Cyclodextrin Production by *Bacillus lehensis* Isolated from Cassava Starch: Characterisation of a Novel Enzyme

Kate Cristina BLANCO[1], Flávio Faria DE MORAES[2], Natalia Sozza BERNARDI[1], Mary Helen Palmuti Braga VETTORI[1], Rubens MONTI[3] and Jonas CONTIERO[1]

[1]Department of Biochemistry and Microbiology, Biological Sciences Institute, UNESP – University Estadual Paulista, Rio Claro, Brazil; [2]Chemical Engineering Department, UEM – University Estadual de Maringá, Maringá, Brazil; [3]Department of Food and Nutrition, Faculty of Pharmaceutical Sciences, UNESP – University Estadual Paulista, Araraquara, Brazil

Abstract

Blanco K.C., de Moraes F.F., Bernardi N.S., Vettori M.H.P.B., Monti R., Contiero J. (2014): **Cyclodextrin production by *Bacillus lehensis* isolated from cassava starch: Characterisation of a novel enzyme.** Czech J. Food Sci., **32**: 48–53.

The properties of a previously unknown enzyme, denominated cyclodextrin glycosyltransferase, produced from *Bacillus lehensis,* were evaluated using affinity chromatography for protein purification. Enzyme characteristics (optimum pH and temperature; pH and temperature stability), the influence of substances on the enzyme activity, enzyme kinetics, and cyclodextrin production were analysed. Cyclodextrin glycosyltransferase was purified up to 320.74-fold by affinity chromatography using β-cyclodextrin as the binder and it exhibited 8.71% activity recovery. This enzyme is a monomer with a molecular weight of 81.27 kDa, as estimated by SDS-PAGE. Optimum temperature and pH for cyclodextrin glycosyltransferase were 55°C and 8.0, respectively. The Michaelis-Menten constant was 8.62 g/l during maximum velocity of 0.858 g/l·h.

Keywords: cyclodextrin glycosyltransferase; affinity chromatography; purification

Starch is composed of amylose and amylopectin polysaccharides with D-glucopyranose residues linked by α-1,4 and α-1,6 glycoside bridges with helical and branched structures, respectively (Eliasson 2004). The industrial use of starch and its derivatives generates residues that require treatment (Zhang *et al*. 2011). For such, enzymes are safer and healthier than chemical additives (Eliasson 2004). Enzymes are complex proteins and their high degree of specificity in catalysts is manifested only in their native structure (Wolfgang 2007). The enzyme cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) breaks α-1,4 amylopectin starch bonds and catalyses the group transfer of starch to produce cyclodextrins (CDs) (Wolfgang 2007). CDs are molecules with a truncated conical shape of six (γ-CD), seven (β-CD), eight (α-CD), or more glucose units formed by α-1,4 bonds, with a hydrophobic cavity formed by primary and secondary OH groups (Qu *et al*. 2002; Del Valle 2004). CDs are widely used in different industrial fields based on complexes with organic, inorganic, organo-metallic, and metallo-organic compounds. CDs are currently studied in research to treat diseases such as AIDS (Storsberg *et al*. 2003) and adrenoleukodystrophy (Pillai *et al*. 2009).

A wide variety of microorganisms are used to produce CGTase, especially *Bacillus* (Freitas *et al*. 2004; Blanco *et al*. 2009). Moreover, novel CGTase-producing strains have been identified for this purpose (Blanco *et al*. 2012). The purification degree of commercial enzymes depends on the application (Wolfgang 2007). However, the commercial use of CGTase is only possible after the definition of the purification method and enzyme characterisation. The microorganism *Bacillus lehensis* CGII, isolated from the wastewater of cassava flour mills and identified

by Blanco *et al.* (2012) (GenBank accession number HQ399547), exhibits high levels of CGTase production whose features, however, remain unknown.

The aim of the present study was to describe the purification and characterisation of CGTase from *Bacillus lehensis* CGII, which have not been reported previously in the literature.

MATERIAL AND METHODS

Bacterium and enzyme production. *Bacillus lehensis* CGII was isolated from wastewater samples from a cassava flour mill in Brazil. This bacterium is filed under the accession number HQ399547 (Blanco *et al*. 2012). The *B. lehensis* CGII strain was inoculated in an Erlenmeyer flask containing 100 ml of optimised growth medium (Blanco *et al*. 2012) and incubated at 35 ± 1°C for 72 h on a rotary shaker (New Brunswick Scientific Co, Edison, USA) at 150 rpm with an initial pH of 9.2. The cell-free supernatant was used for enzyme purification.

Enzyme assay and protein determination. Enzyme activity was determined after 72 h of fermentation as well as after each purification step using the method described by Makela *et al*. (1988). Total protein was determined using bovine serum albumin as the standard, following Lowry's method (Lowry *et al*. 1951).

Cyclodextrin glycosyltransferase purification. The centrifuged crude enzyme was precipitated using 80% ammonium sulphate and re-suspended in 40 ml of 5mM HCl-Tris buffer, pH 8. The precipitated enzyme was dialysed using a 12-KDa cellulosic membrane against 2 l of 50mM HCl-Tris buffer, pH 8, for 10 h, with the buffer changed every two hours. The dialysed enzyme was chromatographed on a DEAE-Sepharose 6B column (1.5 × 40 cm), stabilised with 30mM Tris-HCl chromatography buffer, pH 8, containing 5mM $CaCl_2$.

For β-CD immobilisation in agarose gel, 1 g of Sepharose 6B (Pharmacia Fine Chemical Inc., Piscataway, USA) was activated with 2 ml of 99% 1.4-dioxane solution (1 ml) and 0.6M NaOH containing 1% $NaBH_4$ and 2 ml of 0.1M NaOH containing 20% (w/v) of the β-CD. This mixture was stirred mechanically for 16 h at 45°C for solubilisation. The lyophilised enzyme (87.76 U/ml and 0.8 mg/ml) solution (0.1%) in chromatography buffer was applied to the upper end of the column. CGTase was eluted with chromatography buffer containing 10 mg/ml of β-CD at a rate of 0.4 ml/min, and fractions of 2.4 ml were collected. All fractions were subjected to spectrophotometric analysis (280 nm). The fractions showing enzyme activity were submitted to electrophoresis. All purification steps were carried out at 4°C.

Electrophoresis. The molecular weight of CGTase from *B. lehensis* CGII was estimated by sodium dodecyl sulphate-polyacrylamide (SDS) gel electrophoresis (PAGE) using 8–18% (w/v) gradient polyacrylamide, based on the method described by Laemmli (1970).

Characterisation of purified CGTase

Optimum pH and temperature. Optimum pH was determined using 0.05M buffers in the 3.0–10.0 range. The enzyme activity of purified CGTase was measured in glycine-HCl (pH 3.0), sodium acetate (4.0–5.0), phosphate (6.0), Tris-HCl (7.0, 8.0, and 9.0), and glycine-NaOH (10.0) at 55°C. For the determination of optimum temperature, the purified enzyme was analysed in the temperature range from 35°C to 85°C in Tris-HCl buffer, pH 8.

CGTase stability. CGTase thermal stability assays were performed with the enzyme incubated at 55°C for 110 minutes. Stability in the 3.0 to 10.0 pH range was determined with the enzyme solution incubated for 12 and 24 hours.

Effect of substances on CGTase. The effect of compounds (Table 2) on the enzyme activity of purified CGTase was investigated using concentrations of 2mM, 0.25M, 0.5M, and 1M for 12 min at 55°C in 50mM Tris-HCl buffer, pH 8. In all experiments, CGTase activity without the addition of inhibitors was considered 100%.

Kinetic parameters of CGTase. For the measurement of kinetic parameters, 5 ml of the purified enzyme were mixed with 5 ml of starch solution (3, 3.5, 4, 5, 6, 7, 8, 9, and 10mM) and 50mM Tris-HCl buffer, pH 8.0 at 55°C for 30 min in the same buffer solution. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined by a Lineweaver-Burk plot.

Cyclodextrin production. The reaction conditions were the following: 5 ml of purified enzyme solution (0.035 mg/ml) in 5 ml of 1% (w/v) potato starch solution. The two solutions were prepared in 50mM Tris-HCl buffer. The reaction was incubated at 55°C, pH 8.0. The samples were withdrawn at 0, 22, 35, and 42 h and inactivated at 100°C. Centrifugation was performed at 8800 *g* for 10 min and the supernatant was analysed using a high-performance liquid chromatography (HPLC) system equipped with a UV detector in a 210-nm column (4.6 × 250 mm; Phenomenex, Torrance, USA) at 26°C. The samples were applied to the column following membrane filtration

Table 1. Purification of CGTase from *Bacillus lehensis* CGII

Purification step	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Activity yield (%)	Purification fold
Crude extract	10.332	1.032	9.99	100	1
Ammonium sulphate precipitation	87.760	0.800	109.70	42.79	10.98
DEAE-Sepharose column 6B	112.470	0.035	3204.27	8.71	320.74

(0.22 μm; Millipore; Massachusetts, USA). The mobile phase was acetonitrile/water (50:50, v/v), with a flow rate of 0.7 ml/min and an injection volume of 20 μ.

RESULTS AND DISCUSSION

CGTase purification. The two-step purification method (ammonium sulphate precipitation and Sepharose 6B column biospecific-affinity chromatography) proved efficient for CGTase from *Bacillus lehensis* (Table 1).

The amount of protein decreased from 3097 mg to 120.8 mg and specific activity increased from 9.99 U/mg to 109.7 U/mg (Table 1), indicating that non-CGTase proteins were excluded due to the reduction in the interactions between water and the polar groups of the proteins. The purification factor and recovery of original enzyme activity after precipitation were 10.98 and 42.79%, respectively, indicating that the enzyme was purified 10 fold and 42% of the enzyme activity was maintained following the precipitation process (Table 1). Dialysis was performed prior to the next step to remove the salt.

At the beginning of the purification procedure, absorbance was measured at 280 nm, with protein detection represented by the continuous line in Figure 1. No enzyme activity was detected in the first peak. In the enzyme elution step with β-CD inserting, enzyme activity (represented by dots) exhibited a narrow peak, which characterises a high resolution result achieved by the selectivity and efficiency of the chromatographic technique.

The purified enzyme was obtained with a final yield of 8.71% (collected from fractions 27 to 37) due to losses stemming from the number of purification steps; losses also occurred during enzyme centrifugation of the fermented broth and during the precipitation process by ammonium sulphate followed by dialysis. Due to the high selectivity of the purification technique for affinity chromatography, CGTase was recovered with purification levels of the order of 320.74 fold, demonstrating an increase of CGTase concentration. Specific activity rates were found to be from 9.99 to 3204.27. The elution by the addition of β-CD to the buffer competes for binding with the target protein. CGTase was purified due to specificity between CGTase and β-CD by the interaction with a binding site on the enzyme.

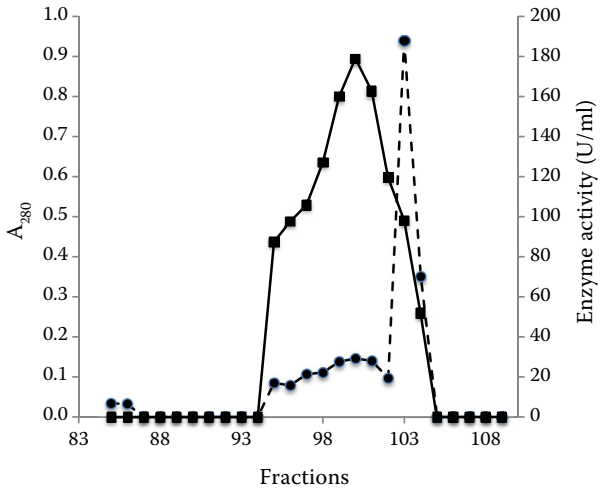

Figure 1. Sepharose 6B affinity chromatography of a CGTase from *Bacillus lehensis* CGII. Line absorbance at 280 nm; CGTase activity represented by the line with square dots

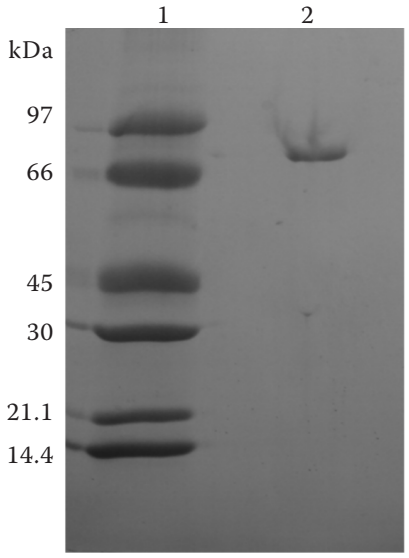

Figure 2. Analysis of CGTase purification by sodium dodecyl sulphate polyacrylamide gel electrophoresis
Lines: 1 – Molecular mass standard proteins, 2 – sample from DEAE-sepharose 6B column after protein precipitation and dialysis

Fractions corresponding to the peaks with enzyme activity were combined and molecular weight was demonstrated by electrophoresis homogeneity in SDS-PAGE, with only one band of 81 262.466 Da in the electrophoresis gel in Figure 2. Protein molecular weight was calculated using a standard curve constructed with a molecular mass log and the relative mobility of each protein used. The molecular mass of CGTase from *B. lehensis* CGII is similar to that from *Thermococcus* sp and the enzyme proved to be a monomeric protein (SDS-PAGE) with a molecular weight of 83 KDa.

Enzyme properties. Enzymes are biological catalysts that function under specific conditions. Therefore, after obtaining the pure CGTase enzyme, the optimum temperature and pH were tested in a 12-min reaction. Moreover, the stability was tested in a range of temperature and pH values for 12 h and 24 hours. Catalytic activity and the influence of different substances on the activity and stability of the CGTase corresponding to 100% was 100.54 U/ml.

Optimum pH and temperature and CGTase stability. Figure 3a shows an increase in the thermodynamic reaction rate at an optimum temperature of 55°C, followed by a decrease in the thermal denaturation of CGTase. According to Starnes (1990), CGTases from microorganisms have optimum temperatures between 30°C and 95°C. Moreover, optimum temperatures between 45°C and 65°C are frequent in studies on the characterisation of CGTase. For example, Kitahata *et al*. (1974) reported an optimum temperature of 55°C for a CGTase from *Bacillus circulans*.

Optimum pH is the result of the effect of factors such as affinity and stability of the enzyme-substrate complex and it also depends on the type of buffer used. Figure 3c displays enzyme activity in different buffers. In this study, pH 8 was determined as the best for the enzyme activity. The optimum pH of CGTase varies depending on the producer strain (Starnes 1990; Sato *et al*. 1993). In enzyme applications, pH and thermal stability are important properties for the storage of enzymes. The CGTase enzyme may be incorporated in industrial starch processing, such as the industrial production of CDs. The enzyme activity dropped in each time interval tested, with a decrease of more than 50% after 80 min (Figure 3b). Figure 3d shows the enzyme activity after 12 and 24 h at different pH values (3, 4, 5, 6, 7, 8, and 9). Losses in enzyme function occurred from 12 h to

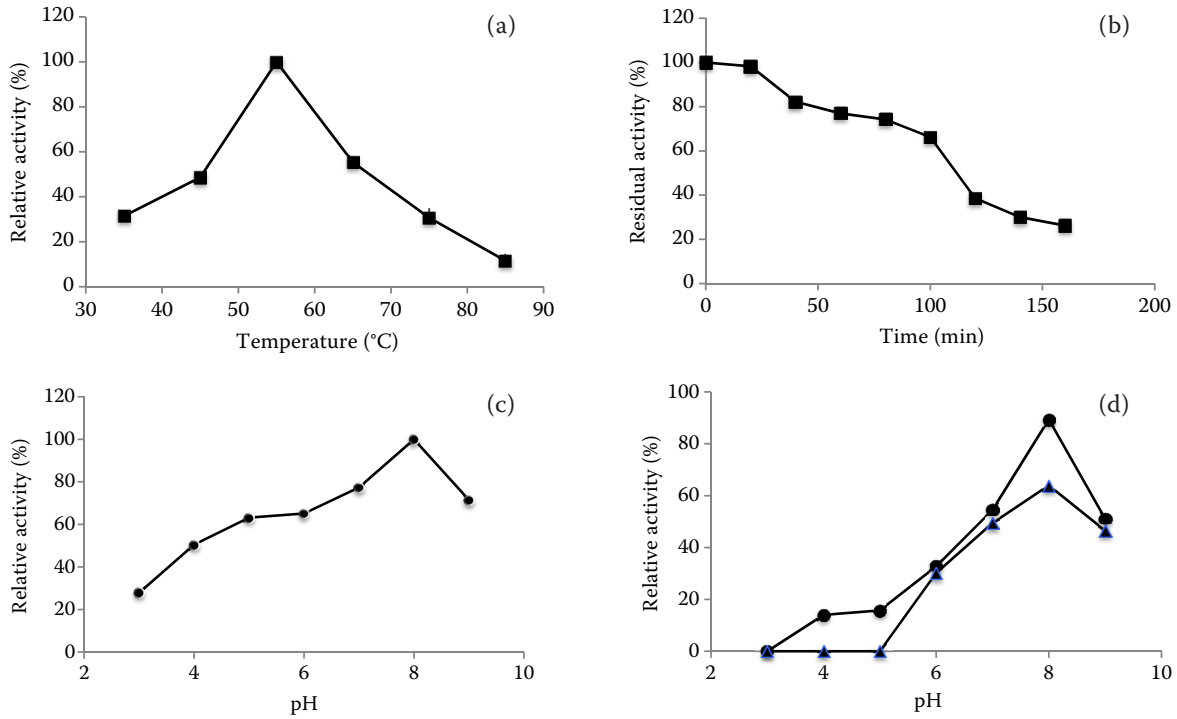

Figure 3. Temperature (a) and pH (c) influence on CGTase activity from *B. lehensis* CGII. pH stability of enzyme activity (d) was determined after incubation for 12 (●) and 24 h (▲) at pH from 3.0 to 10.0; temperature stability (b) was determined after incubation for 0 to 110 min at the optimum temperature of 55 °C. The CGTase activity corresponding to 100% was 100.54 U/ml

24 hours. Enzyme stability regards the capacity of such substances to retain their catalytic ability under different conditions over time. The action of CGTase is determined by its ability to convert starch into CD. However, CGTase loses its enzyme function due to pH and thermal instability.

Effect of substances on CGTase. Table 2 displays the inhibitor/activator effect of substances on CGTase from *B. lehensis*. Stability is an important factor for enzyme application in blends with different substances. Hence, it is important to understand the effect of these substances on the enzyme activity. In the present study, dextran and $CaCl_2$ were the substances that most potentiated the effect of enzyme action. With dextran, this likely occurred due to the fact that this substance forms aggregates with the enzyme, changing the conformation of the enzyme-substrate structure and providing improved enzyme action. Dextran may be used industrially as a stabiliser and gelling agent, inhibiting crystallisation. As to $CaCl_2$, CGTase likely requires Ca^+ as a co-factor. A number of studies, such as that carried out by Suntinanalert (1997), have demonstrated that CGTase may be stabilised by adding $CaCl_2$ (10mM). However, in characterising a purified

Table 2. Effect of inhibitory and activating substances on CGTase from *Bacillus lehensis* CGII

Metal ions and reagents (2mM)	Residual activity (%)
No reagent	100 ± 0.0043
$Ca(CH_3COO)_2$	23.08 ± 0.0085
$CaCO_3$	78.78 ± 0.0104
$CaCl_2$	111.60 ± 0.0024
$CuSO_4 \cdot 5H_2O$	83.92 ± 0.0032
Dextran 0.25M	104.49 ± 0.0067
Dextran 0.5M	108.8 ± 0.0051
Dextran 1M	109.82 ± 0.0069
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0
$FeCl_3$	0
$FeSO_4 \cdot 7H_2O$	0
FeO	0
$Fe_2(SO_4)3 \cdot nH_2O$	0
Ethylenediamine tetraacetic acid	44.24 ± 0.0042
$MgSO_4 \cdot 7H_2O$	25.03 ± 0.0051
$MnSO_4 \cdot H_2O$	50.39 ± 0.0041
Na_2SO_4	92.04 ± 0.0034
Sodium dodecyl sulphate	34.48 ± 0.0045
$ZnSO_4 \cdot H_2O$	88.59 ± 0.0038

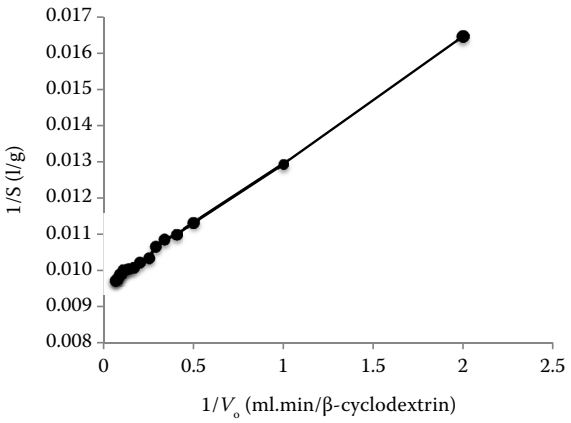

Figure 4. Lineweaver-Burk plot of initial velocity ($1/V_o$) and reciprocal starch concentration (1/[S]) for CGTase from *Bacillus lehensis* CGII

CGTase from the alkaliphilic *Bacillus pseudalcaliphilus* 20RF, Atanasova (2011) found no significant difference in enzyme activity with the addition of $CaCl_2$. The ions Cu^{4+}, Fe^{2+}, Mg^{4+}, Mn^{4+}, Na^{2+}, and Zn^{4+} as well as ethylenediamine tetraacetic acid and sodium dodecyl sulphate inhibit the enzymatic activity of CGTase, indicating that the presence of these ions interferes with the catalytic activity of CGTase from *B. lehensis*. It is likely that these substances alter the structural conformation of CGTase.

Cyclodextrin production. Cassava starch was treated with the purified enzyme to produce a mixture of α-, β- and γ-CDs under the optimum conditions determined in the characterisation of CGTase (Figure 3). The CDs produced with purified CGTase were confirmed by HPLC and maximum starch conversion into CDs by CGTase from *Bacillus lehensis* CGII strain was obtained in 35 hours. The production ratio of α-, β-, and γ-CDs (g/l) was 0.32:6.33:1.02, respectively.

Kinetic parameters. The Michaelis-Menten model was used to characterise the system and a maximum velocity rate (V_{max}) of 8.62 g/l was determined. At this velocity, approximately all enzyme molecules are in enzyme-substrate form and the amount of free enzyme is insignificant. Moreover, the K_m is equivalent to the starch concentration necessary for half V_{max} of 0.862 g/l.h, indicating high affinity between CGTase and starch, as a smaller K_m value denotes greater enzyme-substrate affinity. The plot in Figure 4 displays the apparent K_m and V_{max}.

CONCLUSION

The present study described the purification procedure for CGTase from *Bacillus lehensis*. CGTase was

purified in two steps, using a precipitation technique with ammonium sulphate, followed by biospecific affinity chromatography. The molecular weight of the enzyme is 81.27 kDa. With the purified enzyme, the specific characteristics of optimum pH and temperature, stability in a range of pH and temperature values, kinetics and substances that influence the enzyme activity were described. Enzyme characterisation is a useful tool for obtaining knowledge on the properties and possible applications of cyclodextrin glycosyltransferase produced by *Bacillus lehensis* CGII isolated from cassava starch.

References

Atanasova N., Kitayska T., Bojadjieva I., Yankov D., Tonkova A. (2011): A novel cyclodextrin glucanotransferase from alkaliphilic *Bacillus pseudalcaliphilus* 20RF: Purification and properties. Process Biochemistry, **46**: 116–122.

Blanco K.C., De Lima C.J.B., De Oliveira P.A.P.L.V., Pião A.C.S., Contiero J. (2009): Cyclodextrin glycosyltransferase production by the *Bacillus* sp., subgroup *alcalophilus* using a central composite design. Research Journal of Microbiology, **4**: 45.

Blanco K.C., Lima C.J.B., Monti R., Martins J., Bernardi N.S., Contiero J. (2012): *Bacillus lehensis* – an alkalitolerant bacterium isolated from cassava starch wastewater: optimization of parameters for cyclodextrin glycosyltransferase production. Annals of Microbiology, **62**: 329–337.

Del Valle E.M.M. (2004): Cyclodextrins and their uses: a review. Process Biochemistry, **39**: 1033–1046.

Eliasson A.-C. (ed.) (2004): Starch in Food: Structure, Function and Applications. Woodhead Publishing Limited, Cambridge.

Freitas T.L.D., Monti R., Contiero J. (2004): Production of CGTase by a *Bacillus alkalophilic* CGII strain isolated from wastewater of a manioc flour industry. Brazilian Journal of Microbiology, **35**: 255–260.

Kitahata S., Okada S. (1974): Action of cyclodextrin glycosyltransferase from *Bacillus megaterium* strain No. 5 on starch. Agricultural and Biological Chemistry, **38**: 2413–2417.

Laemmli U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680–685.

Lowry O.H., Rosebrough N.J., Farr A.L.R.R. (1951): Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, **193**: 265–75.

Makela M.J., Korpela T.K., Puisto J., Laakso S.V. (1988): Nonchromatographic cyclodextrin assays: evaluation of sensitivity, specificity, and conversion mixture applications. Journal of Agricultural and Food Chemistry, **36**: 83–88.

Pillai B.K., Jasuja R., Simard J.R., Hamilton J.A. (2009): Fast diffusion of very long chain saturated fatty acids across a bilayer membrane and their rapid extraction by cyclodextrins: implications for adrenoleukodystrophy. Journal of Biological Chemistry, **284**: 33296–33304.

Qu Q.I., Tucker E., Christian S.D. (2002): Sulfoalkyl ether β-cyclodextrin derivatives: synthesis and characterizations. Journal of Inclusion Phenomena and Macrocyclic Chemistry, 213–221.

Sato M., Matsuo T., Orita N., Yagi Y. (1991): Synthesis of novel sugars, oligoglucosyl-inositols, and their growth stimulating effect for *Bifidobacterium*. Biotechnol Letters, **13**: 69–74.

Starnes R.L. (1990): Industrial potential of cyclodextrin glycosyl transferases. Cereal Foods World, **35**: 1094–1099.

Storsberg J., Aert H.V., Roost C.V., Ritter H. (2003): Cyclodextrins in polymer synthesis: A simple and surfactant free way to polymer particles having narrow particle size distribution. Macromolecules, **36**: 50–53.

Suntinanalert P., Petmaneechote J., Damnian N., Hutadilok-Towatana N. (1997): Purification and properties of cyclodextrin glycosyltransferase (CGTase) from an alkaline-tolerant *Bacillus* sp. PS304. Journal of the Science Society of Thailand, **23**: 381–388.

Wolfgang E.A. (2007): Enzymes in Industry: Production and Applications. 3rd Ed. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Zhang C., Zhang C., Liu J., Han L. (2011): Research progress on corn starch wastewater treatment process. New Technology of Agricultural, **1**: 620–625.

Received for publication November 1, 2012
Accepted after corrections May 28, 2013

Corresponding author:

Prof Dr Jonas Contiero, UNESP – Universidade Estadual Paulista, Instituto de Biociências de Rio Claro, Departamento de Bioquímica e Microbiologia, Rio Claro, Av. 24-A, 1515 Bela Vista, Rio Claro, SP, Brazil;
E-mail: jconti@rc.unesp.br