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Purification and Characterization of Novel Cyclodextrin Glucanotransferase from *Bacillus* sp. SK13.002

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Bacillus sp. SK13.002 was found to be a novel alkaliphilic cyclodextrin glucanotransferase (CGTase) producing strain. Two CGTase isozymes (CGTase-1 and CGTase-2) could be obtained following precipitation with ammonium sulphate as well as DEAE-Sepharose and Superdex 75 gel chromatography. After purification, the specific activity was increased from 0.49 U/mg (for the crude enzyme) to 9.47 and 8.83 U/mg for CGTase-1 and CGTase-2, respectively. Results obtained from SDS-PAGE showed that the enzyme was purified to homogeneity, with the molecular weight of CGTase-1 and CGTase-2 being estimated to 67.5 and 46.8 kDa and confirmed by LC/MS to be 67.6 and 47.3 kDa, respectively. CGTase-1 exhibited optimum temperature and pH of 65 ºC and 8.0, whereas those of CGTase-2 were 60 ºC and 6.5, respectively. Both CGTase isozymes were found stable at relatively high temperatures and in a very wide range of pH values. Following incubation with 5 % (w/v) soluble starch at 65 ºC and pH 8.0 for 24 h, CGTase-1 produced 36.9 g/L cyclodextrins comprising 81 % β- cyclodextrin. Present findings provided an efficient method for the industrial production of β- cyclodextrin from starch.

Key Words: Cyclodextrin glucanotransferase, Isozyme, *Bacillus*, Cyclodextrin, Purification, Characterization.

INTRODUCTION

Cyclodextrin glucanotransferases (CGTases; EC 2.4.1.19) are industrially important enzymes that produce cyclodextrins from starch. They are extracellular induced enzymes and predominantly produced by *Bacillus* species, although a variety of other bacterial species has been reported to produce CGTase[1]. CGTase catalyzes mainly transglycosylation reactions (cyclization, coupling and disproportionation) but can also exhibit, to a lesser extent, α-amylase-like activity, which hydrolyzes starch into short linear saccharides. The main products of CGTases are α-, β- and γ-cyclodextrins, composed of 6, 7, or 8 glucose residues. Cyclodextrins have numerous applications in the pharmaceutical, cosmetic, textile and food industries, as well as in bioremediation and separation processes. However, most of the CGTases used today convert starch into a mixture of α-, β- and γ-cyclodextrins in different ratios. CGTases can synthesize predominantly one type of cyclodextrin have great commercial importance because separation of one type of cyclodextrin from the products mixture is time-consuming, costly and tedious[2].

Also, for the industrial application of a CGTase, the enzyme thermal stability and pH profile are important properties because the first step in the starch liquefaction is performed at a high temperature under the appropriate pH[3]. For cyclodextrin production, normally a thermostable α-amylase is added during the liquefaction step carried out at high-temperature (95-105 ºC) and later the solution is cooled to 50-55 ºC for the cyclodextrin production by a CGTase[2]. Unfortunately when α-amylase is used, the cyclodextrin yield can be reduced because short maltodextrin oligosaccharides formed during this process can inhibit CGTase action[4]. However, most of CGTases were produced by mesophilic microorganisms and exhibited low thermostability. Therefore, thermostable CGTases may be useful in the industrial liquefaction of starch, thereby eliminating the need to pre-treat with other amylolytic enzymes[5]. Several processes using simultaneous starch liquefaction by thermostable CGTases from extremophiles have been published[6,7].

CGTases from different microorganisms have also different characteristics, including molecular weight, optimum pH, temperature and stability[8-12]. Usually CGTase consists of only a single enzyme, while CGTase isozymes are very scarce[13]. Moreover, to the best of our knowledge, CGTase isozymes have not been purified to homogeneity yet.

In present study, a novel alkaliphilic strain *Bacillus* sp. SK13.002 was found capable to produce CGTase. The enzyme was successfully purified and its main characteristics were investigated.

EXPERIMENTAL

Soluble starch, cyclodextrin, bovine serum albumin and all other analytical grade chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). Protean IEF cell system and electrophoresis equipment were obtained from Bio-Rad Laboratories Inc. Protein standard markers, DEAE-Sepharose CL 6B Fast Flow and Superdex 75 chromatography equipments were supplied by Sangon Biotech Co., Ltd., (Shanghai, China).

Bacterial strain and culture conditions for CGTase production: *Bacillus* sp. SK13.002 was originally isolated from a soil sample in our laboratory. The 16S rRNA 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 (g/L), soluble starch 10, soy peptone 10, yeast extract 5, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.2 and Na_2CO_3 8. A 3 % (v/v) culture inoculum was transferred into a 250 mL conical flask containing 33 mL fermentation medium and incubated at 37 ºC for 96 h with continuous orbital shaking at 200 rpm. The cells and insoluble materials were removed by centrifugation at 5000 g for 15 min at 4 ºC and the supernatant was used as crude enzyme.

CGTase activity assay (hydrolytic activity): The starch hydrolyzing activity of CGTase was assayed using the method of Shiosaka and Bunya[14], based on the decrease of blue colour intensity of the amylose-iodine complex formed after reaction of CGTase with 0.3 % (w/v) soluble starch in 20 mM sodium acetate buffer (pH 5.5) at 40 ºC for 10 min. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed a 10 % decrease of the initial absorbance per min under the assay conditions.

Purification of CGTase

CGTase concentration and precipitation: Concentrated enzyme solution was obtained using Millipore ultrafiltration system (Millipore Company, U.S.A.) with a molecular weight cut-off point of 10 kDa. The concentrated enzyme solution was then precipitated with 70 % saturated solid ammonium sulphate at 4 ºC. The mixture was centrifuged at 10,000 rpm and 4 ºC for 15 min and the pellets were dissolved in 20 mM Tris-HCl buffer, pH 8 before dialysis against the same buffer overnight at 4 ºC.

Ion-exchange chromatography: Five mL of the enzyme sample obtained after precipitation with ammonium sulphate and dialysis was loaded onto DEAE-Sepharose CL-6B Fast Flow column, which was equilibrated with 20 mM Tris-HCl buffer, pH 8. The column was washed with the same buffer at a flow rate of 1.5 mL/min to remove unbound proteins. For elution, gradient concentrations of NaCl (0-1.0 M) were used at a flow rate of 1.5 mL/min. The collected fractions were analyzed for protein content and CGTase activity.

Gel filtration chromatography: The CGTase active fraction obtained from ion-exchange chromatography were concentrated and dialyzed in 20 mM Tris-HCl buffer, pH 8. One mL of sample was loaded onto Superdex 75 gel column previously equilibrated with the same buffer. Elution was carried out using the same buffer at a flow rate of 0.5 mL/min.

Determination of protein content and molecular weight: The protein contents of fractions obtained at different purification steps were determined according to Lowry *et al.*[15] using bovine serum albumin as standard. The molecular weight of purified enzyme was estimated by SDS-PAGE[16] using 4 % acrylamide for the stacking gel and 12 % acrylamide for the separating gel. Lysozyme (14.4 kDa), Trypsin inhibitor (20.1 kDa), Carbonic anhydrase (31 kDa), Rabbit actin (43 kDa), bovine serum albumin (66.2 kDa) and rabbit phosphorylase B (97.4 kDa) were used as standard protein molecular weight markers. The gel was stained with 1 % Coomassie Brilliant Blue R-250 (Bio-Rad). The molecular weight of purified enzyme was confirmed using liquid chromatography coupled with mass spectrometry (Waters, Milford, USA).

Isoelectric point (pI) determination: Isoelectric focusing was performed in a protean IEF cell system (Bio-Rad) following the manufacturer's instructions. Reference proteins used for the determination of pI were phycocyanin (4.45, 4.65, 4.75), β-lactoglobulin B (5.1), bovine carbonic anhydrase (6.0), human carbonic anhydrase (6.5), equine myoglobin (6.8, 7.0), human hemoglobin A (7.1), human hemoglobin C (7.5), lentil lectin (7.8, 8.0, 8.2) and cytochrome c (9.6). Gels were stained with Coomassie Brilliant Blue R-250.

Optimum pH and temperature: The optimum pH of purified CGTase was determined by assaying the activity at 40 ºC using various buffers at different pH values, *i.e.*, 0.2 M sodium acetate, (pH 3.0-5.5), 0.2 M sodium phosphate, (pH 6.0-8.5) and 0.2 M glycine-NaOH (pH 9.0-11.0). The optimum temperature of purified CGTase was determined by carrying out the enzymatic reaction at different temperatures ranging from 30 to 80 ºC.

Thermal and pH stability: The pH stability was determined by incubating 0.1 mL purified enzyme solution with 0.2 mL of each one of the buffers mentioned previously at 60 ºC, without substrate for 2 h and the remaining activity of the enzyme was assayed as mentioned above. The thermal stability was measured by incubating 0.1 mL pure CGTase at temperatures ranging from 60 to 75 ºC for up to 2 h. The residual activity was assayed as described previously and calculated taking into account the initial activity as control.

Effects of metal ions on CGTase activity: The activity was assayed using different metals at 1 mM dissolved in 0.2 M sodium phosphate buffer (pH 8) for CGTase-1 and 0.2 M sodium acetate buffer (pH 6.5) for CGTase-2. The residual activity was expressed as a percent of the enzyme activity obtained without metal (control).

Analysis of CGTase reaction products: CGTase was applied in the production of cyclodextrins by incubating purified enzyme and 5 % (w/v) soluble starch as substrate for 24 h at pH 8 and 65 ºC (CGTase-1) as well as pH 6.5 and 60 ºC (CGTase-2). Samples were collected at regular time intervals and inactivated by heating in boiling water for 5 min. Reaction products were analyzed by HPLC with an Asahipak NH2P-50 4E Shodex column (4.6 mm id × 250 mm, Tokyo, Japan) at 30 ºC and a refractive index detector. A mobile phase consisting of acetonitrile/water (70/30, v/v) was used at a flow rate of 1.0 mL/min.

RESULTS AND DISCUSSION

Purification of CGTase: Two individual isozymes of the CGTase produced by *Bacillus* sp. SK13.002, designated as CGTase-1 and CGTase-2, were successfully separated and purified from the crude enzyme. Multiple steps were applied for CGTase purification, which included ultrafiltration, precipitation with ammonium sulphate, DEAE-Sepharose and Superdex 75 gel filtration chromatography (Table-1).

The DEAE-Sepharose CL-6B Fast Flow column chromatography of CGTase resulted in 15.1 purification fold and 26.7 % recovery yield (Table-1). As shown in Fig. 1., CGTase was eluted with a gradient concentration of salt (from about 0.2 to 0.3 M NaCl), whereas other tightly bound proteins were eluted with 1.0 M NaCl. The active fraction collected at this purification step exhibited two major bands on SDS-PAGE (Fig. 3).

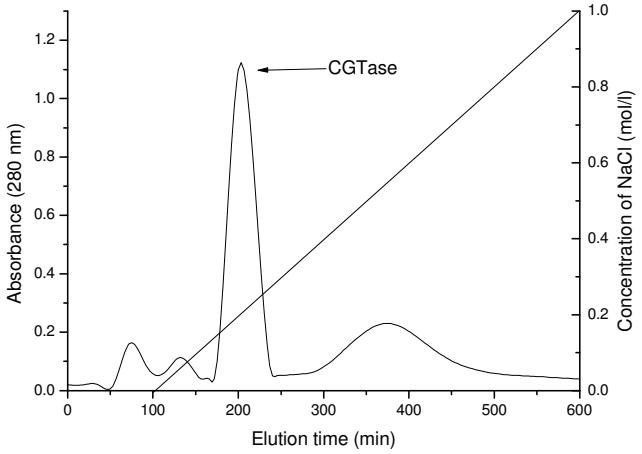

Fig. 1. Ion-exchange chromatogram of CGTase. The enzyme solution obtained after precipitation with ammonium sulphate and dialysis was loaded onto DEAE-sepharose CL-6B fast flow column previously equilibrated with 20 mM Tris-HCl (pH 8) buffer. Elution was carried out using 0-1 M NaCl at a flow rate of 1.5 mL/min.

The active fraction obtained after ion-exchange chromatography was further purified using Superdex 75 gel filtration chromatography. Two isozymes, CGTase-1 and CGTase-2 (Fig. 2), were observed as two individual single bands on SDS-PAGE (Fig. 3). The purification process increased the specific activity from 0.49 U/mg for the crude enzyme to 9.47 and 8.83 U/mg for CGTase-1 and CGTase-2, respectively (Table-1).

Characterization of purified CGTase

Determination of CGTase molecular weight : As shown in Fig. 3. the two CGTase isozymes CGTase-1 and CGTase-2 obtained after gel filtration chromatography were purified to homogeneity (Fig. 3). The molecular weight of CGTase-1 and CGTase-2 were estimated by SDS-PAGE to be 67.5 and 46.8 kDa, respectively. Moreover, results from LC/MS showed 67.6 and 47.3 kDa respectively for CGTase-1 and CGTase-2. Most of the previously reported CGTases from various *Bacillus* strains had a molecular weight range of 68-88 kDa[17-21]. However, a molecular weight as low as 38 kDa was observed for CGTase produced by *Bacillus* sp. strain[22], whereas molecular weights as high as 103[23] and 110 kDa[24] have been reported on CGTases from *B. circulans* and *B. agaradhaerens*, respectively.

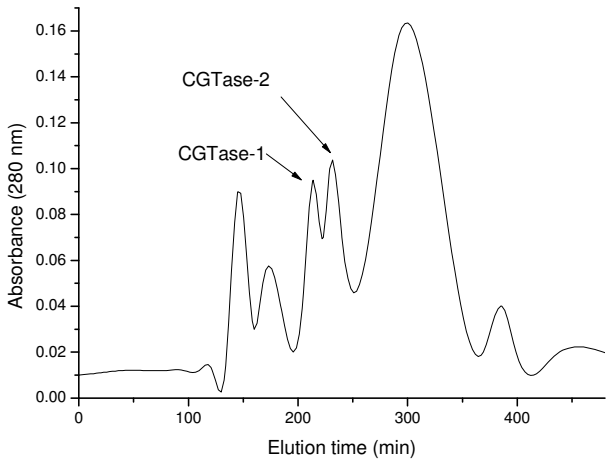

Fig. 2. Gel filtration chromatogram of CGTase. The active fraction obtained from ion-exchange chromatography was concentrated and dialyzed before being loaded onto Superdex 75 gel column previously equilibrated with 20 mM Tris-HCl (pH 8.0) buffer. Separation was performed using the same buffer at a flow rate of 0.5 mL/min.

The isoelectric points (pI) of CGTase-1 and CGTase-2 from *Bacillus* sp. SK13.002 were about 8.1 and 5.0, respectively. CGTase from *B. circulans* IFO 3329 exhibited a pI of 8.8[25], whereas that of CGTase produced by *B. firmus* was 9.6[21]. Although CGTases from a few microorganisms such as *Bacillus* sp.[26] and *B. circulans* E 192[27] have also been reported to exist as isozymes with different pI values, *Bacillus* sp. SK13.002 can be considered as another unique strain that produces two CGTase isozymes.

Effects of temperature on CGTase activity and stability: The effect of temperature on CGTase activity was investigated by incubating the reaction mixture at different temperatures. The optimum temperatures were found to be 65 and 60 ºC for CGTases-1 and CGTase-2, respectively (Fig. 4). Furthermore, the remaining activity after incubation at 60 ºC for 2 h was as high as 76.5 and 79.1 % respectively for CGTases-1 (Fig. 5A) and CGTase-2 (Fig. 5B). Following incubation at 65 ºC for 2 h, CGTase-1 and CGTase-2 could retain respectively 42.1

TABLE-1
PURIFICATION STEPS OF CGTase FROM *Bacillus* sp. SK13.002

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery yield (%)
Crude enzyme	3281.0	6656.3	0.49	1.0	100
Ultrafiltration	2979.1	1610.4	1.85	3.8	90.8
$(NH_4)_2SO_4$ precipitation	1374.7	313.9	4.38	8.9	41.9
DEAE-Sepharose CL-6B	876.0	118.4	7.40	15.1	26.7
Superdex 75 CGTase-1	298.4	31.5	9.47	19.3	9.1
CGTase-2	328.3	37.2	8.83	18.0	10.0

and 57.1 % of their initial activity, indicating that both isozymes have prominent thermal stability.

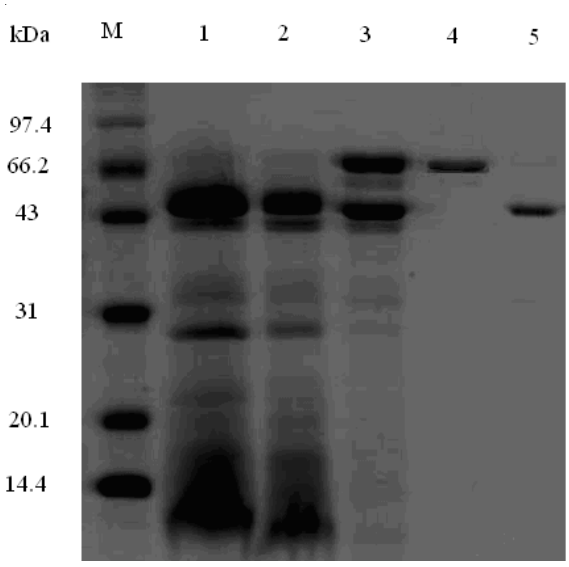

Fig. 3. SDS-PAGE of CGTase at different purification steps. Lane M, standard protein markers; Lane 1, enzyme sample obtained after precipitation with ammonium sulphate; Lane 2, enzyme sample obtained after dialysis; Lane 3, DEAE-Sepharose enzyme fraction; Lane 4, Gel filtration enzyme fraction (CGTase-1); Lane 5, Gel filtration enzyme fraction (CGTase-2).

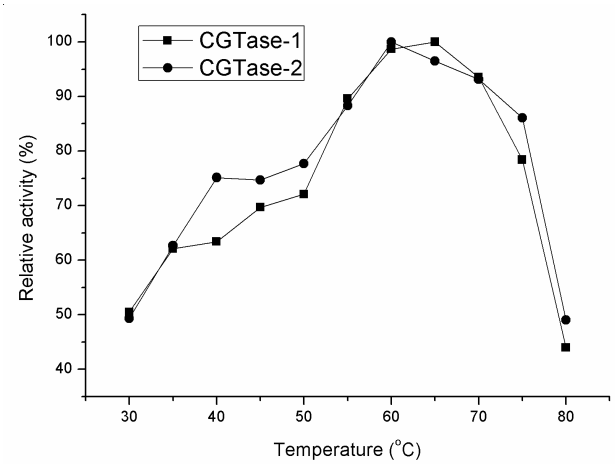

Fig. 4. Optimal temperature for CGTase

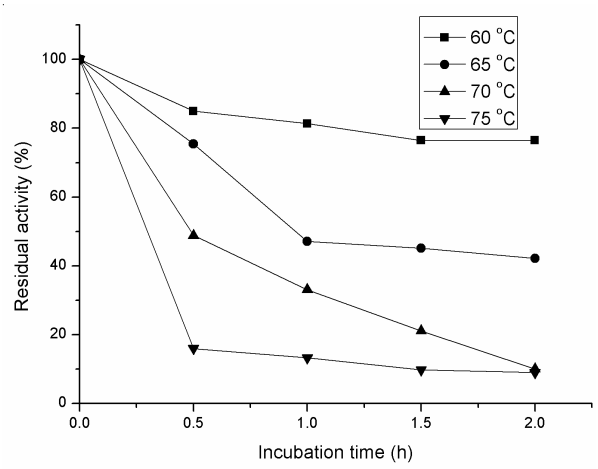

Fig. 5A. Thermal stability for CGTase-1

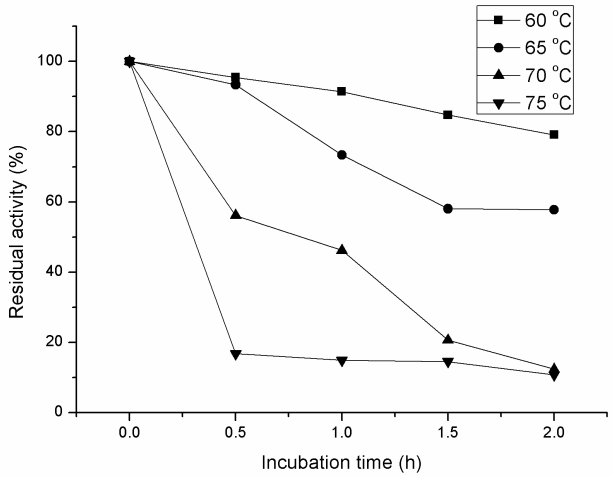

Fig. 5B. Thermal stability for CGTase-2

Effects of pH on CGTase activity and stability: The effect of pH on CGTase activity was tested using various buffers at different pH. The optimum pH values were found to be 8.0 and 6.5 for CGTase-1 and CGTase-2, respectively (Fig. 6). Both CGTase isozymes exhibited a wide pH range to perform their reactions. In addition, results showed that CGTase-1 and CGTase-2 have very prominent pH stability. After exposure at pH ranging from 4 to 10 for 2 h, the remaining activity was higher than 96 %. Larsen *et al*., reported that CGTase from *Paenibacillus* sp. F8 remained stable for 1h between pH 6 and 9[28], whereas other CGTases could remain stable between pH 8 and 10[29,30]. The wide range of reaction pH observed for both CGTase-1 and CGTase-2 makes *Bacillus* sp. SK13.002 a novel CGTase-producing strain.

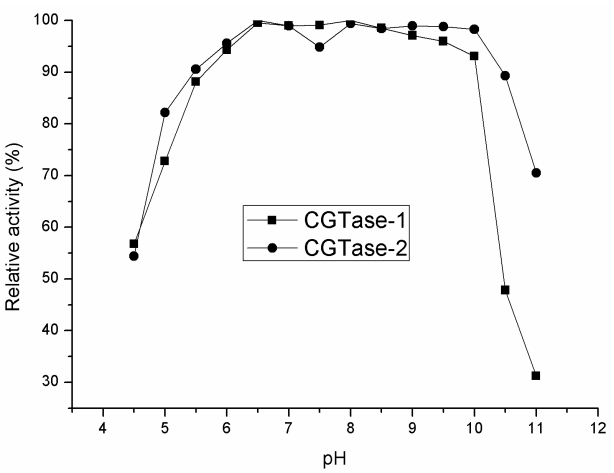

Fig. 6. Optimal pH for CGTase

Effects of metal ions on CGTase activity: The effects of different metal ions on CGTase activity are summarized in Table-2. Ca^{2+} could slightly enhance the activity of CGTase-2. Fe^{3+}, Cu^{2+} and Zn^{2+} inhibited the activity of both CGTase-1 and CGTase-2, with Cu^{2+} being the strongest inhibitor. Mg^{2+}, Fe^{2+}, Mn^{2+}, Na^{+} and K^{+} had almost no effect on the activity of both CGTase isozymes; Co^{2+} and Ni^{+} did not affect the activity of CGTase-2, but could inhibit to some extent the activity of CGTase-1. Previous studies stated that CGTase from *Bacillus* sp. G[18], *Bacillus* sp. AL-6[19]and *Bacillus autolyticus* 11149[18]

were activated by the presence of Ca^{2+}, whereas CGTase from *Bacillus halophilus* was not affected by Ca^{2+} [31]. However, other CGTases were found to be inhibited strongly by Cu^{2+} and Zn^{2+} [8].

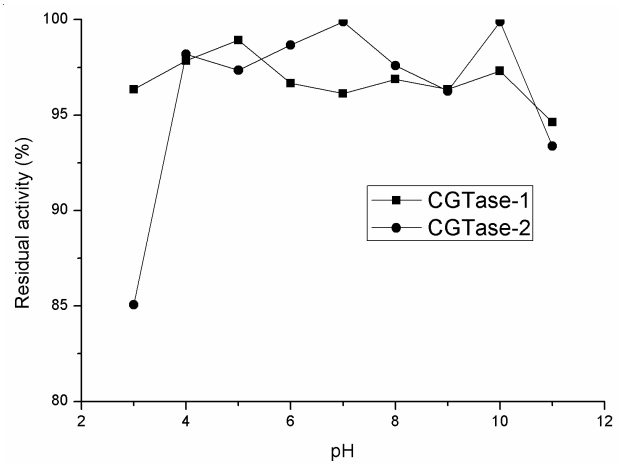

Fig. 7. pH stability for CGTase

TABLE-2
EFFECTS OF DIFFERENT METAL IONS ON CGTase ACTIVITY

Metal ion	Residual activity (%)	
	CGTase-1	CGTase-2
None	100	100
$FeSO_4$	95.60	98.20
$NiSO_4$	85.05	96.46
$ZnSO_4$	45.40	67.30
$CuSO_4$	11.52	6.86
$MnSO_4$	96.68	98.06
Na_2SO_4	96.12	99.24
K_2SO_4	96.90	98.27
$MgSO_4$	99.00	99.76
$CoCl_2$	87.82	98.72
$CaCl_2$	98.56	102.36
$FeCl_3$	67.44	87.24

Cyclodextrin production: *Bacillus* sp. SK 13.002 CGTase produced mainly β-cyclodextrin with minor amounts of γ-cyclodextrin from soluble starch without formation of α-cyclodextrin. Most of the CGTases produced a mixture of α-, β- and γ-cyclodextrin at different ratios and the separation of one cyclodextrin type from the mixture is therefore time-consuming, costly and tedious. The time course for cyclodextrins production, which is presented in Fig. 8. indicated that the production rate increased rapidly during the first 12 h of reaction time but raised slowly afterwards. According to Jemli *et al.*[10], the maximum product concentration obtained with most of β-CGTases ranged from 3 to 40 g/L. Following incubation of CGTase-1 with 5 % (w/v) soluble starch at 65 ºC and pH 8 for 24 h, the cyclodextrin production yield was about 36.9 g/L. However, when CGTase-2 was incubated with 5 % (w/v) soluble starch at 60 ºC and pH 6.5 for 24 h, the conversion yield was only 13.3 g/L. For CGTase-1, both β- and γ-cyclodextrin could be produced, with β-cyclodextrin representing 81 % of total cyclodextrin. These findings provided an effective method for the industrial production of β-cyclodextrin from starch directly without using any other amylolytic enzymes.

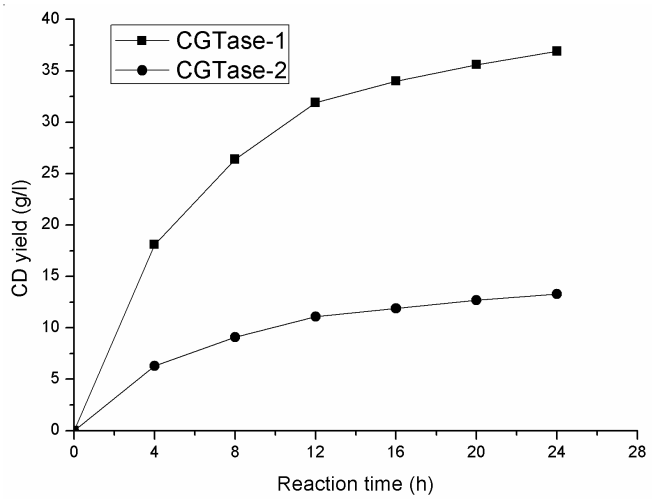

Fig. 8. Time-course for cyclodextrin production

Conclusion

A novel alkaliphilic strain *Bacillus* sp. SK13.002 showed a high ability to produce cyclodextrin glucanotransferase (CGTase). Two CGTase isozymes, namely CGTase-1 and CGTase-2, could be obtained after purification. Both CGTase-1 and CGTase-2 were found to be good candidates for the industrial production of β-cyclodextrin from soluble starch, with CGTase-1 providing a higher cyclodextrin yield. However, determination of the amino acid residues and sequence regions as well as cloning and expression of the CGTase gene from *Bacillus* sp. SK13.002 are required to gain further information about this enzyme.

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