Research Article

The Cyclodextrin Glycosyltransferase of *Paenibacillus pabuli* US132 Strain: Molecular Characterization and Overproduction of the Recombinant Enzyme

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The gene encoding the cyclodextrin glycosyltransferase (CGTase) of *Paenibacillus pabuli* US132, previously described as efficient antistaling agent and good candidate for cyclodextrins production, was cloned, sequenced, and expressed in *Escherichia coli*. Sequence analysis showed that the mature enzyme (684 amino acids) was preceded by a signal peptide of 34 residues. The enzyme exhibited the highest identity (94%) to the β-CGTase of *Bacillus circulans* no. 8. The production of the recombinant CGTase, as active form, was very low (about 1 U/mL) in shake flasks at 37°C. This production reached 22 U/mL after 22 hours of induction by mainly shifting the postinduction temperature from 37 to 19°C and using 2TY instead of LB medium. High enzyme production (35 U/mL) was attained after 18 hours of induction in fermentor using the same culture conditions as in shake flask. The recombinant enzyme showed $V_{\max}$ and K_m values of 253 ± 36 μmol of β-cyclodextrin/mg/min and 0.36 ± 0.18 g/L, respectively.

1. INTRODUCTION

Cyclodextrin glycosyltransferases (CGTases; E.C.2.4.1.19) are starch degrading enzymes which generate cyclic oligosaccharides termed cyclodextrins (CDs). These oligosaccharides exist as three major types commonly known as α-CD, β-CD, and γ-CD having 6, 7, and 8 glucose residues, respectively. They have torus-shaped structures able to encapsulate a wide range of molecules, thereby modifying their physical and chemical properties. Consequently, CDs are extensively used in pharmaceutical, chemical, agricultural, cosmetic, and food industries [1, 2].

CGTases belong to the glycoside hydrolase family 13 (α-amylase family) [3]. Members of this family contain a catalytic $(\beta/\alpha)_8$ barrel and use an α-retaining mechanism but they display a variety of reaction specificities [4]. Whereas α-amylases typically catalyze starch hydrolysis reaction, CGTases catalyze mainly three transglycosylation reactions (cyclization, coupling, and disproportionation) besides a weak hydrolytic activity [5]. Both α-amylases and CGTases share three domains (A, B, and C) while CGTases possess two additional domains (D and E). Domains A and B form

the catalytic core, domains C and E are involved in substrate binding whereas domain D has unknown precise function [6, 7].

Understanding the enzymatic features has been explored by cloning, sequencing, and comparing various *cgtase* genes [8–11]. The sequence data analysis has revealed the key amino acids residues that determine the reaction mechanism and product specificity, thereby enhancing genetic engineering techniques to provide modified CGTases [7, 12, 13].

E. coli is frequently adopted as host strain for heterologous protein expression since it is well characterised with various expression systems. However, the formation of inclusion bodies remains a significant barrier for expression of heterologous proteins in *E. coli*. Refolding of inclusion bodies into soluble and active form needs high cost and tedious jobs. Hence, maximizing the yield of soluble and active recombinant proteins in vivo by altering the culture conditions is an attractive alternative [14]. However, no universal approach has been established to minimize the formation of inclusion bodies and some empirical conditions must be screened on an individual basis. To date, there are only a few reports that succeeded the overproduction of

CGTase in *E. coli* by varying fermentation conditions [15–17].

In a previous study, the CGTase of *Paenibacillus pabuli* US132 was reported as good candidate for cyclodextrins production and efficient antistaling agent [18]. In this work, we described the molecular cloning of the US132 *cgtase* gene as well as the amino acid sequence inspection and the comparison with other related CGTases. We also reported the production enhancement of the recombinant active enzyme in *E. coli* by varying mainly the cultivation conditions.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, and culture media

Paenibacillus pabuli US132 strain, previously isolated [18], was used as source of chromosomal DNA. *E. 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) was used for all plasmid constructions. *E. coli* ER2566 (F$^-$ λ^- *fhu* A2 [*lon*] *omp*T *lac*Z:T7 *gene*1 *gal* *sul*A11 Δ(*mcr*C-*mrr*)114:IS10 R(*mcr*-73:*mini*Tn10-*Tet*S)2 R(*zgb*-210:Tn10) (*Tet*S) *end*A1 [*dcm*]) and *E. coli* JM109 (F$'$ *traD36 proA$^+$B$^+$ lacI^{q}z* ΔM15/Δ(*lac-proA*B e14$^-$ (McrA$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_k^- m_k^+) *supE44 relA1*) were used for protein expression. The plasmids pSJ8 and pSJ9 (this work) carrying the *cgtase* US132 gene, derived from pCR2.1 (Invitrogen) and pTrc99a vector [19], respectively. *E. coli* recombinant strains were grown on LB, M9, 2TY [20], or M9ZB medium [16] containing 100 μg/mL ampicillin. *Paenibacillus pabuli* US132 strain was grown as previously described [18].

2.2. Cloning of the US132 cgtase gene and sequence analysis

To amplify an internal *cgtase* gene fragment, we used the highly conserved aa regions previously reported for CGTase enzymes as members of the α-amylase family [4, 21]. Furthermore, we aligned several *cgtases* DNA sequences and we located the corresponding CGTases conserved regions. Hence, a degenerate pair of primers, S209 (5$'$-GAYTTTGCRCCCAAYCAT-3$'$) and S211 (5$'$-ATCATGATTGTCRATRAA-3$'$), corresponding respectively to the conserved regions I (DFAPNH) and IV (FIDNHD), were designed. The chromosomal DNA isolated from *Paenibacillus pabuli* US132 strain was used as template for gene amplification by *Pfu* DNA polymerase (Fermentas). The PCR parameters were 94°C for 120 seconds followed by 35 cycles of 94°C for 30 seconds, 50°C for 45 seconds, and 72°C for 120 seconds. The PCR product was purified using Wizard SV Gel and PCR Clean-Up System (Promega). To amplify the entire US132 *cgtase* gene, we have conceived two primers, S260 (5$'$-TCCAATATTTCTTACGAT-3$'$) and S262 (5$'$-GTTACGTTACTCCGGG-3$'$), flanking the closely matching *cgtase* gene (accession no. L25256). The resulting PCR fragment was cloned into the pCR2.1 vector to give pSJ8 plasmid harboring the US132 *cgtase* gene under the control of the IPTG inducible T7 promoter. The pSJ8 was digested with *Hind*III and *Eco*RV and the resultant *cgtase* gene was subcloned in the pTrc99a, under the control of the Trc promoter (IPTG inducible), to give pSJ9 plasmid. Nucleotide sequences of the US132 *cgtase* gene carried by three independent pSJ8 plasmids obtained from different PCR reactions were determined. DNA sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit and the automated ABI Prism3100-Avant Genetic Analyser (Applied Biosystems). Sequence analysis and comparison were performed with BioEdit and CLUSTAL W programs. Homology search was performed using BLAST search algorithm. For the promoter determination, the prokaryotic promoter prediction program NNPP2.2A (http://www.fruitfly.org/cgi-bin/seqtools/promoter.pl) was used. The US132 *cgtase* sequence was submitted to the EMBL data bank under Accession no. AM748796.

2.3. Heterologous expression and recombinant US132 CGTase purification

The heterologous expression of the US132 CGTase was investigated using different recombinant strains (DH5α/pSJ8, ER2566/pSJ8, DH5α/pSJ9, ER2566/pSJ9 or JM109/pSJ9), various temperature cultivation (37°C or 19°C during all operating time and 37°C followed by a shift to 19°C after IPTG induction) and different medium composition (LB, M9, 2TY or M9ZB). An overnight culture of the studied strain, cultivated at 37°C, was used to inoculate 50 mL of basal medium (in 250 mL shake flask) with an initial OD$_{600\,nm}$ of 0.1. Protein expression was induced by addition of different IPTG concentration (16–240 μg/mL) at OD$_{600\,nm}$ between 0.8 and 1. Effects of the postinduction time on the US132 CGTase production were also investigated. All experiments were performed at least twice.

Batch fermentation was carried out in a 7 L fermentor INFORS AG CH-4103 (Bottmingen, Switzerland) containing 4.5 L of 2TY medium. The fermentor was inoculated, with an initial OD$_{600\,nm}$ of 0.1, by an overnight culture of the ER2566/pSJ8 strain grown at 37°C in 2TY broth. The pH, aeration and agitation were maintained constant at 7.4, 1.5 vvm and 500 rpm, respectively, during all cultivation. The temperature was shifted from 37 to 19°C after induction with 16 mg/L of IPTG when OD$_{600\,nm}$ reached 0.8–1.

The CGTase extract was prepared from the periplasm by the modified osmotic-shock procedure of Ausubel et al. [22]. Cells were harvested by centrifugation at 8000 × g for 10 minutes and the pellets were suspended in 30 mM Tris-HCl buffer (pH 8) containing 20% sucrose and 1 mM EDTA. After agitation for 10 minutes at 25°C, cells removed by centrifugation were rapidly suspended in ice-cold water, incubated for 10 minutes at 0°C and clarified by centrifugation at 12000 × g for 30 minutes. The supernatant was recovered as periplasmic fraction and used for CGTase assay. Residual cells were sonicated at 4°C for 6 minutes (pulsations 3 s, amplify 90) using a Vibra-Cell 72405 Sonicator and insoluble fraction (cells debris) was recuperated by centrifugation at 12000 × g for 30 minutes.

For the purification of the recombinant US132 CGTase, the ER2566/pSJ8 periplasmic fraction was heat-treated at 60°C for 15 minutes in presence of 10 mM calcium followed

by centrifugation at $16000 \times$ g for 30 minutes at 4°C. The supernatant was then purified using hydrophobic interaction chromatography and starch adsorption as previously described for the native enzyme [18].

2.4. CGTase assay and kinetic parameters determination

The CGTase activity, determined as dextrinisation activity, was monitored at 60°C for 10 min as described in a previous work [18].

The kinetic parameters (K_m and V_{max}) were determined by incubating the purified CGTase at 60°C in 50 mM sodium acetate buffer (pH 6.5) and using various concentrations of soluble starch ranging from 0.4 to 1.6 g/L. Samples were taken at regular time intervals and the reaction was stopped by boiling for 5 minutes. The amount of generated β-CDs was detected according to a phenolphthalein method [23]. The initial speed was calculated for each substrate concentration and then represented according to the Lineweaver-Burk method [24]. The K_m and V_{max} were determined graphically using Hyper32 program (http://homepage.ntlworld.com/john.easterby/hyper32.html).

3. RESULTS AND DISCUSSION

3.1. Identification of the P. pabuli US132 cgtase gene

To clone the US132 *cgtase* gene, an internal PCR fragment of about 600 bp was firstly amplified using US132 genomic DNA and two primers corresponding to the conserved regions I and IV in CGTases as described in Materials and Methods section. The nucleotide sequence of this internal fragment (582 bp) showed the highest homology (95% identity) with homologous region of the putative *Bacillus* Q *cgtase* gene (accession no. L25256). This result incited us to conceive two primers from the 5′ and 3′ flanking regions of the *Bacillus* Q *cgtase* gene in order to amplify the entire US132 *cgtase* gene using the chromosomal DNA of *P. pabuli* US132 as template. The PCR amplification, using *Pfu* DNA polymerase, gave a nucleotide fragment of approximately 2400 bp. The cloning of this fragment in pCR2.1 vector, under the control of the T7 promoter, provided pSJ8 plasmid and conferred starch degrading activity for *E. coli* host strains.

The nucleotide sequence analysis of the fragment containing the US132 *cgtase* gene revealed the presence of a single open reading frame (ORF) with two potential initiation ATG codons (Figure 1). A putative Shine-Dalgarno site (5′-AGAAGGGTGG-3′), exhibiting a good rationally complementarity with the 3′ end of the US132 16S rRNA gene [18], was 7 bases upstream the first ATG codon. Consequently, this latter is most likely the true initiator codon. Thus, the US132 *cgtase* ORF, consisted of 2157 bp, encoded a protein having 718 amino acids. By using the promoter prediction program, a potential −10 region (5′-TCTGCA-3′) and −35 region (5′-TTGGCG-3′) were found at 101 and 124 bp upstream the initiator codon, respectively. The first 34 amino acids are considered as signal peptide according to the SignalP server (http://www.cbs.dtu.dk/services/signalP). Therefore, the mature enzyme was consisted of 684 amino acids with an estimated molecular mass of about 74.3 kDa.

The alignment of the mature US132 CGTase sequence to the data bank showed 93, 92, 74, and 73% identity with the CGTases of *Bacillus* sp. (accession no. CAA46901), *Bacillus licheniformis* (accession no. CAA33763), *Bacillus circulans* strain 251 (accession no. CAA55023), and *Bacillus* sp.1011 (accession no. 1I75A), respectively. The highest homology (97% similarity and 94% identity) was found with the CGTase of *Bacillus circulans* strain no. 8 (accession no. 1CGT). The alignment showed a difference of 39/684 amino acids which does not radically affect the product specificity. Indeed, both enzymes are specific for the β-CD production and are classified as β-CGTases since they generated a mixture of CDs composed of α-, β-, and γ-CD 20:58:13 and 10:64:20 ratios for the CGTase of strain US132 and *B. circulans* strain no. 8 [25], respectively. However, as far as we know, no data was available concerning biochemical properties of the CGTase of *B. circulans* strain no. 8 (pH, thermoactivity, thermostability) allowing the comparison of these two enzymes.

From the multiple sequence alignment (Figure 2), the five structural domains (A, B, C, D, and E) [11, 26] could be identified in the US132 CGTase. Furthermore, four highly conserved regions labelled I–IV and located in the catalytic domain were found. These conserved regions would constitute the active center of the enzyme since it contained the three catalytic residues Asp229, Glu257, and Asp328 referring to the CGTase of *B. circulans* strain no. 8 numbering. The conserved amino acids in the acceptor binding site, namely Lys47, Tyr89, Asn94, Phe183, Asn193, Leu194, Tyr195, Asp196, Phe259, Phe283, and Asp371, reported as the main determinants for cyclization reaction [12, 27], were also identified.

3.2. Production enhancement of the active recombinant US132 CGTase

The purification of the US132 CGTase from the native strain (*Paenibacillus pabuli*) was extremely hampered by the viscosity of the crude supernatant, probably due to the presence of polysaccharides of unknown nature (data not shown). In the aim to overcome this problem and also to overproduce the US132 CGTase, we have tested the enzyme production from the initial recombinant *E. coli* strain (DH5α/pSJ8). This study showed that this latter strain, cultivated in LB medium at 37°C and induced by 16 μg/mL IPTG for 18 hours, exhibited a very low activity of about only 0.3 U/mL. For this reason, we have studied the effect of several parameters on the production enhancement of the active recombinant CGTase.

3.2.1. Effect of the association promoter/host strain

Using the same culture conditions (LB medium, 37°C and induction by 16 μg/mL IPTG for 18 hours), the association *E. coli* ER2566/pSJ8 increased the production of the active recombinant enzyme by 3-fold (Table 1) since the ER2566

Table 1: Effect of the association promoter/host strain on the production of the active recombinant US132 CGTase.

Plasmids (promoters)	pSJ8 (T7)		pSJ9 (Trc)		
E. coli strains	DH5α	ER2566	DH5α	ER2566	JM109
Enzyme production (U/mL)	0.32 ± 0.08	0.92 ± 0.08	0.35 ± 0.05	1.07 ± 0.03	0.85 ± 0.05

Table 2: Effect of the operating temperature on the production of the active US132 CGTase by E. coli ER2566/pSJ8 and ER2566/pSJ9.

Strains	Operating temperature		
	37°C	37–19°C	19°C
ER2566/pSJ8	0.93 U/mL	15 U/mL	13 U/mL
ER2566/pSJ9	1 U/mL	4 U/mL	3.6 U/mL

strain contains a chromosomal IPTG inducible copy of the T7 RNA polymerase gene. Despite this improvement, the production of the active US132 CGTase remained negligible (0.92 U/mL) compared to that obtained by the native strain (18 U/mL) [18]. To further enhance this production, the gene was placed downstream the Trc promoter in pSJ9 and the enzyme production was investigated in various strains (DH5α, ER2566 and JM109). As shown in Table 1, the association E. coli ER2566/pSJ9 gave the highest activity of only about 1 U/mL which is nearly the same with that obtained by ER2566/pSJ8 strain.

This study showed that the use of different promoters and E. coli host strains did not significantly enhance the production of the active recombinant CGTase, which is probably due to the formation of inclusion bodies often reported for E. coli heterologous expression [14, 28].

3.2.2. Effect of the operating temperature

A well-known technique to prevent aggregation of recombinant proteins in vivo consists to reduce the operating temperature [15, 16, 29]. Therefore, in order to improve the recombinant US132 CGTase production, ER2566/pSJ9 and ER2566/pSJ8 strains were cultivated at different temperatures and were induced by 16 μg/mL of IPTG for 18 hours: (a) 37°C during all operating time, (b) 37°C followed by a temperature shift to 19°C after IPTG induction, and (c) 19°C during all operating time. This study showed that the conditions (b) and (c) allowed the enhancement of the active enzyme production in the periplasm for the two constructions pSJ8 and pSJ9 (Table 2). The highest production (15 U/mL) was reached by E. coli ER2566/pSJ8 when the culture was performed at 37°C followed by temperature shift to 19°C after IPTG induction. SDS-PAGE analysis of periplasmic and insoluble fractions, obtained from E. coli ER2566/pSJ8, showed that the cultivation of the strain at 37°C expressed most the US132 CGTase as insoluble inclusion bodies with a small amount of soluble protein (Figure 3) explaining the low activity detected. However, the decrease of the operating temperature (37–19°C or 19°C) strongly increased the expression of the US132 CGTase as periplasmic soluble form (Figure 3). The enhancement of the

production of soluble and active enzyme at low cultivation temperature could be explained by: (i) the reduction of the high rate of protein synthesis, which prevents misfolding [14], (ii) the enhancement of chaperones expression, which allows correct protein folding [30].

Based on all obtained data, we retained for further investigation the strain E. coli ER2566/pSJ8 and a culture temperature at 37°C followed by a shift to 19°C after induction.

3.2.3. Effect of medium composition and postinduction time

The composition of growth medium could have significant metabolic effects on both cells growth and protein production [31]. To investigate the effect of this parameter, the strain E. coli ER2566/pSJ8 was cultivated in 2TY, M9ZB, and M9 besides LB medium used in all previous experiences. The US132 CGTase production monitored after 18 hours of induction showed that the use of M9ZB and 2TY medium increased the production by about 1.1-fold (16.5 U/mL) and 1.3-fold (20 U/mL), respectively, in comparison to that obtained by LB broth. However, the use of M9 medium decreased the production to attain only 8 U/mL. The enhancement of the US132 CGTase production was likely correlated with cell density since the $OD_{600\,nm}$ reached about 3.7, 4.8, 6, and 2 for the LB, M9ZB, 2TY, and M9 medium, respectively (Figure 4). Moreover, this difference in the production level of the recombinant active enzyme, using different medium, could be also influenced by the growth phase at which induction took place on.

Otherwise, extensive attempts to improve the enzyme production using different IPTG concentrations and stressing additives (sorbitol, mannitol, and ethanol) did not increase the US132 CGTase production (data not shown) as reported for other recombinant proteins [16, 28, 32].

The effect of postinduction time on the US132 CGTase production was examined by analysis of samples taken, from ER2566/pSJ8 culture performed in 2TY medium, at different times after IPTG addition. Following the IPTG induction, the culture temperature is shifted from 37 to 19°C. The evolution of both cell growth and active enzyme production (Figure 5) showed that the CGTase production reached its maximum (22 U/mL) after 22 hours of induction when cells are at full stationary phase. When culture attained the lysis phase the production of the active enzyme decreased by about 27% probably due to the proteases release.

3.2.4. Batch fermentation scale

The fermentation, using the ER2566/pSJ8, was scaled up in 7 L fermentor strain under the conditions previously

TABLE 3: Purification steps of the recombinant US132 CGTase.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	7000	35	200	100	—
Heat treatment	6650	14.8	450	95	2.2
HIC*	4340	1.55	2800	62	14
Starch adsorption	1400	0.28	5000	20	25

* HIC: Hydrophobic Interaction Chromatography.

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AGCGTTGTGATCATAACTCGAGGGCCATGCTTTTAAACCAGAGCGGTTTAGGACAACCCCGGTAACTCCCGAAGGAGATACCGGGGTTGT    90

TGGCGTGTCAAAAGGAGGGAAGTCTGCAATCCACTGCATATCATCCCGTTGTATTGAAAGCGCTGTTATTCAAGAGCCAGGTTGGTTGTT    180
  −35                        −10

TATCTAAAATACTACATTCACGAGAAGGGTGGATTACTATGTTTCAAATGGCCAAACGCGTACTCCTCAGCACAACACTGACACTCAGTT    270
                          RBS         M  F  Q  M  A  K  R  V  L  L  S  T  T  L  T  L  S    17

TACTTGCAGGGGGCGCACTTCCCTTTCTGCCTGCTTCCGCGATCTATGCCGATGCGGATACCGCGGTTACCAACAAGCAGAACTTTAGTA    360
L  L  A  G  G  A  L  P  F  L  P  A  S  A  I  Y  A  D  A  D  T  A  V  T  N  K  Q  N  F  S    47

CAGACGTCATCTATCAGATCTTTACGGACCGCTTCCTGGACGGCAATCCTTCCAACAACCCCACCGGAGCTGCCTACGATGCCACTTGTA    450
T  D  V  I  Y  Q  I  F  T  D  R  F  L  D  G  N  P  S  N  N  P  T  G  A  A  Y  D  A  T  C    77

GCAACCTGAAGCTGTATTGTGGCGGAGACTGGCAGGGTTTGATCAACAAAATCAACGATAACTATTTTAGCGATTTGGGCGTTACGGCAT    540
S  N  L  K  L  Y  C  G  G  D  W  Q  G  L  I  N  K  I  N  D  N  Y  F  S  D  L  G  V  T  A    107

TGTGGATCTCCCAACCGGTTGAAAATATCTTCGCCACGATCAACTACGGCGGCGTGATCAACACAGCGTATCATGGCTACTGGGCACGTG    630
L  W  I  S  Q  P  V  E  N  I  F  A  T  I  N  Y  G  G  V  I  N  T  A  Y  H  G  Y  W  A  R    137

ATTTCAAGAAGACCAATCCGTATTTCGGAACAATGGCTGACTTTCAAAATCTGATTACAACCGCTCATGCCAAAGGTATCAAGATCGTAA    720
D  F  K  K  T  N  P  Y  F  G  T  M  A  D  F  Q  N  L  I  T  T  A  H  A  K  G  I  K  I  V    167

TTGACTTTGCACCGAACCATACGTCTCCTGCCATGGAAACCGATACGTCCTTTGCTGAGAATGGCAAGTTGTACGATAACGGTAATCTTG    810
I  D  F  A  P  N  H  T  S  P  A  M  E  T  D  T  S  F  A  E  N  G  K  L  Y  D  N  G  N  L    297

TTGGCGGATACACGAATGATACGAATGGATATTTCCATCACAACGGCGGCTCCGATTTTTTCCTCCCTGGAGAATGGTATCTACAAAAACC    900
V  G  G  Y  T  N  D  T  N  G  Y  F  H  H  N  G  G  S  D  F  S  S  L  E  N  G  I  Y  K  N    227

TCTATGACCTCGCTGACCTGAATCACAATAACAGTACCATTGACCAATATTTCAAAGATGCGATCAAGCTCTGGCTTGATATGGGCGTGG    990
L  Y  D  L  A  D  L  N  H  N  N  S  T  I  D  Q  Y  F  K  D  A  I  K  L  W  L  D  M  G  V    257

ACGGCATTCGCGTTGATGCGGTGAAACATATGCCTCTCGGCTGGCAAAAGAGCTGGATGTCCTCCATCTATGCACATAAACCTGTATTTA    1080
D  G  I  R  V  D  A  V  K  H  M  P  L  G  W  Q  K  S  W  M  S  S  I  Y  A  H  K  P  V  F    287

CCTTCGGTGAATGGTTCCTGGGATCTGCTGCATCCGATGCGGATAACACGGAATTTGCCAACGAATCCGGAATGAGCCTGCTTGATTTCC    1170
T  F  G  E  W  F  L  G  S  A  A  S  D  A  D  N  T  E  F  A  N  E  S  G  M  S  L  L  D  F    317

GCTTTAACTCTGCTGTACGTAACGTCTTCCGGGATAACACATCCAACATGTATGCTCTGGATTCGATGATTACGGGCACAGCCGCAGATT    1260
R  F  N  S  A  V  R  N  V  F  R  D  N  T  S  N  M  Y  A  L  D  S  M  I  T  G  T  A  A  D    347

ACAATCAGGTGAATGATCAAGTTACGTTCATCGACAACCATGATATGGATCGGTTCAAAACAAGTGCCGTGAACAATCGTCGTCTGGAAC    1350
Y  N  Q  V  N  D  Q  V  T  F  I  D  N  H  D  M  D  R  F  K  T  S  A  V  N  N  R  R  L  E    377

AGGCGCTGGCCTTCACGCTGACTTCACGCGGCGTACCTGCCATCTATTATGGTACCGAGCAGTATCTGACGGGTAACGGGGACCCTGATA    1440
Q  A  L  A  F  T  L  T  S  R  G  V  P  A  I  Y  Y  G  T  E  Q  Y  L  T  G  N  G  D  P  D    407

ATCGGGCCAAAATGCCTTCCTTCTCCAAAACAACGACTGCCTTCAACGTGATCAGCAAGCTGGCACCTCTGCGCAAATCGAATCCGGCTA    1530
N  R  A  K  M  P  S  F  S  K  T  T  T  A  F  N  V  I  S  K  L  A  P  L  R  K  S  N  P  A    437

TCGCATATGGATCCACCCAGCAGCGCTGGATCAGCAATGATGTGTACGTCTATGAACGCAAATTTGGCAAAGTGTAGCTGTTGTTGCGG    1620
I  A  Y  G  S  T  Q  Q  R  W  I  S  N  D  V  Y  V  Y  E  R  K  F  G  K  S  V  A  V  V  A    467

TGAACCGTAATCTCTCCACACCAGCAAGCATTGCGAATCTAAGCACTTCACTGCCAACAGGCAACTACACCGATGTACTGGGCGGCGCGC    1710
V  N  R  N  L  S  T  P  A  S  I  A  N  L  S  T  S  L  P  T  G  N  Y  T  D  V  L  G  G  A    497

TGAACGGTAATAACATCACTTCCACCAACGGCAACGTCTCTTCATTCACGCTGGCAGCAGGAGCAACCGCGGTCTGGCAGTATACCACGA    1800
L  N  G  N  N  I  T  S  T  N  G  N  V  S  S  F  T  L  A  A  G  A  T  A  V  W  Q  Y  T  T    527

GCGAAACGACACCAACCATCGGGCATGTGGGTCCTGTCATGGGCAAACCGGGTAATGTCATTACGATTAGCGGACGTGGATTCGGCTCCA    1890
S  E  T  T  P  T  I  G  H  V  G  P  V  M  G  K  P  G  N  V  I  T  I  S  G  R  G  F  G  S    557

CCAAAGGCACGGTATACTTCGGTACATCAGCCGTTACCGGTGCAGCCATTACATCCTGGGAAGATACACAGATTAAAGTGACGATCCCAG    1980
T  K  G  T  V  Y  F  G  T  S  A  V  T  G  A  A  I  T  S  W  E  D  T  Q  I  K  V  T  I  P    587

CTGTTGCCGCAGGCAATTATGCTGTAAAAGTAGCTGCGAATGGCGTCAACAGCAATGCGTATAACAACTTCACCATCCTTACGGGTGATC    2070
A  V  A  A  G  N  Y  A  V  K  V  A  A  N  G  V  N  S  N  A  Y  N  N  F  T  I  L  T  G  D    617

AGGTCACTGTACGATTTGTCATTAACAATGCTTCCACCACACTGGGTCAGAACATCTATCTGACAGGTAACGTGGCAGAACTCGGCAACT    2160
Q  V  T  V  R  F  V  I  N  N  A  S  T  T  L  G  Q  N  I  Y  L  T  G  N  V  A  E  L  G  N    647

GGAGTACAGGTGCAACAGCTATTGGGCCTGCTTTCAATCAGGTTATCCATGCCTACCCAACTTGGTACTATGATGTGAGCGTGCCAGCCG    2250
W  S  T  G  A  T  A  I  G  P  A  F  N  Q  V  I  H  A  Y  P  T  W  Y  Y  D  V  S  V  P  A    677

GGAAACAACTGGAGTTCAAATTCTTCAAGAAAATAGGTGCGACCATTACGTGGGAAGGTGGTTCCAACCACACATTCACCACACCAGCCA    2340
G  K  Q  L  E  F  K  F  F  K  K  N  G  A  T  I  T  W  E  G  G  S  N  H  T  F  T  T  P  A    707

GCGGTACAGCCACTGTCAATGTGAAACTGGCAATAAGATAATCCGTCTACAAGCTAA    2396
S  G  T  A  T  V  N  V  N  W  Q  *    718
```

FIGURE 1: Nucleotide and deduced amino acid sequences of the CGTase of *Paenibacillus pabuli* US132. The putative ribosome-binding site (RBS) is double underlined. The putative promoter regions (−10 and −35) are shaded. The two possible initiator codons are boxed. The underlined amino acid sequence was the predicted signal peptide and the vertical arrow indicated the possible signal peptide cleavage site. Full nucleotide and amino acid sequences data reported in this paper were submitted to the GenBank under the accession number AM748796.

```
                                                                                          ▼
PP132    DADTAVTNKQNFSTDVIYQIFTDRFLDGNPSNNPTGAAYDATCSNLKLYCGGDWQGLINKINDNYFSDLG   70
BC8      DPDTAVTNKQSFSTDVIYQVFTDRFLDGNPSNNPTGAAYDATCSNLKLYCGGDWQGLINKINDNYFSDLG   70
Bli      DADTAVTNKQNFSTDVIYQVFTDRFLDGNPSNNPTGAAFDGTCSNLKLYCGGDWQGLVNKINDNYFSDLG   70
BC251    APDTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGTCSNLRLYCGGDWQGIINKINDGYLTGMG   70
Bsp1011  APDTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGSCTNLRLYCGGDWQGIINKINDGYLTGMG   70
         .**:*:****.*******:***** ****:*****::*.:.:**:***********:*****.*::.:*
                                      Ca²⁺ binding site
              ▼                       ▼                                       I
PP132    VTALWISQPVENIFATINYGGVINTAYHGYWARDFKKTNPYFGTMADFQNLITTAHAKGIKIVI|DFAPNH|  140
BC8      VTALWISQPVENIFATINYSGVTNTAYHGYWARDFKKTNPYFGTMADFQNLITTAHAKGIKIVI|DFAPNH|  140
Bli      VTALWISQPVENIFATINYSGVTNTAYHGYWARDFKKTNPYFGTMADFQNLVTTAHAKGIKIII|DFAPNH|  140
BC251    VTAIWISQPVENIYSIINYSGVNNTAYHGYWARDFKKTNPAYGTIADFQNLIAAAHAKNIKVII|DFAPNH|  140
Bsp1011  ITAIWISQPVENIYSVINYSGVNNTAYHGYWARDFKKTNPAYGTMQDFKNLIDTAHAHNIKVII|DFAPNH|  140
         :.**:********.:::.***.**.** ******************.:**:.*:.:****.**.**:.*
                                                              A1 ←——→ B
                                           ▼            ▼▼▼
PP132    TSPAMETDTSFAENGKLYDNGNLVGGYTNDTNGYFHHNGGSDFSSLENGIYKNLYDLADLNHNNSTIDQY  210
BC8      TSPAMETDTSFAENGRLYDNGTLVGGYTNDTNGYFHHNGGSDFSSLENGIYKNLYDLADFNHNNATIDKY  210
Bli      TSPAMETDTSFAENGRLYDNGTLVGGYTNDTNGYFHHNGGSDFSSLENGIYKNLYDLADLNHNNSTIDTY  210
BC251    TSPASSDQPSFAENGRLYDNGTLLGGYTNDTQNLFHHNGGTDFSTTENGIYKNLYDLADLNHNNSTVDVY  210
Bsp1011  TSPASSDDPSFAENGRLYDNGNLLGGYTNDTQNLFHHYGGTDFSTIENGIYKNLYDLADLNHNNSSVDVY  210
         ****.:.  ******:*****.*:******:.* *** **:**:* **********:***:****:::* *
                       II                              III            B ←—┴—→ A2
PP132    FKDAIKLWLDMGVD|GIRVDAVKH|MPLGWQKSWMSSIYAHKPVFTFG|EWFL|GSAASDADNTEFANESGMSL  280
BC8      FKDAIKLWLDMGVD|GIRVDAVKH|MPLGWQKSWMSSIYAHKPVFTFG|EWFL|GSAASDADNTDFANKSGMSL  280
Bli      FKDAIKLWLDMGVD|GIRMDAVKH|MPQGWQKNWMSSIYAHKPVFTFG|EWFL|GSAAPDADNTDFANESGMSL  280
BC251    LKDAIKMWLDLGID|GIRMDAVKH|MPFGWQKSFMAAVNNYKPVFTFG|EWFL|GVNEVSPENHKFANESGMSL  280
Bsp1011  LKDAIKMWLDLGVD|GIRVDAVKH|MPFGWQKSFMATINNYKPVFTFG|EWFL|GVNEISPEYHQFANESGMSL  280
         :*****:***:*:* *****:****** **** .:: :********** :*********. .::. ***:******
                                                                      IV
             ▼
PP132    LDFRFNSAVRNVFRDNTSNMYALDSMITGTAADYNQVNDQVT|FIDNHD|MDRFKTSAVNNRRLEQALAFTL  350
BC8      LDFRFNSAVRNVFRDNTSNMYALDSMINSTATDYNQVNDQVT|FIDNHD|MDRFKTSAVNNRRLEQALAFTL  350
Bli      LDFRFNSAVRNVFRDNTSNMYALDSMLTATAADYNQVNDQVT|FIDNHD|MDRFKTSAVNNRRLEQALAFTL  350
BC251    LDFRFAQKVRQVFRDNTDNMYGLKAMLEGSAADYAQVDDQVT|FIDNHD|MERFHASNANRRKLEQALAFTL  350
Bsp1011  LDFRFAQKARQVFRDNTDNMYGLKAMLEGSEVDYAQVNDQVT|FIDNHD|MERFHTSNGDRRKLEQALAFTL  350
         *****    :*.*:****.***.*   *   .:*: *: **** *******.:  .*:  :.**:*********
              ▼
PP132    TSRGVPAIYYGTEQYLTGNGDPDNRAKMPSFSKTTTAFNVISKLAPLRKSNPAIAYGSTQQRWISNDVYV  420
BC8      TSRGVPAIYYGTEQYLTGNGDPDNRAKMPSFSKSTTAFNVISKLAPLRKSNPAIAYGSTQQRWINNDVYV  420
Bli      TSRGVPAIYYGTEQYLTGNGDPDNRAKMPSFSKSTTAFNVISKLAPLRKSNPAIAYGSTQQRWINNDVYI  420
BC251    TSRGVPAIYYGTEQYMSGGTDPDNRARIPSFSTSTTAYQVIQKLAPLRKCNPAIAYGSTQERWINNDVLI  420
Bsp1011  TSRGVPAIYYGSEQYMSGGNDPDNRARLPSFSTTTTAYQVIQKLAPLRKSNPAIAYGSTHERWINNDVII  420
         ***********:***::*..******:.****:.**:.*: ********.*.********:.:****.**::
                                                               A2 ←——→ C
PP132    YERKFGKSVAVVAVNRNLSTPASIANLSTSLPTGNYTDVLGGALNGNNITS-TNGNVSSFTLAAGATAVW  489
BC8      YERKFGKSVAVVAVNRNLSTSASITGLSTSLPTGSYTDVLGGVLNGNNITS-TNGSINNFTLAAGATAVW  489
Bli      YERKFGKSVAVVAVNRNLTTPTSITNLNTSLPSGTYTDVLGGVLNGNNITS-SGGNISSFTLAAGATAVW  489
BC251    YERKFGNSVAVVAVNRNLNAPASISGLVTSLPQGSYNDVLGGILNGNTLSVSGGGAASNFTLAAGGTAVW  490
Bsp1011  YERKFGNNVAVVAINRNMNTPASITGLVTSLPRGSYNDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVW  490
         ******..*****:.***.:.:::**: .:.**.* .*** .***:.. :.:    *.**:.:***.*****
PP132    QYTTSETTPTIGHVGPVMGKPGNVITISGRGFGSTKGTVYFGTSAVTGAAITSWEDTQIKVTIPAVAAGN  559
BC8      QYTTAETTPTIGHVGPVMGKPGNVVTIDGRGFGSTKGTVYFGTTAVTGAAITSWEDTQIKVTIPSVAAGN  559
Bli      QYTASETTPTIGHVGPVMGKPGNVVTIDGRGFGSAKGTVYFGTTAVTGSAITSWEDTQIKVTIPPVAGGD  559
BC251    QYTAATATPTIVHVGPMMAKPGVTITIDGRGFGSKGTVYFGTTAVSGADITSWEDTQIKVKIPAVAGGN  560
Bsp1011  QYTTDATTPIIGNVGPMMAKPGVTITIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQVKIPAVPGGI  560
         ***:. :***.: :.***:*:*.::* :******  ***:.*.* ***.:*: : **.*.*:.**.*.* *
         C ←——→ D
PP132    YAVKVA-ANGVNSNAYNNFTILTGDQVTVRFVINNASTTLGQNIYLTGNVAELGNWSTGATAIGPAFNQV  628
BC8      YAVKVA-ASGVNSNAYNNFTILTGDQVTVRFVINNASTTLGQNLYLTGNVAELGNWSTGSTAIGPAFNQV  628
Bli      YAVKVA-ANGVNSNAYNDFTILSGDQVSVRFVINNATTALGENIYLTGNVSELGNWTTGAASIGPAFNQV  628
BC251    YNIKVANAAGTASNVYDNFEVLSGDQVSVRFVINNATTALGQNVYLTGSVSELGNWDP-AKAIGPMYNQV  629
Bsp1011  YDIRVANAAGAASNIYDNFEVLSGDQVTVRFVINNATTALGQNVFLTGNVSELGNWDP-NNAIGPMYNQV  629
         * ::**  *..:.**.* :**.:.*****:.*.** .:*:.: *.**:.***.****  :.*. *:.**:*
              D ←—┴—→ E
PP132    IHAYPTWYYDVSVPAGKQLEFKFFKKNGATITWEGGSNHTFTTPASGTATVNVNWQ-            684
BC8      IHQYPTWYYDVSVPAGKQLEFKFFKKNGSTITWESGSNHTFTTPASGTATVTVNWQ-            684
Bli      IHAYPTWYYDVSVPAGKQLEFKFFKKNGATITWESGSNHTFTTPTSGTATVTINWQ-            684
BC251    VYQYPNWYYDVSVPAGKTIEFKFLKKQGSTVTWEGGSNHTFTAPSSGTATIINVNWQP           686
Bsp1011  VYQYPTWYYDVSVPAGQTIEFKFLKKQGSTVTWEGGANRTFTTPTSGTATVNVNWQP           686
         :: **.**.***********:**:**:***:*:*:*.*::*:.*.*:*:*********:.:**
```

FIGURE 2: Comparison of the deduced amino acid sequence of US132 CGTase with those of related CGTases. The five domains found in CGTase proteins are indicated by letters A to E under the sequences. The four highly conserved regions are boxed and the catalytic residues are shaded. Arrows indicate the main amino acid residues of cyclization activity Lys47, Tyr89, Asn94, Phe183, Asn193, Leu194, Tyr195, Asp196, Phe259, Phe283, and Asp371. The numbering starts after the respective signal sequence, with identity (∗), strongly similar (:), and weakly similar (·). PP132: CGTase of *Paenibacillus pabuli* US132 strain; BC8: CGTase of *Bacillus circulans* strain no. 8; Bli: CGTase of *Bacillus licheniformis*; BC251: CGTase of *Bacillus circulans* strain 251; Bsp1011: CGTase of *Bacillus* sp.1011.

optimised: 2TY medium and temperature shift from 37 to 19°C after IPTG induction. Interestingly, the production of the US132 CGTase in fermentor reached a maximum (35 U/mL) after only 18 hours of induction while this maximum (22 U/mL) was obtained after 22 hours in Erlenmeyer. It should be mentioned that the cell density obtained in the two cases was almost the same (about $OD_{600\,nm} = 6$). This finding suggested that the improvement of the active enzyme production was not only related to the cell growth but it could also depend on the stability of fermentation parameters (pH and pO_2), which probably affect translation and correct folding of recombinant proteins as well as proteolysis according to Makrides [28].

3.3. Purification and kinetics parameters determination of the recombinant US132 CGTase

For the purification of the recombinant US132 CGTase, we have used the periplasmic fraction of the ER2566/pSJ8 strain cultivated in the fermentor in the cultivation conditions described above. This periplasmic fraction was firstly
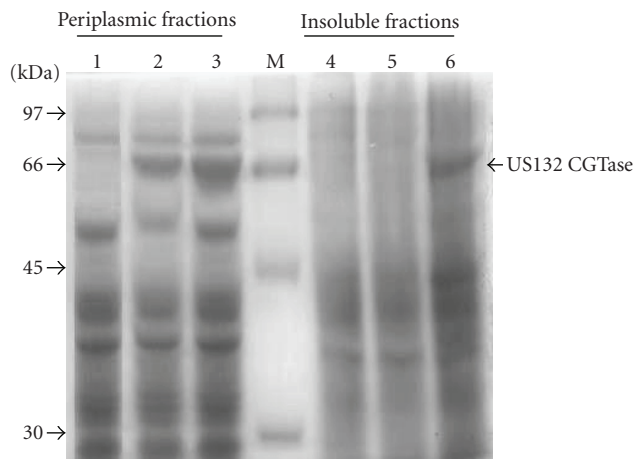
FIGURE 3: Influence of the operating temperature on the production of soluble US132 CGTase by *E. coli* ER2566/pSJ8 strain. Protein samples loaded onto the 10% SDS-polyacrylamide gel were stained with Coomassie Brilliant Blue. Lanes 1, 2, and 3: periplasmic fractions obtained from cells cultivated at 37, 19, and 37–19°C, respectively. Lane M: low molecular weight marker. Lanes 4, 5 and 6: insoluble fractions obtained when cells are cultivated at 37–19, 19, and 37°C, respectively.
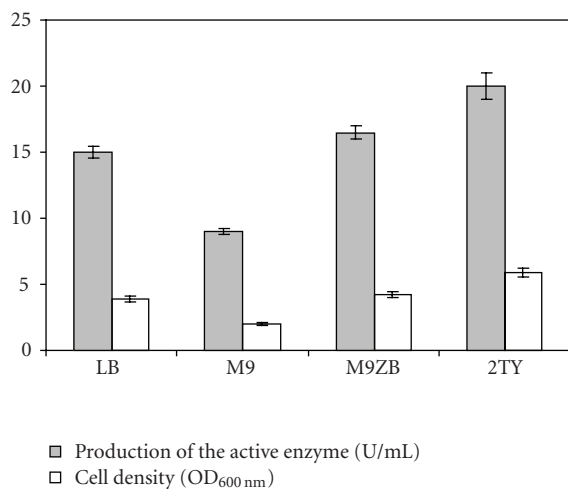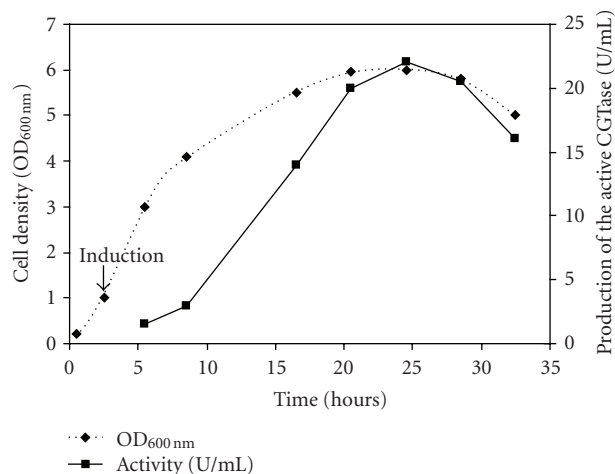

FIGURE 5: Evolution of bacterial cell growth and the active US132 CGTase production generated by *E. coli* ER2566/pSJ8 cultivated in 2TY medium. Induction was performed by 16 mg/L IPTG when culture reached $OD_{600 nm}$ between 0.8 and 1. Following the IPTG induction, the culture temperature is shifted from 37 to 19°C.

FIGURE 4: Influence of the growth medium on the active US132 CGTase production and the cell density of *E. coli* ER2566/pSJ8 strain. The temperature was shifted from 37 to 19°C after induction with 16 mg/L of IPTG when $OD_{600 nm}$ reached 0.8–1.
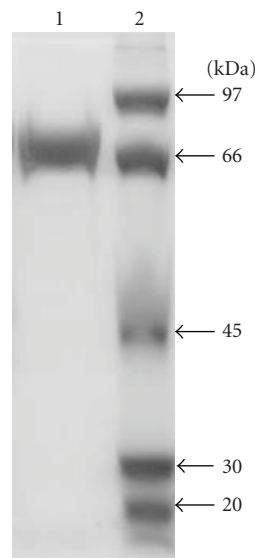

FIGURE 6: SDS-PAGE analysis of the purified US132 CGTase from the recombinant *E. coli* ER2566/pSJ8 strain. Lane 1: purified US132 CGTase, Lane 2: molecular weight marker.

heat-treated at 60°C in order to remove *E. coli* thermolabile proteins. The extract was then purified using hydrophobic interaction chromatography (HIC) and starch adsorption as described for the native enzyme [18]. The recombinant enzyme was purified to homogeneity 25-fold with a yield of 20% (Table 3, Figure 6). This recombinant US132 CGTase retained the same biochemical properties (data not shown) as the native enzyme (thermoactivity, thermostability, pH stability, CDs production) [18].

The kinetic parameters were determined by incubating the purified recombinant US132 CGTase in presence of vari-ous soluble starch concentrations and using the Lineweaver-Burk method. The V_{max} and K_m values were evaluated to be 253 ± 36 μmol of β-cyclodextrin/mg/min and 0.36 ± 0.18 g/L, respectively. K_m values determined, using also soluble starch as substrate, for CGTase from *Bacillus agaradhaerens* [33], *Bacillus circulans* E192 [34], *Bacillus firmus* [35], and *Bacillus* sp. TS1-1 [11] were 21.2, 5.7, 1.21, and 0.52 g/L, respectively. Since a small value of K_m exhibited a high affinity for the substrate, the values shown above suggested that US132 CGTase was more specific towards starch than the other reported CGTases.

4. CONCLUSION

The molecular characterization of the gene encoding the US132 CGTase showed that the enzyme is a novel β-CGTase exhibiting 95% of identity with the β-CGTase from *Bacillus circulans* strain no. 8.

The production of the active US132 CGTase by *E. coli* DH5α under usual culture conditions (37°C and LB) was very low (0.3 U/mL) suggesting the formation of inclusion bodies. The investigation of different culture parameters showed that mainly the shift of the operating temperature from 37 to 19°C increased the production of the active CGTase to reach 22 U/mL. This production was further enhanced to reach 35 U/mL by using batch fermentation while the native strain produced only 18 U/mL.

The recombinant US132 CGTase, purified to homogeneity, shared the same biochemical properties with the native enzyme. The determination of the kinetic parameters (V_{max} and K_m) of this recombinant enzyme showed that the US132 CGTase had the highest affinity towards soluble starch in comparison to other reported CGTases.

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