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A β-cyclodextrin glycosyltransferase from a newly isolated *Paenibacillus pabuli* US132 strain: Purification, properties and potential use in bread-making

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Abstract

A bacterial strain designed US132, isolated from a Tunisian soil was selected for its production of a potent cyclodextrin glycosyltransferase (CGTase) activity. This strain was identified as *Paenibacillus pabuli* by sequencing of the 16S rDNA and the 16S-23S internal transcribed spacer (ITS). The US132 CGTase, purified to homogeneity by hydrophobic interaction chromatography and starch adsorption, is a monomer of approximately 70 kDa. This enzyme exhibited a maximal activity at 65 °C, in presence of 10 mM calcium, and was most active at pH range 5.5–9 with an optimum at 6.5. Using 10% (w/v) of potato starch, this CGTase produced a high level of cyclodextrins reaching 42 g/l with a β-cyclodextrin ratio of 63%. Furthermore, this enzyme can be used in the bread-baking process since its addition in the dough mix improved significantly the loaf volume and decreased the firmness of bread during storage.
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Keywords: β-CGTase; *Paenibacillus pabuli*; Purification; Cyclodextrins production; Bread-making

1. Introduction

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is the unique enzyme able to convert starch and related sugars into cyclodextrins (CDs) via a cyclization reaction. CDs are non reducing cyclic structures consisting of 6, 7 or 8 glucose residues, joined by α-(1,4) linkages, for α-, β- and γ-cyclodextrin, respectively. These compounds have an exclusive ability to act as molecular containers by entrapping hydrophobic molecules in their internal cavity. This property has been used for stabilization, solubilization and masking odors and tastes of a wide variety of interesting compounds used in food, pharmaceutical, cosmetic, agricultural and chemical industries [1–3]. Besides the ability of CGTases to catalyze the intramolecular transglycosylation reaction (cyclization), they are also able to perform two intermolecular transglycosylation reactions: coupling, in which a cyclodextrin ring is cleaved and transferred to a linear acceptor substrate and disproportionation, wherein two linear oligosaccharides are converted into linear oligosaccharides of different sizes. In addition, these enzymes possess a weak hydrolyzing activity in which water is the glycosyl acceptor [4,5].

All known CGTases convert starch into a mixture of α-, β- and γ-CD in different ratios. Depending on the main cyclodextrin produced, CGTases are classified as α-, β- or γ-CGTases. Among the three types of cyclodextrins, β-CD is of high interest due to the size of its non-polar cavity which is suitable to accommodate many molecules such as aromatics and drugs; its low solubility in water which facilitate its separation from the reaction mixture. Furthermore, β-CD inclusion complexes are easily prepared and stable [1,6]. The reported β-CGTases generate various CDs yield and proportion, which depends on the microbial source of the enzyme and the bioconversion conditions [4,6–10]. To improve the CDs production yield and selectivity, organic complexing agents can be added [6,10–12]. However, the application of organic solvents has several disadvantages limiting the use of CDs in food and pharmaceutical industries [3,10]. Hence, it is of interest to set out a CGTase activity producing high concentration of β-CD in the absence of organic solvent.

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Additionally, CGTases were reported to improve baked product characteristics such as loaf volume [13,14] and to delay the bread staling during storage [14–16]. Staling phenomenon, caused by the retrogradation of starch, refers to various undesirable changes that occur in the loaf during storage, thereby lowering the consumer's acceptance. The increase of crumb firmness, which means that bread becomes gradually hard and brittle during storage, is the major aspect of staling. Owing to their ability to reduce starch retrogradation, some amylases are described as effective anti-staling agent [14–17]. Among these, the maltogenic (*exo*-hydrolytic) α-amylase (EC 3.2.1.133) from *Bacillus stearothermophilus*, commercially available under the trade name Novamyl (Novozymes A/S) [18], is widely used in industry of bread-baking.

In this paper, we report the purification and the characterization of a CGTase, from the newly isolated *Paenibacillus pabuli* US132 strain, producing a high concentration of cyclodextrins, mainly formed by β-CD. The effect of this enzyme on the loaf volume and the crumb firmness, compared with that of Novamyl, is also reported and discussed.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture media

The strain US132 was isolated from Tunisian soil as a potent β-CGTase producer. *Escherichia coli* DH5α (F^- *sup*E44 φ80 δ*lac*ZΔ M15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^-) *deo*R thi-1 λ^- gyrA96 *rel*A1) [19] was used as host strain. The plasmids pJS3 and pJS7, derivatives of pGEM-T easy vector (Promega), carried, respectively, the 16S rRNA gene and the 16S-23S internal transcribed spacer (ITS) of the US132 strain. *E. coli* recombinant strains were grown on Luria Bertani (LB) plates [20] containing ampicillin (100 μg/ml). For US132 strain a medium composed of 1% potato starch, 1% peptone, 0.5% yeast extract, 0.5% beef extract and 0.1% NaCl was used. The initial pH of culture media was adjusted to 8.

2.2. Isolation and identification of the strain US132

The soil samples for screening were taken from different regions in Tunisia. The microorganisms were isolated on agar plates of LB medium containing 1% starch. After incubation at appropriate temperature for 24 h, the plates were stained with iodine vapor. All colonies exhibiting a clear zone, indicating starch degradation, were isolated for further investigation. The bacterium designated US132 displayed a large halo of starch degradation versus a very low level of reducing sugars. Hence, it was retained for its production of a CGTase activity and was used as source of chromosomal DNA. PCR amplifications of the 16S rRNA gene and the 16S-23S ITS were performed using primers designed from conserved regions within the rRNA operon of *E. coli* [21]. The PCR products were purified and cloned into pGEM-T easy vector. Then, the corresponding nucleotide sequences were determined by the BigDye Terminator v3.1 Cycle Sequencing Kit and the automated ABI Prism® 3100-Avant Genetic Analyser (Applied Biosystems). Homology search was carried out using BLAST search algorithm. The nucleotide sequences of the 16S rRNA gene and the 16S-23S ITS have been submitted to the EMBL data bank under accession numbers AM087615 and AM087616, respectively.

2.3. Purification of the US132 CGTase

The bacterial cells of the fermentation broth were removed by centrifugation at 6000 × *g* for 15 min at 4 °C and the cell-free supernatant was used as source of the enzyme. After the addition of $(NH_4)_2SO_4$ at 35% saturation, the resultant mixture was applied to Phenyl Sepharose™ 6 Fast Flow column (Amersham Biosciences) previously equilibrated with 50 mM sodium acetate buffer (pH 6.5) saturated by 35% $(NH_4)_2SO_4$. The enzyme was eluted with a decreasing linear gradient, 35–0% of $(NH_4)_2SO_4$ in sodium acetate buffer, at a flow rate of 10 ml/min. The fractions containing CGTase activity were pooled, concentrated and dialyzed against sodium acetate buffer at 4 °C. The dialyzed solution was adsorbed to 7% insoluble potato starch in the presence of 1 M $(NH_4)_2SO_4$ at 4 °C as described by Martins and Hatti-Kaul [7] with some modifications. The mixture was centrifuged at 5000 × *g* for 10 min and the pellet was washed with cold water until the absorbance at 280 nm reached zero. To allow enzyme elution, the pellet was incubated with 50 mM sodium acetate buffer (pH 6.5) at 40 °C for 30 min followed by centrifugation at 5000 × *g* for 10 min. The elution was repeated twice and the fractions were concentrated and stored at 4 °C. The purity of the US132 CGTase was checked by using SDS-PAGE and HPLC size exclusion column (Shodex KW-802.5, Bio-Rad).

2.4. Enzyme activity assays

The amount of the reducing sugars released by the action of amylases on starch was determined according to the DNS method of Miller [22].

The CGTase activity was determined as starch-degrading activity and monitored by measuring the decrease in absorbance (blue value) of iodine–amylose complex [23]. The reaction mixture contains 50 μl of appropriately diluted enzyme, 10 mM calcium and 500 μl of 1% potato starch (Sigma) dissolved in 100 mM sodium acetate buffer (pH 6.5), in a total volume of 1 ml. In standard conditions, assays were incubated for 10 min at 65 °C and the reaction was stopped by addition of 1 ml of 1.5 M acetic acid; then 1 ml of iodine reagent (0.02% I_2, 0.2% KI) was supplemented to the mixture and the absorbance was measured at 700 nm. One activity unit was defined as the amount of enzyme able to decrease 10% of amylose–iodine complex optical density per min under the assay conditions.

The *exo*-hydrolytic activity introduced in dough mix was assayed using the Betamyl Kit commercialized by Megazyme (Wicklow, Ireland). The procedure employs as substrate the *p*-nitrophenyl maltopentaoside (PNPG5) and an excess level of high purity α-glucosidase in the presence of stabilizers which significantly reduce the rate of cleavage of PNPG5 by α-glucosidase. The assay was performed by incubating 100 μl of appropriately diluted enzyme with 100 μl of substrate mix-

ture for 10 min at 40 °C and pH 6.5. Released *p*-nitrophenol is determined by measuring the absorbance at 410 nm after adding 1.5 ml of Trizma base solution (pH 11). One Betamyl unit is defined as the amount of enzyme able to release 1 μmol of *p*-nitrophenol from PNPG5 per min and under the defined assay conditions.

2.5. Effect of temperature and pH on enzyme activity and stability

The effect of temperature on the activity of the purified CGTase was done by incubation of the enzyme at different temperatures ranging from 40 to 80 °C. The reaction was performed according to the method of CGTase assay described above. The effect of pH on the activity of the purified enzyme was determined at pH ranging from 4 to 10.5 at 65 °C in the presence of 10 mM Ca^{2+}. The buffers used are: sodium acetate 0.1 M (pH 4–6.5), Tris–HCl 0.1M (pH 7–9) and glycine–NaOH 0.1 M (pH 9–10.5). The activities at optimal temperature and optimum pH were defined as 100%.

The thermostability was determined by incubating 15 U of the pure enzyme for 1 h at different temperatures ranging from 40 to 65 °C. The pH stability of the enzyme was also determined by incubating 15 U of the pure enzyme at 40 °C for 1 h in different pH buffers ranging from 4 to 10.5. The CGTase residual activity was measured at optimum temperature and pH of the enzyme and the activity of untreated sample was defined as 100%.

2.6. Analysis of cyclodextrins by HPLC

The concentrations of α-, β- and γ-CD were measured by HPLC using Aminex HPX-42A saccharide analysis column (Bio-Rad), water as mobile phase (at a flow rate of 0.6 ml/min) at 80 °C and refractive index (RI) as detector (10A from Shimadzu). The purified enzyme was incubated at 55 °C in presence of 10% of pregelatinized potato starch (Sigma), 50 mM sodium acetate buffer pH 6.5 and 10 mM Ca^{2+}. Samples were taken at regular time intervals, boiled for 5 min, filtered through a 0.45 μm membrane and injected into HPLC system. Quantification of CDs was carried out using α-, β- and γ-CD standards at 5 g/l each.

2.7. Protein quantification and electrophoresis

Protein concentration was determined using Bradford's method with bovine serum albumin as the standard [24]. Samples from different purification steps were migrated in 10% SDS-PAGE according to the method of Laemmli [25]. Protein bands were visualized by Coomassie brilliant blue R-250 (Bio-Rad) staining.

2.8. Bread-baking and analysis of crumb firmness

The bread-dough recipe composed of 100 g wheat flour, 4 g glucose, 5 g yeast, 2 g salt and 62 g water. Bread loaves were baked in the following setups: negative control using the bread mix alone, positive control using the bread mix supplemented with 97 Betamyl units of Novamyl (Novozymes AS, Denmark) and bread mix supplemented with an equivalent amount of US132 β-CGTase Betamyl units. The introduced Betamyl units correspond to 45 ppm of Novamyl which was recommended by Novozymes as the best concentration for an anti-staling effect [26]. All the ingredients were mixed using Mac Duffy mixer. A straight dough schema (dough temperature of 26 °C, fermentation at 35 °C during 80 min) was performed with bread of 516 g of dough. The bread loaves were baked at 215 °C during 25 min. The firmness of crumb was evaluated using a texturometer (TAXT-2) at days 1, 6 and 10 during the storage of bread.

3. Results and discussion

3.1. Identification of the bacterial strain

The newly isolated US132 strain is Gram positive, rod shaped and spore forming bacteria. To identify this strain, the 16S rRNA gene was amplified, cloned and sequenced. The alignment of the 1508 bp correspondent sequence (accession number AM087615) showed the highest sequence similarity with the 16S rRNA gene of *Paenibacillus* genus members as follows: 99% with *Paenibacillus* sp. IDA5358 (accession number AY289507); 99% with *Paenibacillus pabuli* (accession numbers AB045104 and AB073191); 99% with *Paenibacillus amylolyticus* (accession numbers AB073190 and D85396) and 97% with *Paenibacillus xylanilyticus* strain XIL14 (accession number AY427832).

For a more precise classification, the 16S-23S ITS was amplified according to the approach reported by Gürtler and Stanisich [21]. The amplification gave different bands of various sizes with a major band of about 500 bp (data not shown). The nucleotide sequence (accession number AM087616) of this cloned fragment contains the 3′ end 16S rDNA (3 bp), the 16S-23S ITS (304 bp) and the 5′ end 23S rDNA (192 bp). The alignment of the US132 ITS to the data base bank showed a unique similarity of about 87% with counterpart region of *P. pabuli* ATCC 43899^T (accession number AF478102) [27]. Based on all obtained data, we propose the assignment of our isolate as *P. pabuli* strain US132.

3.2. Purification of the enzyme

The US132 CGTase was successfully purified to homogeneity through two principal steps: Hydrophobic Interaction Chromatography (HIC) and starch adsorption, as described in Section 2. The results of purification steps are summarized in Table 1 and the purity of the enzyme was about 23-fold greater than that of the crude supernatant. The yield of the purification steps was about 20% with respect to the culture supernatant. The specific activity of the purified enzyme was about 4000 U/mg. This preparation was a homogeneous enzyme with a high purity as it exhibits a unique elution peak on gel filtration chromatography (Fig. 1A) and a single protein band of approximately 70 kDa on SDS-PAGE (Fig. 1B). The purified enzyme treated either by the β-mercaptoethanol or thermal denaturation showed the same

Table 1
Purification steps of the CGTase from *Paenibacillus pabuli* strain US132

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude supernatant	56,000	320	175	100	–
HIC	27,000	15.6	1730	48.2	9.9
Starch adsorption	11,200	2.8	4000	20	22.9

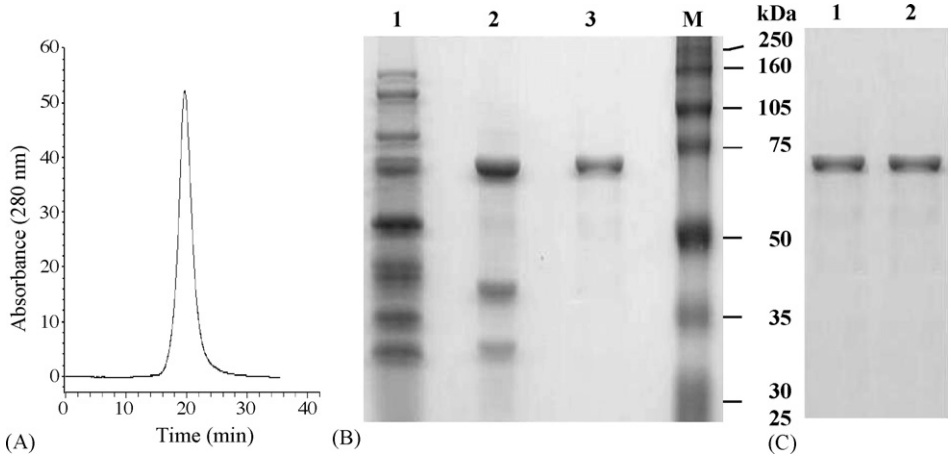

Fig. 1. Size exclusion HPLC chromatography and electrophoretic analysis of the purified US132 β-CGTase. (A) Size exclusion HPLC chromatography using the Shodex column. (B) Determination of molecular weight on SDS-PAGE. Lane 1, crude supernatant; Lane 2, sample after HIC elution; Lane 3, purified CGTase after starch adsorption; M, protein markers (each line contained between 10 and 25 μg of protein). (C) Electrophoretic patterns of the purified CGTase on SDS-PAGE. Lane 1, treated by β-mercaptoethanol; Lane 2, treated by thermal denaturation.

relative mobility on SDS-PAGE (Fig. 1C). Hence, it can be concluded that the US132 CGTase is a monomeric protein. This enzyme presents common properties with the majority of the reported CGTases which are monomeric with a molecular mass ranging from 60 to 110 kDa [3,6,7,28].

3.3. Effect of temperature on enzyme activity and stability

In the absence of calcium, the temperature profile of the purified US132 CGTase showed maximal activity at 60 °C. The addition of 10 mM calcium improved the enzyme thermostability since the optimum was shifted to 65 °C with an enhancement of the relative activity by about 10% (Fig. 2). The effect of calcium on the activity was observed especially at temperature over 55 °C, suggesting that this ion acts as a thermal stabilizer rather than an activator.

The study of the US132 CGTase thermostability showed that, in the absence of any additives, the enzyme retained about 65 and 21% of its activity at 50 and 55 °C, respectively, and was inactive at 60 °C after 1 h of incubation (Fig. 3). The addition of 10 mM Ca^{2+} or 1% starch or 1% β-CD improved considerably the US132 CGTase thermostability. In fact, the enzyme was stable up to 55 °C and preserved about 65% of its activity at 60 °C. Hence, these additives behave as protective agents improving the stability of the enzyme at high temperature. This enhancement of the thermostability in presence of calcium or

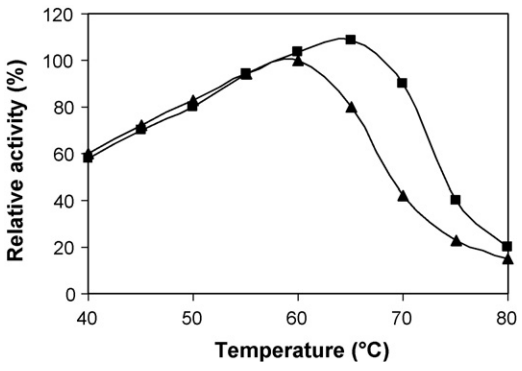

Fig. 2. Effect of temperature on US132 β-CGTase activity in the absence of calcium (▲) and in the presence of 10 mM calcium (■).

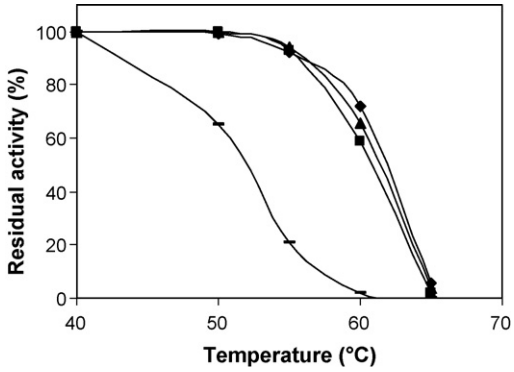

Fig. 3. Effect of temperature on the US132 β-CGTase stability. The enzyme was incubated at various temperatures for 1 h at pH 6.5 (B), pH 6.5 in presence of 10 mM $CaCl_2$ (■), pH 6.5 in presence of 1% of starch (▲), pH 6.5 in presence of 1% β-CD (♦).

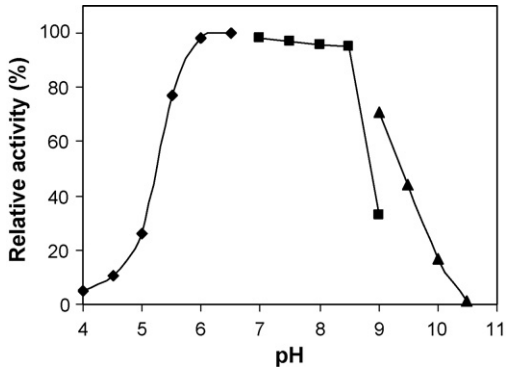

Fig. 4. Effect of pH on the activity of US132 β-CGTase. The buffers used were: sodium acetate (pH 4.0–6.5) (♦), Tris–HCl (pH 7–9) (■) and glycine–NaOH (pH 9–10.5) (▲).

substrate or product was already reported for others CGTases [6,7,9,29].

3.4. pH activity and stability profiles

The US132 CGTase was active in a wide pH range of 5.5–9 with an optimum at 6.5 in sodium acetate buffer (Fig. 4). It should be mentioned that the buffer composition has an important effect on the activity. Indeed, the enzyme displayed about 70% versus only 30% of its optimal activity (obtained in sodium acetate buffer) at pH 9 in glycine–NaOH and Tris–HCl buffers, respectively. Similar effect of buffer composition was reported for the *Bacillus agaradhaerens* CGTase [7]. The study of stability of the US132 CGTase at different pH showed that it was completely stable in the pH range of 6–9 after 1 h of incubation at 40 °C (Fig. 5). Outside this range, the residual activity of the enzyme declined drastically.

3.5. Cyclodextrins production

The kinetic of CDs production by the action of the purified US132 CGTase on 10% potato starch was investigated by HPLC as mentioned in Section 2. A maximum conversion rate of about 42% to CDs was obtained with 100 U enzyme/g of substrate after 42 h of reaction (Fig. 6). At this point, the US132 CGTase

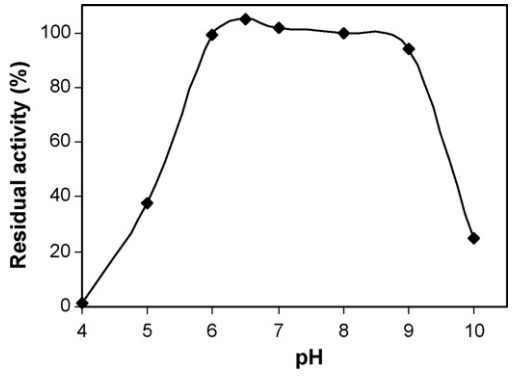

Fig. 5. Effect of pH on the stability of the US132 β-CGTase. The enzyme was incubated at 40 °C for 1 h in sodium acetate (pH 4.0–6.5), Tris–HCl (pH 7–8.5) and glycine–NaOH (pH 9–10.5) buffers.

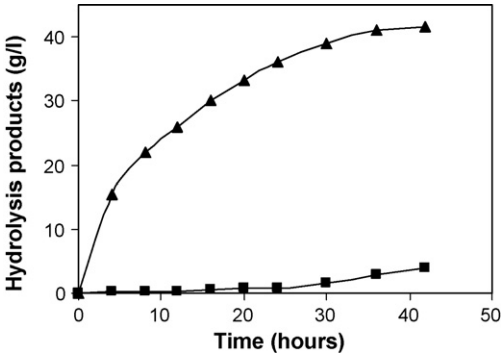

Fig. 6. Profiles of CDs (▲) and reducing sugars (■) production by the action of 100 U enzyme/g of substrate in presence of 10% potato starch.

generated a mixture of CDs in the ratio of 22:63:15 for α-, β- and γ-CD, respectively. Hence, the enzyme was specific for the β-CD production and can be classified as β-CGTase. Moreover, this CGTase displayed a weak hydrolytic activity since the production of reducing sugars reached only about 4 g/l versus 42 g/l of CDs after 42 h of incubation (Fig. 6).

The CDs yield obtained by the action of CGTase is very important for a potential industrial application. In order to determine the suitable enzyme concentration for a maximal productivity, a study was carried out by varying the amount of enzyme per gram of the substrate (50–300 U/g). Reactions mixtures containing different concentrations of enzyme were incubated in the same condition during 42 h. The conversion rate of starch into CDs was about 34, 42, 30 and 28% for 50, 100, 200 and 300 U/g, respectively (Table 2). This result indicated that the enhancement of CDs production was not closely correlated with the increase in the enzyme concentration; thereby the best concentration to be used is 100 U/g of substrate. The reduction of CDs production with the augment of the enzyme concentration was already reported for others CGTases [7,12]. In our case, this diminution was associated with an increase in the amount of the liberated reducing sugars, indicating that excess enzyme favors the catalysis of hydrolyzing, coupling and disproportionation activities.

The maximum of CDs concentration generated by the majority of the reported β-CGTases fluctuated from about 3 to 40 g/l (Table 3). Only the β-CGTase from *Bacillus firmus* is reported to produce about 75 g/l of CDs under optimized conditions; including the use of 15% of pregelatinized tapioca starch [7]. Hence, in our knowledge, the CDs production generated by the action of US132 β-CGTase was the best one after that of β-CGTase of *B. firmus*. In addition, the CDs productivity of US132 CGTase could be improved by changing starch source and using higher substrate concentration as reported for others CGTases [9,30]. Alternatively, this productivity could be also enhanced via an ultrafiltration membrane bioreactor for continuous removal of product from reaction mixture [12].

The β-CGTase from *P. pabuli* strain US132 could be considered as an interesting candidate for the production of β-CD since it generated a high level of cyclodextrins without any toxic complexing agents and produce mainly β-CD (about 63% of total CDs). Likewise, the CDs yield and ratio generated by US132

Table 2
Effect of enzyme concentration on CDs and reducing sugars production

Enzyme concentration (U/g)	Conversion rate into CDs (%)	Product ratio (%)			Reducing sugars amount (g/l)
		α	β	γ	
50	34	19	64	17	3
100	42	22	63	15	4
200	30	35	50	15	12
300	28	43	41	16	18

Table 3
Comparison of the CDs productivity obtained by some reported β-CGTases

β-CGTase from	Maximum of CDs concentration (g/l)	Product ratio (%)			References
		α	β	γ	
Paenibacillus illinoisensis ST-12K	3.2	24	69	7	[6]
Bacillus sp. G1	4.5	0	89	11	[10]
Bacillus agaradhaerens LS-3C	6	11	89	0	[7,8]
Bacillus sp. 7–12	34	12	47	41	[28]
T. thermosulfurigenes EM1	35	28	58	14	[3]
Bacillus circulans strain 251	40	15	65	20	[4,11]
Bacillus firmus	75.4	2	92	6	[30]
Paenibacillus pabuli US132	42	22	63	15	This work

CGTase were very similar to those generated by the β-CGTase of *Bacillus circulans* strain 251 used at the industrial scale for the production of β-CD [4,31].

3.6. Effect on loaf volume and bread firmness

The effect of the US132 β-CGTase on bread features was investigated and compared to that obtained by the commercial anti-staling enzyme, Novamyl. This study showed that the US132 β-CGTase addition to the dough mix increased the loaf volume by 11% compared with that obtained by the negative control to which no enzyme was added. However, the volume of bread was increased only by 4% when Novamyl was used (Table 4). The improvement of the loaf volume is usually believed to be ascribable to the enhancement of yeast metabolism in presence of short oligosaccharides generated by the action of added amylases. Conversely, in this study, no important change in yeast growth was observed since the proof time remained almost constant for all experimental loaves (Table 4). Hence, this result strongly suggests that the increase of loaf volume is not closely related to the fermentation conditions but it depends on the interaction of the generated cyclodextrins with the dough matrix. Indeed, Mutsaers and Van Eijk [13] reported that the production of β-CD, by CGTase added into the dough, increases the swelling power and solubility of wheat starch granules during gelatinization, thereby improving the volume of baked products. Moreover, it was reported by Gujral et al. [32] that cyclodextrins enhance the solubility of hydrophobic proteins and are involved in better entrapment of the CO_2, leading to increased volume and better texture.

In order to estimate the anti-staling effect of the US132 CGTase, the evolution of the crumb firmness in experimental loaves was carried out during a storage period of 10 days (Fig. 7). Interestingly, this study showed that the firmness of the loaf supplemented with the β-CGTase was much lower than that of the negative control and was approximately similar to that of the positive control containing Novamyl. Consequently, US132 β-CGTase was as effective as the commercial anti-staling enzyme in preventing bread firmness. The anti-staling effect of this CGTase can be attributed to the sufficient hydrolysis of starch avoiding amylopectin crystallization. Furthermore, released cyclodextrins could form complexes with lipids, present

Table 4
US132 β-CGTase and Novamyl effects on the loaf volume and on the proof time

	Negative control	Novamyl	US132 β-CGTase
Specific volume (cm^3/g)	4.97	5.14	5.51
Proof time (min)	79	79	78

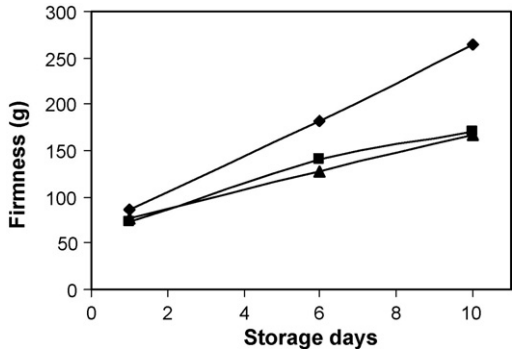

Fig. 7. Evolution of crumb firmness in experimental loaves during storage period: negative control (♦), loaf treated by Novamyl (■) and loaf treated by US132 CGTase (▲).

in the flour, and thus decreased the retrogradation of the amylose [33]. Alternatively, cyclodextrins could interfere with the starch-gluten interactions which are also reported as responsible for bread staling [34].

4. Conclusions

CGTase from *P. pabuli* strain US132 was purified and characterized. The enzyme described here is a good candidate for two industrial fields: CDs production and bread-making. The US132 CGTase produced high level of CDs, without any organic solvent, reaching 42 g/l with β-CD as predominant product (63% of total CDs). This selectivity of US132 CGTase towards β-CD could be improved by protein engineering strategies, which further enhance the industrial importance of this CGTase. On the other hand, the use of this CGTase improved substantially the loaf volume and delayed the bread staling. In the aim to make the enzyme more useful and appropriate even in countries where CDs are not allowed as food additives, mutants with reducing cyclization activity could be also envisaged.

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