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Characterisation of cyclodextrin glucanotransferase from *Bacillus circulans* ATCC 21783 in terms of cyclodextrin production

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Abstract - Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) from *Bacillus circulans* ATCC 21783 was purified by ultrafiltration and a consecutive starch adsorption. Total enzyme yield of 75.5% and purification factor of 13.7 were achieved. CGTase was most active at 65 ºC, possessed two clearly revealed pH-optima at 6.0 and 8.6 and retained from 75 to 100% of its initial activity in a wide range of pH, between 5.0 and 11.0. The cyclising activity was enhanced by 1 mM $CaCl_2$ or 4 mM $CoCl_2$. The enzyme was thermostable up to 70 ºC, and 64% of the original activity remained at 70 ºC after 30 min heat treatment. Up to 41% conversion into cyclodextrins was obtained from 40 g l^{-1} starch without using any additives. This CGTase produced two types of cyclodextrins, beta and gamma, in a ratio 73:27 after 4 h reaction time at 65 ºC. This feature of the enzyme could be of interest for industrial cyclodextrin production.

Key words: *Bacillus circulans* ATCC 21783, cyclodextrin glucanotransferase, cyclodextrins, enzyme stability, starch conversion.

INTRODUCTION

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is known to catalyse four different reactions: cyclisation, coupling, disproportionation and hydrolysis and mainly produced by members of the genus *Bacillus* (Tonkova, 2006). This enzyme is able to convert starch and related α-1,4-glucans into cyclodextrins (CDs), cyclic non-reducing oligosaccharides, composed from six or more glucose units joined by means of α-1,4-glucosidic bonds (Terada *et al.,* 1997). As separation of different CDs is costly and time-consuming, CGTase that synthesizes predominantly one type of CD is of interest. Generally, the majority of the *Bacillus* CGTases convert starch into β-CD as the main product (Sabioni and Park, 1992; Marechal *et al.,* 1996; Chung *et al.,* 1998; Gawande *et al.,* 1999; Park *et al.,* 1999; Martins and Hatti-Kaul, 2002; Sian *et al.*, 2005). The ability of CDs to form inclusion complexes with many organic and inorganic molecules, changing their physical and chemical properties makes CGTase an important enzyme for the medicine, food, cosmetic, pharmaceutical industries and in environmental protection (Szejtli, 1997; Singh *et al.,* 2002). CDs found a wide application in food industry based on the formation of inclusion complexes with fats, flavours and colours. They are used for the removal of cholesterol from eggs and dairy products, phenolic compounds from fruits and vegetable juices (Hedges, 1998), for a detection of aflatoxin in food samples (Cepeda *et al.,* 1996; Fente *et al.,* 2001). CDs and especially the β-CDs are applied in environmental protection by a solubilisation of organic contaminants and removal of highly toxic substances from soil, water and atmosphere (Szejtli, 1997; Singh *et al.,* 2002). Beta-CDs play a major role in the bioremediation of chronically contaminated soils by increasing of water solubility and reducing the toxicity of fungicides making them more susceptible to biodegradation. CDs are also used for the synthesis of organic nanopolymers (pore size 0.7-1.2 nm), possessing the ability to absorb organic contaminants and by this way to reduce their quantity to parts-per-trillion levels (Li and Ma, 2000). In addition to production of CDs through the cyclisation reaction, CGTase can be used for its coupling and disproportional reactions for the synthesis of modified oligosaccharides by alternative acceptor substrates and limit dextrins (Van der Veen *et al.,* 2000).

Our previous studies on batch and semicontinuous cultivation of free and agar- or membrane-immobilised cells of alkalitolerant *Bacillus circulans* ATCC 21783 for β-CGTase production in a bubble column reactor showed a significantly higher CGTase yield compared to another *Bacillus* producers (Vassileva *et al.,* 2003a, 2003b). The further continuous enzyme synthesis realised by free and immobilised cells in a stirred and a bubble column reactors (four different systems for continuous CGTase production) provided 3-5-fold higher CGTase yield and 1.5-2.9-fold higher enzyme productivity in comparison to known strains of *Bacillus* applied for the same aim (Vassileva *et al.*, 2005). The purpose of the present work was to be obtained crude and purified CGTase from *Bacillus circulans* ATCC 21783

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and to be studied some enzyme properties in terms of cyclodextrin production.

MATERIALS AND METHODS

Bacterial strain and media. *Bacillus circulans* ATCC 21783 was supplied by the National Bank of Microorganisms and Cell Cultures (Sofia, Bulgaria) and cultivated in nutrient medium containing (g l^{-1}): soluble starch (Fluka, Poland), 2; peptone (Oxoid, Basingstoke, UK), 5; yeast extract (Oxoid), 5; $MgSO_4$, 0.2 and K_2HPO_4, 1. Sterile sodium carbonate was used to adjust the medium to pH 9.8-10.0 after autoclaving.

Enzyme purification. The active culture liquid was obtained after 24 h cultivation in a laboratory bioreactor Bioflo (New Brunswick, USA) with working volume 350 ml under the following growth conditions: inoculum concentration 2% (v/v, $OD_{650\ nm}$ 1.0-1.3), 40 ºC, air flow rate 1 v/v/min and agitation rate 400 rpm (Vassileva *et al.,* 2005). It was centrifuged at 4000 *x g* for 20 min and the supernatant solution was concentrated and partially purified by ultrafiltration using Millipore Minitan™ Ultrafiltration System (Bedford, Massachusetts, USA; membranes PM 30 kDa). The concentrate obtained was used as a crude enzyme preparation.

Further purification of the crude enzyme was performed by adsorption to insoluble corn starch followed by elution with buffer containing β-CD. To 21 ml appropriate diluted crude enzyme containing 0.57 mg ml^{-1} total protein were added 5% (w/v) insoluble corn starch and ammonium sulphate (15% saturation) with a continuous agitation to allow enzyme adsorption. This procedure extended 1 h at 8 ºC. The mixture was centrifuged at 4500 *x g* for 10 min and the residue was washed twice with 10 ml of cold water. Because the crude enzyme possesses high activity in both, acidic and alkaline ranges of pH, the CGTase was separated from the starch by two elution procedures. Firstly, the residue was incubated with 5 ml 1 mM β-CD in 50 mM phosphate buffer, pH 6.0, for 30 min at 37 ºC with shaking followed by centrifugation. The elution with the same buffer (3 ml) was repeated once. After that, the second additional elution procedure was performed by the same manner, but with 1 mM β-CD in 50 mM Tris buffer, pH 8.6. The pooled eluates (8 ml for pH 6.0 and 8 ml for pH 8.6) were dialysed against 50 mM phosphate buffer, pH 6.0 and 50 mM Tris buffer, pH 8.6, at 8 ºC.

Enzyme characteristics. The enzyme activity was studied over the pH range of 4.0 to 11.0, using the following buffers: 0.1 M Na-acetate-CH_3COOH (pH 4.0-5.6), 1/15 M potassium-sodium phosphate (pH 6.0-8.0) and 0.1 M glycine-NaOH-NaCl (pH 8.6-11). The temperature profile and thermal stability of the enzyme were studied over the range 40-80 ºC and 50-70 ºC, respectively. The effect of various metal ions and reagents on CGTase activity was determined after incubation at 25 ºC for 30 min.

Gel electrophoresis. The crude and the purified enzyme fractions (pH 6.0 and 8.6) were analysed by SDS-PAGE on a 7-17% polyacrylamide gradient gel. The samples (1.5 ml) were vacuum dried in a speed vac (Savant SC110-A, Holbrook, NY, USA) and resuspended with 3 M urea (100 µl). This solution was one fold diluted by a sample buffer (Tris/HCl 10 mM, glycerol 20% (v/v), SDS 1% (w/v), β-mercaptoethanol 1% (v/v), bromophenol blue 0.05% (w/v) pH 6.8) and boiled for 3 min. Crude enzyme (10 µl), purified enzyme samples (15 µl) and molar mass standards (5 µl) were deposed onto the polyacrylamide gel. The molar mass standards applied were rabbit myosin (205 kDa), phosphorylase B (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and bovine carbonic anhydrase (29 kDa). The migration was realised under a 500 volt tension and a 35 mA current intensity delivered by a generator (Shimadzu C-R 3A Chromopac, Columbia, MD, USA) during 2.5 h. Proteins were stained 1 h in a 0.25% Coomassie blue R 250 methanol/acetic acid/H_2O solution.

Assay of the cyclising activity. CGTase cyclising activity was determined by the method of Kaneko *et al.* (1987) based on the reduction in the colour intensity of phenolphthalein after complexation with β-CD. One unit of CGTase activity was defined as the amount of enzyme that formed 1 µg of β-CD min^{-1} under standard conditions (substrate starch soluble acc. to Zulkowsky, Fluka; phosphate buffer pH 6.0; 60 ºC, 20 min reaction time; after determination of the temperature optimum at 65 ºC).

Total protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Cyclodextrin production. CDs formed were measured by HPLC systems at a room temperature. Starch (40 g l^{-1}, Fluka) and 60 U crude CGTase per gram substrate were incubated at 65 ºC, pH 6.0. The reaction was stopped after 4 h by placing the samples in a boiling water bath for 5 min. They were cooled, centrifuged at 6000 *x g* for 15 min, filtered through a membrane filter (0.45 µm, Millipore) and analysed for CDs using YMC-Pack-ODS-AQ column. The mobile phase was methanol:water (7:93, v/v) with a flow rate 1.2 ml min^{-1}. Injection volume was 20 µl.

RESULTS AND DISCUSSION

Purification of the CGTase

The bacterial cells from the culture liquid were harvested by centrifugation and the clear supernatant was concentrated by ultrafiltration using a 30 kDa membrane. A 14.7-fold concentration and 1.3-fold purification of CGTase with an enzyme recovery of 87% was achieved. The crude CGTase obtained was with a specific activity 1413 U mg^{-1} and a total protein content 2.1-2.4 mg ml^{-1}.

The further starch adsorption was performed with four time diluted crude enzyme. A significant enzyme yield and high purification factor were achieved (Table 1). Some purification variants by starch adsorption procedure were performed. Different quantities of a crude enzyme and ammonium sulphate as well as three variants with a change of the order of the eluting buffers were used. The highest enzyme yield (63.9, total 75.5%) and 13.7-fold purification were achieved under the experimental conditions described in Materials and Methods.

Purified CGTases to a homogeneous protein for CD production are not often applied on industrial scale (Horikoshi, 1999). Therefore, a large part from the experiments was carried out with the crude preparation.

TABLE 1 - Purification of the diluted crude CGTase by starch adsorption

Enzyme samples	Volume (ml)	Activity (U ml^{-1})	Protein (mg ml^{-1})	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme	21	715 ± 0.07	0.570 ± 0.03	1254.4	1	100
Eluate pH 6.0	5+3	1200 ± 0.07	0.070 ± 0.02	17142.9	13.7	63.9
Eluate pH 8.6	5+3	218 ± 0.04	0.030 ± 0.02	7266.7	5.8	11.6
Total			0.100			75.5

Data are mean values ± SD, n = 3.

Temperature profile of the enzyme

The cyclisation activity of the crude CGTase was measured at various temperatures in phosphate buffer (pH 6.0). The enzyme was optimally active at 65 ºC (Fig. 1). The performance of the enzyme reaction at 70ºC showed a high relative activity (90.4%), which is a positive feature of the CGTase since the applying of high temperature in CD production affects significantly the CD yield.

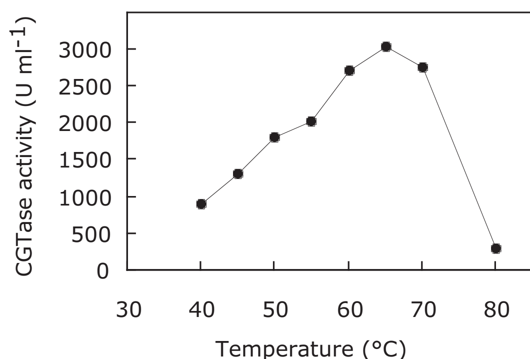

FIG. 1 - Temperature profile of the crude CGTase. The enzyme activity was measured at various temperatures in phosphate buffer (pH 6.0). Data are mean values ± SD from 0.07 to 0.21, n = 3.

Effect of pH on CGTase activity and stability

The pH profile of the crude CGTase was studied under varying pH values ranging from 4.0 to 9.6 at 60 ºC (Fig. 2). The crude enzyme exhibited two pH optima, in the acidic range at pH 6.0 and in the alkaline range at pH 8.6, which suggests two CGTases, each one active in a different pH range. Maximum enzyme activity occurred at pH 6.0. Most of the reported *Bacillus* CGTases show optimal cyclising activity in pH range 5.0-7.0 (Boveto *et al*., 1992; Sabioni and Park, 1992; Marechal *et al*., 1996; Yim *et al.,* 1997; Chung *et al.,* 1998). Only CGTase from *Bacillus agaradhaerens* LS-3C is optimal active at pH 9.0 (Martins and Hatti-Kaul, 2002). Two pH optima (5.5 and 8.5) have been established for CGTase from alkaliphilic *Bacillus firmus* (Higuti *et al.,* 2003) and *Bacillus firmus* NCIM5119 (Gawande *et al*., 1999).

The pH stability of the enzyme was determined by preincubating the enzyme in various buffers for 15 min and 1 h at 25 ºC and then the residual activities were measured using the standard assay conditions at 65 ºC (at the established temperature optimum). Untreated enzyme was used as a control (100% activity). The residual CGTase activities obtained were from 75 to 100% over the wide range of pH, between 5.0 and 11.0 (Fig. 3). At pH 4.0 the residual activities were 73.3% and 46.6% after 15 min and 1 h pretreatment, respectively. CGTases from other strains of *Bacillus circulans* have been reported to possess more limited pH range for stability, from 6.5-7.0 to 8.0-8.5 (Boveto *et al*., 1992; Marechal *et al*., 1996). The high activity of the enzyme from *Bacillus circulans* ATCC 21783 in a wide pH range provides the performance of CD production process without maintaining a constant pH.

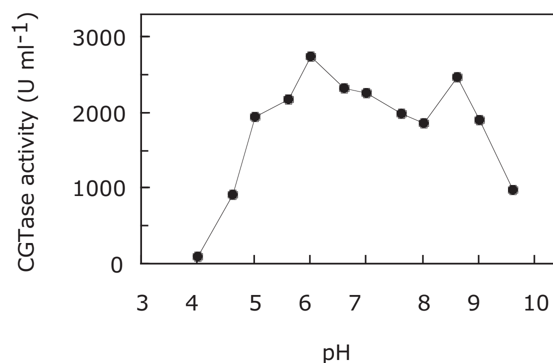

FIG. 2 - pH-profile of the crude CGTase. The enzyme activity was measured in buffers of various pHs at 60 ºC. Data are mean values ± SD from 0.06 to 0.07, n = 3.

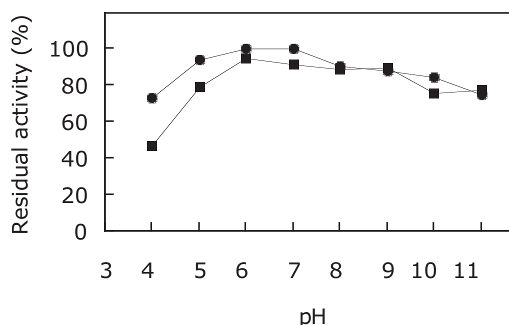

FIG. 3 - pH-stability of the crude CGTase. The enzyme was stored in buffers of various pHs at 25 ºC for 15 min (•) and 1 h (▪), and the residual activities were measured under standard assay conditions at 65 ºC. Data are mean values ± SD from 0.035 to 0.049, n = 3.

Effect of various reagents on cyclising activity

The enzyme solution was preincubated at 25 ºC for 30 min in the presence of 1 mM of ions or reagents, and then cyclising activity was measured in phosphate buffer (pH 6.0) at 65 ºC (Table 2). Negligible or slight inhibition was established when the reaction mixture contained 1 mM N-bromosuccinimide, sodium azide or EDTA. A similar effect of these reagents are reported for the CGTase from alkaliphilic *Bacillus firmus* even at a concentration of 5 mM (Yim *et al*., 1997) or an increased enzyme activity (117%) in the presence of 4 mM EDTA (Fujita *et al.,* 1990), unlike CGTase from alkaliphilic *Bacillus agaradhaerens* which is strongly inhibited by N-bromosuccinimide (Martins and Hatti-Kaul, 2002). It is known that the inhibitory effect of N-bromosuccinimide is due to the oxidation of tryptophan. The results obtained indicated that tryptophan is not essential amino acid residue for the enzyme activity, which is in agreement with the report of Uitdehaag *et al*. (2002) concerning the catalytic mechanism of CGTase from *Bacillus circulans*, strain 251. An increased CGTase activity was established in the presence of 1 mM $CaCl_2$ or 1 mM $CoCl_2$ (Table 2; increased β-CD formation by 25 and 19%, respectively). The addition of these ions in higher concentrations showed loss of CGTase activity in the presence of 2-8 mM $CaCl_2$, while $CoCl_2$ in concentrations 1-4 mM maintained a 19-20% increase in cyclising activity (Table 3). Similar effect of 2-10 mM $CaCl_2$ is reported for CGTase of alkaliphilic *Bacillus firmus* (Higuti *et al*., 2003) unlike other strain *Bacillus firmus* maintaining 100% residual activity in the presence of 1-5 mM Ca^{2+} (Yim *et al*., 1997). Generally, the effect of metal ions and reagents on the cyclising activity of the enzyme is depending on the bacterial producer (Fujita *et al*., 1990; Yim *et al.,* 1997; Higuti *et al*., 2003).

TABLE 2 - Effect of metal ions and chemical reagents on the crude CGTase

Metal ions and reagents (1 mM)	Relative CGTase activity (%)
$CaCl_2$	125.0 ± 0.29
$CoCl_2$ $6H_2O$	119.2 ± 0.17
$ZnSO_4$ $7H_2O$	102.3 ± 0.15
$FeSO_4$ $7H_2O$	99.6 ± 0.14
$CuSO_4$ $5H_2O$	98.4 ± 0.08
$MgSO_4$	106.0 ± 0.27
NaCl	80.4 ± 0.33
KCl	116.7 ± 0.07
$AgNO_3$	111.6 ± 0.15
$(NH_4)6Mo_7O_{24}$ H_2O	104.0 ± 0.26
Na-EDTA $2H_2O$	93.3 ± 0.42
NaN_3	89.5 ± 0.71
N-bromosuccinimide	96.4 ±0.57

Data are mean values ± SD, n = 3.

TABLE 3 - Effect of various concentrations of $CaCl_2$ and $CoCl_2$ on the cyclising activity of a crude CGTase

Salts	Final concentration in the reaction mixture (mM)	Relative CGTase activity (%)
$CaCl_2$	2	67.1 ± 0.19
	4	65.7 ± 0.17
	6	64.8 ± 0.25
	8	65.7 ± 0.23
$CoCl_2.6H_2O$	2	119.8 ± 0.14
	4	120.0 ± 0.11
	6	98.6 ± 0.21
	8	88.4 ± 0.12

Data are mean values ± SD, n = 3.

Thermal stability of the crude and purified CGTase

The effect of temperature on CGTase stability was studied by pretreatment at different temperatures for a fixed time under three different conditions: a) crude enzyme; b) crude enzyme with 1 mM $CaCl_2$; c) crude enzyme containing 1 mM $CaCl_2$ and 1% or 4% substrate (Table 4). The residual cyclising activity was measured under optimum reaction conditions (65 ºC, pH 6.0, 20 min reaction time). The enzyme activity of an untreated sample corresponded to 100%. The enzyme without additives was found to be stable: at 50 ºC retained 51.2% of its activity after 4 h preincubation; at 55 ºC residual activity of 48.6% was established after 1 h treatment; at 60 ºC, 1 h incubation led to enzyme inactivation.

The addition of Ca^{2+} to the reaction mixture significantly increased the thermal stability of the crude enzyme (Table 4). At 50 ºC the stabilising effect of these ions was clearly observed after treatment time of 1-3 h. The same influence of these salts was established at 55 and 60 ºC but for shorter treatment time. At 70 ºC the presence of Ca^{2+} did not affect the thermal stability (data not shown). Structural analysis of the CGTases have shown the presence of two calcium ions, one near the N-terminal end and the other near the active site region, which provide stability to the conformational structure of flexible region of the protein molecule including the active site (Harata *et al.,* 1996).

The study of the enzyme thermal stability in the presence of 1 mM Ca^{2+} and 1 or 4% substrate (Fluka, according to Zulkowsky) showed stabilisation of the enzyme especially at higher temperatures of 55-70 ºC (Table 4). After 1-4 h incubation at 55 ºC, the established residual activities were higher a those at 55 ºC treatment without addition of substrate. At 60 ºC for 1 h the enzyme retained 95.0% of its activity in the presence of 1% starch while at the same reaction conditions but at the absence of substrate, the residual activity was only 6.6%. At 70 ºC the higher substrate concentration (4%) was more efficient. After 30 min treatment the residual CGTase activity was 36%, while the absence of substrate, these reaction conditions led to zero CGTase activity. Starch as a substrate is thought to be fixed between two domains like a bridge (Fujiwara *et al*., 1992). CGTase may be stabilised by such a starch bridge. If the starch bridge forms, the thermostability of CGTase may be increased by the addition of starch. Fujiwara *et al*. (1992) have established that substrate binding at domain D (amino acid residues in domain D are highly conserved among different CGTases) is related to enzyme stability but not to cyclisation characteristics.

TABLE 4 - Temperature stability of the crude CGTase

Temperature (ºC)	Additives	Time						
		5 min	10 min	30 min	1 h	2 h	3 h	4 h
50 ºC	none	nd*	95.4**	94.8	97	90.9	86.1	51.2
	1 mM Ca^{2+}	nd	nd	nd	130.7	117.2	90.6	nd
55 ºC	none	nd	88.8	75.9	48.6	37.9	19.8	6.2
	1 mM Ca^{2+}	nd	nd	nd	54.5	45.9	21.9	nd
	1 mM Ca^{2+} + 1% starch	nd	nd	nd	119.4	127.2	112.7	101.0
	1 mM Ca^{2+} + 4% starch	nd	nd	nd	83.6	82.0	78.6	76.2
60 ºC	none	nd	38.5	5.9	1.0	0	0	0
	1 mM Ca^{2+}	nd	83.6	28.2	6.6	nd	nd	nd
	1 mM Ca^{2+} + 1% starch	nd	nd	113.5	95.0	89.2	45.4	nd
	1 mM Ca^{2+} + 4% starch	nd	nd	71.5	66.0	66.0	24.6	nd
70 ºC	none	0	0	0	0	0	0	0
	1 mM Ca^{2+} + 1% starch	57.6	41.2	4.1	nd	nd	nd	nd
	1 mM Ca^{2+} + 4% starch	78.0	78.0	36.0	16.1	nd	nd	nd

* not determined; ** Residual CGTase activity (%). Data are mean values ± SD from 0.13 to 0.27, n = 3.

The thermal stability of the purified CGTase fractions was studied after pretreatment at 60 ºC for 1 h in the presence of 1 mM Ca^{2+} and 1% starch and at 70 ºC for 30 min in the presence of 1 mM Ca^{2+} and 4% substrate. In comparison to the crude enzyme, the purified enzyme fractions were more thermostable, and retained 100% of its activity after a heat treatment at 60 ºC. The temperature incubation at 70 ºC for 30 min provided 64% residual activities of the purified fractions compared to 36% for crude CGTase. Probably, the purified CGTase fractions released from inactive proteins were bound more efficient with substrate, providing enzyme stabilisation. When enzyme reaction was carried out at 70 ºC, the higher starch concentration (4%) was more suitable for the thermal stability of the crude and purified CGTase, probably because the higher temperature and higher substrate content affected the four reactions catalysed from CGTase by formation of maltohexaose and maltoheptaose accelerating the cyclisation reaction (Uitdehaag *et al*., 2000).

Starch liquefaction, which is the first step in the CD production, is performed at a high temperature. Therefore, the thermal and pH stability of CGTase are characteristics of a great significance for the CD yield. CGTase from *Bacillus circulans* ATCC 21783 was compared to the enzymes produced by other *Bacillus* strains (Table 5). It could be noted that the high activity of the studied CGTase in a wide pH range (5.0-11.0) and at temperatures 65-70 ºC is for the benefit of CD production.

TABLE 5 - Comparison of CGTases from *Bacillus* strains

Producer	pH optimum	Temperature optimum (ºC)	pH stability	Thermal stability	Molecular mass, (Da) by SDS-PAGE	References
Bacillus coagulans	6.5	nd*	5.0-10.0	Up to 65 ºC	36 000	Akimaru *et al*., 1991
Bacillus sp. 277	5.0 and 8.5	60 ºC	6.0-10.0	Below 70 ºC	69 000	Cao *et al*., 2005
Bacillus stearothermophilus ET1	6.0	80 ºC	6.0-8.0	70 ºC/1 h (+15 mM Ca^{2+}), 90% residual activity	66 800	Chung *et al*., 1998
Bacillus sp. AL-6	7.5	60 ºC	5.0-8.0	40 ºC/30 min, 100% residual activity	74 000	Fujita *et al*., 1990
Bacillus firmus NCIM 5119	5.5 and 8.5	65 ºC	7.0-11.0	Up to 30 ºC, thermolabile	78 000	Gawande *et al*.,1999
Bacillus agaradhaerens LS-3C	9.0	55 ºC	5.0-11.4	Up to 40 ºC	110 000	Martins and Hatti-Kaul, 2002
Bacillus lentus	6.5-7.5	45-55 ºC	6.8-8.5	Up to 55 ºC	33 000	Sabioni and Park, 1992
Bacillus sp. G1	6.0	60 ºC	7.0-9.0	Up to 60 ºC	75 000	Sian *et al*., 2005
Bacillus ohbensis C-1400	5.0	55 ºC	6.5-10.0	Below 45 ºC	80 000	Sin *et al*.,1991
Bacillus firmus	7.5-8.5	65 ºC	6.5-9.0	Up to 55 ºC	75 000	Yim *et al*.,1997
Bacillus circulans ATCC 21783	6.0 and 8.6	65 ºC	5.0-11.0	60 ºC/1 h (Ca^{2+}+ starch), 100% residual activity; 70 ºC/30 min (Ca^{2+}+ starch), 64% residual activity	Three proteins 97400, 66000 and 41000	Present work

* nd: not data.

Cyclodextrin production

Up to 41% conversion to cyclodextrins was obtained from 40 g l^{-1} starch without using any additives and without an optimisation of the bioconversion conditions, such as a kind, nature and concentration of the substrate, enzyme concentration, temperature, pH and reaction time. The crude CGTase (60 U per gram substrate) formed two types of CDs, namely, 11.99 g l^{-1} β-CD (73.4%) and 4.35 g l^{-1} γ-CD (26.6%) after 4 h enzyme reaction at 65 ºC. This feature of CGTase from *Bacillus circulans* ATCC 21783 to produce two types of CDs and a relative high quantity of γ-CD could be of interest for industrial production.

SDS-PAGE analysis

SDS-PAGE analysis of the purified enzyme fractions eluted at pH 6.0 and 8.6 showed three protein bands, one, strongly revealed, corresponding to a protein marker with molecular mass 66 kDa and two others with molecular mass 97.4 kDa and 41 kDa (Fig. 4). The data from the electrophoresis confirmed the presumption for the availability of several CGTases and the explanation for the lower thermostability of the crude enzyme because of the presence of many proteins besides the main three detected in the purified fractions. Probably, these three proteins correspond to three CGTases with different pH optima, 8.6, 6.0 and 5.0 (Fig. 2, the peak at pH 5.0 is a very weakly revealed). The future separation of these proteins could be of interest for obtaining of three enzyme preparations with different properties and different fields for applications. It is most likely, the CGTase with the highest molecular mass to be more thermolabile and its removal could improve the heat resistance of the other two CGTases.

It can be concluded that the properties of CGTase from *Bacillus circulans* ATCC 21783 enable application of the enzyme for efficient starch conversion into cyclodextrins.

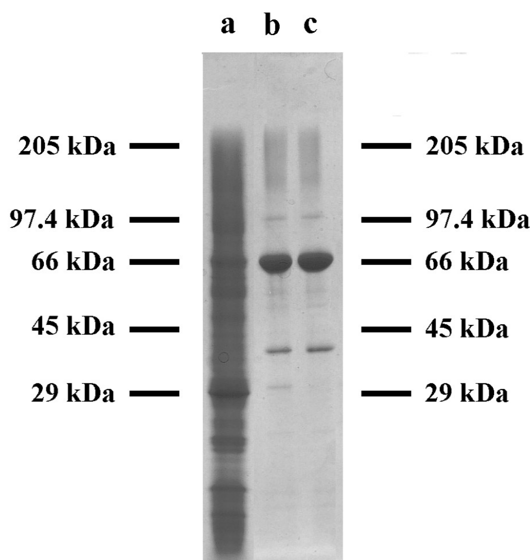

FIG. 4 - SDS-PAGE profile (7-17% acrylamide gradient) of crude enzyme (a), purified fraction, pH 6.0 (b) and purified fraction, pH 8.6 (c). Molecular mass markers: rabbit myosin (205 kDa), phosphorylase B (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and bovine carbonic anhydrase (29 kDa) are noticed on the both sides.

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REFERENCES

Akimaru K., Yagi T., Yamamoto S. (1991). Purification and properties of *Bacillus coagulans* cyclomaltodextrin glucanotransferase. J. Ferm. Bioeng., 71: 322-328.

Boveto L.J., Bacher D.P., Villete J.R., Sicard P.J., Bouquelet S.J.L. (1992). Cyclomaltodextrin glucanotransferase from *Bacillus circulans* E 192. Purification and characterization of the enzyme. Biotechnol. Appl. Biochem., 15: 48-58.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Cao X., Jin Z., Wang X., Chen F. (2005). A novel cyclodextrin glycosyltransferase from an alkalophilic *Bacillus* species: purification and characterization. Food Res. Int., 38: 309-314.

Cepeda A., Franco C.M., Fente C.A., Vazquez B.I., Rodriguez J.L., Prognon P., Mahuzier G. (1996). Postcolumn extraction of aflatoxins using cyclodextrins in liquid chromatography for food analysis. J. Chromatography A, 721 (1): 69-74.

Chung H.J., Yoon S.H., Lee M.J., Kim M.J., Kweon K.S., Lee I.W., Kim J.W., Oh B.H., Lee H.S., Spiridonova V.A., Park K.H. (1998). Characterization of a thermostable cyclodextrin glucanotransferase isolated from *Bacillus stearothermophilus* ET1. J. Agr. Food Chem., 46: 952-959.

Fente C.A., Ordaz J.J., Vazquez B.I., Franco C.M., Cepeda A. (2001). New additive for culture media for rapid identification of aflatoxin-producing *Aspergillus* strains. Appl. Environ. Microbiol., 67 (10): 4858-4862.

Fujita Y., Tsubouchi H., Inagi Y., Tomita K., Ozaki A., Nakamura K. (1990). Purification and properties of cyclodextrin glycosyltransferase from *Bacillus* sp. AL-6. J. Ferment. Bioeng., 70: 150-154.

Fujiwara S., Kakihara H., Sakaguchi K., Imanaka T. (1992). Analysis of mutation in cyclodextrin glucanotransferase from *Bacillus stearothermophlus* which affect cyclization characteristics and thermostability. J. Bacteriol., 174: 7478-7481.

Gawande B.N., Goel A., Patkar A.Y., Nene S.N. (1999). Purification and properties of a novel raw starch degrading cyclomaltodextrin glucanotransferase from *Bacillus firmus*. Appl. Microbiol. Biotechnol., 51: 504-509.

Harata K., Haga K., Nakamura A., Aoyagi M., Yamame K. (1996). X-ray structure of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011. Comparison of two independent molecules at 1.8 Å resolution. Acta Crystallogr. D, 52: 1136-1145.

Hedges R.A. (1998). Industrial applications of cyclodextrins. Chem. Rev., 98: 2035-2044.

Higuti I.H., Grande S.W., Sacco R., Jose do Nascimento A. (2003). Isolation of alkalophilic CGTase-producing bacteria and characterization of cyclodextrin-glycosyltransferase. Brazilian Arch. Biol. Technol., 46: 1-7.

Horikoshi K. (1999). Alkaliphiles: Some applications of their products for biotechnology. Microbiol. Mol. Biol. Rev., 63 (4): 735-750.

Kaneko T., Kato T., Nakamura N., Horikoshi K, (1987). Spectrophotometric determination of cyclization activity of β-cyclodextrin-forming cyclomaltodextrin glucanotransferase. J. Japan. Soc. Starch Sci., 34: 45-48.

Li D.Q., Ma M. (2000). Nanosponges for water purification. Clean Products and Processes, 2: 112-116.

Marechal L.R., Rosso A.M., Marechal M.A., Krymkiewicz N., Ferrarotti S.A. (1996). Some properties of a cyclodextrin-glucanotransferase from *Bacillus circulans* DF 9 R type. Cell. Mol. Biol., 42: 659-664.

Martins R.F., Hatti-Kaul R. (2002). A new cyclodextrin glycosyltransferase from an alkalophilic *Bacillus agaradhaerens* isolate: purification and characterization. Enzyme Microb. Technol., 30: 116-124.

Park H.T., Shin H.D., Lee Y.H. (1999.) Characterization of the β-cyclodextrin glucanotransferase gene of *Bacillus firmus* var. *alkalophilus* and its expression in *E. coli.* J. Microbiol., 9: 811-819.

Sabioni J.G., Park Y.K. (1992). Cyclodextrin glycosyltransferase production by alkalophilic *Bacillus lentus*. Rev. Microbiol., 23: 128-132.

Sian H.K., Said M., Hassan O., Kamaruddin K., Ismail A.F., Rahman R.A., Mahmood N.A.N., Illias R.M. (2005). Purification and characterization of cyclodextrin glucanotransferase from alkalophilic *Bacillus sp.* G1. Proc. Biochem., 40: 1101-1111.

Sin K.A., Nakamura A., Kobayashi K., Masaki H., Uozumi T. (1991). Cloning and sequencing of a cyclodextrin glucanotransferase gene from *Bacillus ohbensis* and its expression in *Escherichia coli*. Appl.Microbiol. Biotechnol., 35: 600-605.

Singh M., Sharma R., Banerjee U.C. (2002). Biotechnological applications of cyclodextrins. Biotechnol. Adv., 20: 341-359.

Szejtli J. (1997). Utilization of cyclodextrins in industrial products and processes. J. Material Chem., 7: 575-587.

Terada Y., Yanase M., Takata H., Takaha T., Okada S. (1997). Cyclodextrins are not the major cyclic α-1,4-glucans produced by the initial action of cyclodextrin glucanotransferase on amylose. J. Biol. Chem., 272 (25): 15729-15733.

Tonkova A. (2006). Microbial starch converting enzymes of the α-amylase family. In: Ray R.C., Ward O.P., Eds, Microbial Biotechnology in Horticulture*,* Vol. 1, Science Publishers, Enfield, New Hampshire, USA, pp. 421-472.

Uitdehaag J.C.M., van Alebeek G., Van der Veen B.A., Dijkhuizen L., Dijkstra B.W. (2000). Structures of maltohexaose and maltoheptaose bound at the donor sites of cyclodextrin glycosyltransferase give insight into the mechanisms of transglycosylation activity and cyclodextrin size specificity. Biochemistry, 39: 7772-7780.

Uitdehaag J.C.M., van der Veen B.A., Dijkhuizen L., Dijkstra B.W. (2002). Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the α-amylase family. Enzyme Microb. Technol., 30: 295-304.

Van der Veen B.A., Uitdehaag J.C.M., Dijkstra B.W., Dijkhuizen L. (2000). Engineering of cyclodextrin glycosyltransferase reaction and product specificity. Biochim. Biophys. Acta, 1543: 336-360.

Vassileva A., Burhan N., Beschkov V., Ivanova V., Tonkova A. (2003a). Immobilization of *Bacillus circulans* ATCC 21783 cells for cyclodextrin glucanotransferase production. In: Proceeding of the XIth International Workshop on Bioencapsulation*,* Strasbourg, France, 25-27 May 2003, pp. 201-204.

Vassileva A., Burhan N., Beschkov V., Spasova D., Radoevska S., Ivanova V., Tonkova A. (2003b). Cyclodextrin glucanotransferase production by free and agar gel immobilized cells of *Bacillus circulans* ATCC 21783. Proc. Biochem., 38: 1585-1591.

Vassileva A., Beschkov V., Ivanova V., Tonkova A. (2005). Continuous cyclodextrin glucanotransferase production by free and immobilized cells of *Bacillus circulans* ATCC 21783 in bioreactors. Proc. Biochem., 40: 3290-3295.

Yim D.E., Sato H.H., Park Y.H., Park Y.K. (1997). Production of cyclodextrin from starch by cyclodextrin glycosyltransferase from *Bacillus firmus* and characterization of purified enzyme. J. Ind. Microbiol. Biotechnol., 18: 402-405.