only produce 60% of the CGTase obtained from the combined nitrogen sources. Therefore, soy peptone is good for both enzyme production and cell growth. Yeast extract alone was only able to produce around 30% CGTase, however, it is normally added in the media to supply growth factors because it is rich in amino acids, trace elements and inorganic salts. Other studies on the influence of nitrogen sources also found peptone and yeast extract to promote the highest production of CGTase (Avci and Donmez, 2009; Ibrahim *et al*., 2005; Blanco *et al*., 2009). In the medium without yeast extract very low or no CGTase activities are usually detected, hence yeast extract is needed for the production of CGTase.

Inorganic nitrogenous compounds are not good sources for *B. licheniformis* SK 13.002 strain CGTase production as they did not support any CGTase synthesis (data not shown). However, *B. circulans* DF 9R produced maximum CGTase when ammonium sulfate was used as sole nitrogen source (Rosso *et al*., 2002).

Effect of metal ions on CGTase production: Metal ions are necessary elements for cell growth and maintenance of the active conformation of enzymes (Villafranca and Nowak, 1992). Although, a large number of inorganic salts exist in peptone, the effects of special metal ions on CGTase production are not commonly conducted by many researchers, except for enzyme stability

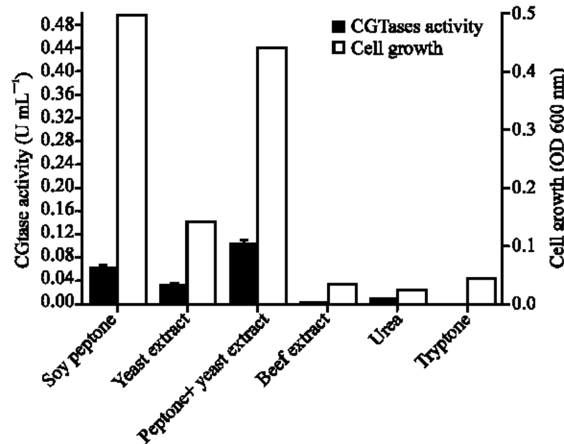

Fig. 3: Effect of different organic nitrogen sources on cell growth and CGTase production by *B. licheniformis* SK 13.002 strain

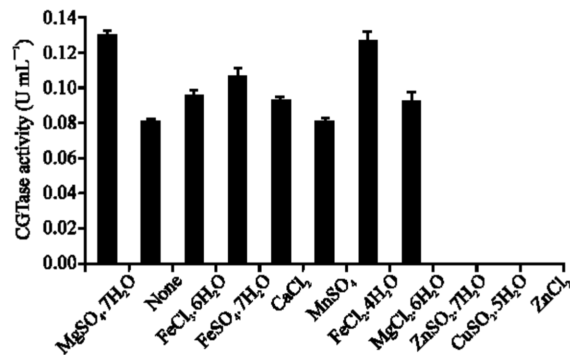

Fig. 4: Effect of different metal ions on CGTase production by *B. licheniformis* SK 13.002 strain. (Statistical significant differences were: $p<0.05$ for $MgSO_4$ and $FeCl_2$; $p<0.05$ for $FeCl_2$ and $FeSO_4$; $p<0.05$ for $FeCl_3$, $FeSO_4$, $CaCl_2$ and $MgCl_2$; $p<0.05$ for None, $FeCl_3$, $CaCl_2$, $MnSO_4$ and $MgCl_2$)

experiments in the presence of these metals. As shown in Fig. 4, $MgSO_4.7H_2O$ was the best for enzyme production to give 0.130±0.003 U mL^{-1} CGTase activity. Previous research by Ibrahim *et al.* (2005), Gawande *et al.* (1998), Gawande and Patkar (1999) and Jin-Bong *et al.* (1990) also found magnesium to be essential for CGTase enzyme production.

However, it is also interesting to note that $FeCl_2.4H_2O$ significantly promoted CGTase production to the same extent like $MgSO_4.7H_2O$ to give a 98% (0.127±0.001) relative CGTase activity amount. There was no significant difference between the effect of $MgSO_4$ and $FeCl_2$ at $p<0.01$. The effect of $FeCl_2$ addition in fermentation media for CGTase production has not been reported before except by Sian *et al.* (2005), who reported that 1 mM $FeCl_2$ enhanced *Bacillus* sp. G1 CGTase residual activity by 189.3% when the metal was preincubated with the enzyme without substrate for 10 min at 25°C. CGTase produced from *Brevibacterium* sp. no. 9605 (Mori *et al.*, 1994) also exhibited a similar property, although not to the same level shown by CGTase from *Bacillus* sp. G1. However, in contrast, Martins and Hatti-Kaul (2002), observed a 10.2% residual activity when *B. agaradhaerens* LS-3C CGTase was preincubated with 1 mM of the same metal at 25°C

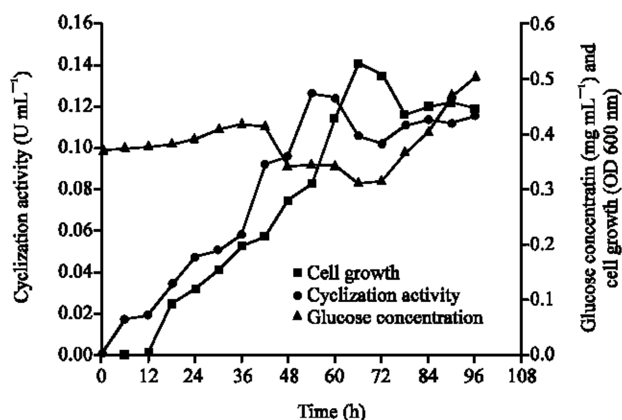

Fig. 5: Cellular growth, CGTase and glucose production by *Bacillus licheniformis* SK13.002 during fermentation

for 1 h. This implies that this metal ion could have an effect on stabilization and induction of CGTase production for some strains.

It is also worth noting that the addition of iron containing metal ions to the fermentation media promoted the biosynthesis of CGTase enzyme by *B. licheniformis* SK 13.002 strain, with $FeSO_4.7H_2O$ and $FeCl_3.6H_2O$ showing 82% (0.106±0.002 U mL^{-1}) and 74% (0.0957±0.004 U mL^{-1}) relative enzyme activities, respectively. CGTase production from either $FeSO_4$ or $FeCl_2$ was found to be statistically similar at $p<0.05$ confidence level. Therefore more work needs to be done to assess the role of iron containing metal ions towards the integrity of CGTases.

$ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $ZnCl_2$ completely inhibited *B. licheniformis* SK 13.002 CGTase production. The same kind of results for $ZnSO_4$ and $CuSO_4$ were observed by Ibrahim *et al.* (2005) and Sian *et al.* (2005). It has also been reported that Cu^{2+} and Zn^{2+} had significant inhibitory effect on CGTase stabilities for *Brevibacterium* sp. No. 9605 (Mori *et al.*, 1994), *Bacillus agaradhaerens* (Martins and Hatti-Kaul, 2002) and *Bacillus* sp. AL-6 (Fujita *et al.*, 1990). The inhibitory effects could be due to the oxidation of amino acid residues essential for the cyclization reaction and transition state stability, especially tryptophan, tyrosine and histidine residues (Machovic and Janecek, 2006; Martins and Hatti-Kaul, 2002).

Time course production of CGTase: As shown in Fig. 5, within 12 h of fermentation, the amount of CGTase production was very small and could hardly be detected. However, it increased steadily until it reached a maximum of 0.141±0.003 U mL^{-1} cyclization activity after 66 h. The cell growth and production of CGTase did not occur at the same rate as the enzyme production lagged behind. After 36 h glucose concentration in the medium reduced steadily as most of it was used for cell growth until it remained constant for some time when cell growth was optimal. After 72 h it started to increase sharply as the enzyme hydrolyzed more starch into short saccharides alongside CD formation until it reached 0.502±0.05 g L^{-1} after 96 h. Although, hydrolysis and cyclization are all performed at a unique active site in the enzyme, they proceed via different kinetic mechanisms (Martins and Hatti-Kaul, 2003; Van der Veen *et al.*, 2000). The hydrolysis side reaction is undesirable, since it produces short saccharides that are responsible for accelerating the breakdown of the cyclodextrin ring during the coupling reaction, thus limiting the final product yields (Kelly *et al.*, 2008). The short saccharides have also been found to have repressive effects on

extracellular enzyme production (Tonkova, 1998). Therefore, the decline in the cyclization activity was attributed to the buildup of glucose in the medium as shown in Fig. 5. When the fermentation time was prolonged for a further 24 h, cyclization activity was not detected while the glucose concentration just remained constant (data not shown).

The α-amylase-like activity of *B. licheniformis* SK13.002 CGTase is thought to be due to partial retention of ancestral enzyme function from evolution over time. Studies of evolutionary relationships within the α-amylase family have provided evidence that CGTase enzymes evolved from α-amylases (Kelly *et al.*, 2009a, b). As all members of GH13 family share both an identical catalytic machinery and mechanism, continuous enzyme evolution within this diverse family has resulted in intermediate enzymes catalyzing new functions while partially retaining ancestral function as a side reaction (Janecek, 1995; Kelly *et al.*, 2008). In some cases this has resulted in misidentification of CGTase enzymes, for example, the *Thermoanaerobacterium thermosulfurigenes* EM1 and *Bacillus* sp. B1018 CGTase enzymes were initially thought to be α-amylases because of their relatively high hydrolytic activity (Itkor *et al.*, 1990; Wind *et al.*, 1995). However, further studies showed that they possessed a clear cyclization activity, as in other CGTases and amino acid alignments with other CGTases also showed a high overall sequence similarity (Kelly *et al.*, 2009a, b; Wind *et al.*, 1995). *Bacillus licheniformis* SK13.002 CGTase is therefore another example of an enzyme at an intermediary stage in between true α-amylases and true CGTases, since besides CDS, significant amount of linear sugars were also formed from starch. It however, appears more likely that it is a CGTase with a few essential mutations modifying product specificity.

While enhanced hydrolytic side reaction of CGTase may have useful applications in the bread baking industry (Jemli *et al.*, 2007), it has been shown to have a detrimental effect on the overall production of cyclodextrins. Increasing cyclodextrin yield and lowering production costs is of particular interest to food, cosmetic and pharmaceutical industries where these circular saccharides have many useful applications. Directed evolution and site-directed mutagenesis studies have revealed that final cyclodextrin product yields could be increased by lowering the hydrolytic side reaction using a combination of error prone polymerase chain reaction (epPCR) and saturated mutagenesis as was done on *Thermoanaerobacterium thermosulfurigenes* EM1 CGTase (Kelly *et al.*, 2008). A single mutation located far from the substrate binding sites improved the cyclization/hydrolysis ratio of the enzyme by lowering the rate of the hydrolytic side reaction up to 15-fold, while cyclization activity was only marginally lowered. However, a mutation on the substrate binding site also showed that cyclization reaction can be completely abolished to remain with only the hydrolysis reaction (Kelly *et al.*, 2007).

Effect of pH on cyclization and hydrolysis activities: The optimum activities of the CGTase measured at varying pH values gave 0.102±0.004 and 0.461±0.003 U mL^{-1} for cyclization and hydrolysis respectively, as displayed in Fig. 6. This meant that hydrolysis activity was four times more than the cyclyzation activity. Optimal cyclization activity observed was at pH 7.0 while optimal hydrolysis activity was at pH 6.0, indicating that this CGTase needs different conditions of pH to effectively carry out both reactions. The starch hydrolysis activity was high in a very broad range of pH values with 99.3% (0.458±0.002 U mL^{-1}) relative activity displayed at pH 5.0 while 83.3% (0.384±0.001 U mL^{-1}) was observed at pH 9.0. However, even when cyclization activity assay was performed at pHs 8.0 and 9.0, there was 75% (0.0762±0.003 U mL^{-1}) and 77% (0.0786±0.002 U mL^{-1}) relative activity, respectively. These established CGTase relative activities in alkaline conditions for both reactions defined this strain as a promising producer of an alkaline

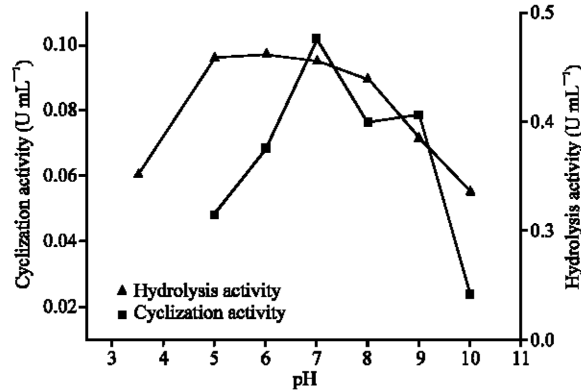

Fig. 6: Effect of pH on cyclization and hydrolysis activities of *B. licheniformis* SK 13.002 CGTase (Hydrolysis activity; Cyclization activity; Buffers used were: Sodium acetate (pH 3.5-6.0), Tris-HCl (pH 7.0-8.0) and Glycine-NaOH (pH 9.0-10.0))

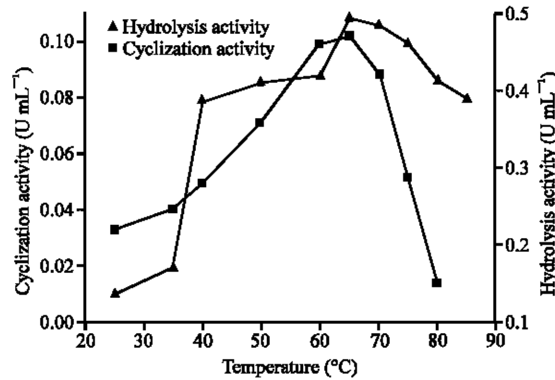

Fig. 7: Effect of temperature on cyclization and hydrolysis activities of *B. licheniformis* SK 13.002 CGTase (Hydrolysis activity; Cyclization activity)

active CGTase. This is an advantage regarding the reduced tendency of starch to retrograde at high pH values (Martins and Hatti-Kaul, 2003).

Effect of temperature on cyclization and hydrolysis activities: The cyclization and hydrolysis activities conducted at different temperatures as shown in Fig. 7, gave optimal values of 0.105±0.004 and 0.494±0.002 U mL^{-1}, at 65°C for both activities, respectively. This meant that hydrolysis activity was still around five times more than the cyclization activity. At 70 and 75°C, the relative cyclization activities were 87% (0.0884±0.002 U mL^{-1}) and 50% (0.0512±0.001 U mL^{-1}), respectively, while those for hydrolysis were 98% (0.484±0.001 U mL^{-1}) and 93% (0.461±0.003 U mL^{-1}), respectively. However, cyclization relative activity at 80°C was only 14% (0.014±0.002 U mL^{-1}) whereas hydrolysis relative activity was still at around 80% (0.402±0.003 U mL^{-1}) for assay at 80-85°C temperature. At elevated temperatures above 60°C, there is improved starch gelatinization, decreased media viscosity, accelerated catalytic reactions and reduced risk of bacterial contamination. An additional benefit of high-temperature catalysis is the inactivation of enzymes originating from food materials which may give rise to undesirable

reactions during processing (Biwer *et al.*, 2002). Therefore *B. licheniformis* SK 13.002 CGTase has a potential for industrial application in processes where high temperature activity is required and can be used in CD production after starch gelatinization without cooling the solution to temperatures lower than 60°C.

The *B. licheniformis* SK 13.002 CGTase enzyme exhibited relatively low cyclization activity of around 0.1 U mL^{-1} as compared to several other CGTases. However, low cyclization activities have also been reported for CGTases from thermophilic *Thermoanaerobacter* sp. P4 (Avci and Donmez, 2009) and *Bacillus sphaericus* (Moriwaki *et al.*, 2009). While this enzyme may be low in protein expression, the high temperature activity property offer protein engineers a genetic template from which to create a highly stable enzyme with the desired properties. Highly thermostable CGTases, therefore, may be useful in the industrial production of CDS, thereby eliminating the need to pre-treat starch with other amylolytic enzymes.

CONCLUSION

A **β**-CGTase from an alkalitolerant *Bacillus licheniformis* SK13.002 strain was produced using different carbon, nitrogen and metal ion sources. Soluble starch, $MgSO_4$ or $FeCl_2$, peptone and yeast extract were needed for CGTase production by this strain. The effect of $FeCl_2$ on the production of CGTase in fermentation has not been reported before. $ZnSO_4$, $ZnCl_2$ and $CuSO_4$ completely inhibited CGTase production. *Bacillus licheniformis* SK 13.002 CGTase could significantly hydrolyze starch into short linear saccharides and this property is thought to be due to partial retention of ancestral enzyme function from evolution over time, hence this CGTase is another example of an enzyme at an intermediary stage in between true α-amylases and "true" CGTases. It also displayed high temperature activities for cyclization and hydrolysis reactions, indicating a potential for application in processes that use high temperature. While enhanced hydrolytic side reaction of CGTase may have useful applications in the bread baking industry, it has a detrimental effect on the overall production of cyclodextrins.

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