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Molecular cloning of a cyclodextrin glucanotransferase gene from alkalophilic *Bacillus* sp. TS1-1 and characterization of the recombinant enzyme

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Abstract

A cyclodextrin glucanotransferase (CGTase) gene from *Bacillus* sp. TS1-1 was isolated and cloned into *Escherichia coli.* Starting from TTG codon, there was an open reading frame composed of 2163 bp (721 amino acids). The NH_2 terminal position encoded a 46-amino acid of a signal peptide and followed by the mature enzyme (675 amino acids). The deduced amino acid sequence of the mature CGTase from *Bacillus* sp. TS1-1 exhibited 98.7% homology with 96% identity to the CGTase sequence from alkalophilic *Bacillus* sp. 1-1. The recombinant CGTase of *Bacillus* sp. TS1-1 expressed in *E. coli* was successfully purified to homogeneity using ammonium sulfate precipitation, followed by α-cyclodextrin-bound-epoxy-activated Sepharose 6B affinity chromatography. The purified CGTase enzymes exhibited a single band with molecular weight of 75 kDa on SDS-PAGE. Biochemical characterization of the enzyme shows an optimum temperature of 60 °C and optimum pH of 6.0. The enzyme was stable between pH 7 and 9 and temperature up to 70 °C. The K_m and V_{max} values calculated were 0.52 mg/ml and 54.35 mg of β-cyclodextrin/ml/min. The yield of the products from soluble starch as the substrate were 86% for β-cyclodextrin and 14% for γ-cyclodextrin after 24 h incubation at 60 °C, without adding any selective agent. The total β-CD produced under the conditions mentioned above was 3.65 g/l.

Keywords: Cyclodextrin glucanotransferase

1. Introduction

The cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is a member of α-amylase family (family 13 of glycosyl hydrolases). Although CGTase is closely related to α-amylase, CGTase differs from α-amylase in that a-amylase usually catalyze hydrolysis reaction using water as acceptor whereby CGTase preferably catalyze transglycosylation reactions in which glucosyl residues are used as acceptor in forming cyclodextrins (CDs) as the main product. CGTase is a multifunctional enzyme [1], besides cyclization it also display intermolecular transglycosylation (coupling, disproportionation) and hydrolytic activity on starch and CDs. Currently, bacteria are still regarded as an important source of CGTases. Since the discovery *of Bacillus macerans* as the first source that is capable of producing CGTases [2], a wide variety of bacteria have been determined as CGTase producers, namely aerobic mesophilic bacteria, aerobic thermophilic, anaerobic thermophilic and aerobic alkalophilic bacteria. Various genera of bacteria that are known as CGTase producer includes *Bacillus* [3], *Klebsiella* [4], *Brevibacterium* [5], *Thermoanaerobacterium* [6] and *Micrococcus* [7]. Most CGTases produce a mixture of α-, β- and γ-CD in different ratios, depending on the origin of the CGTase as well as the reaction conditions. CGTase is classified into three different types, α-CGTase, β-CGTase and γ-CGTase according to the major CD produced [8].

CD molecules have a unique structure with a hydrophobic cavity and hydrophilic at the outer surface and therefore can form inclusion complexes with a wide variety of hydrophobic guest molecules. Their three-dimensional form and size provide

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an important parameter for complex formation with hydrophobic compounds. Thus, specific (α-, β- and γ-)cyclodextrins are required for complexation of specific guest molecules. The formation of inclusion complexes leads to the changes in the chemical and physical properties of the guest molecules. These altered characteristics of encapsulated compounds have led to various applications of cyclodextrins in analytical chemistry [9], agriculture [10], biotechnology [11], food, pharmacy and cosmetics [12].

A major disadvantage of cyclodextrin production by CGTase is that, all known wild type CGTase enzyme produce a mixture of α-, β- and γ-cyclodextrin and are subjected to inhibition by these cyclic products. This shows that the availability of CGTase enzymes capable of producing an increase ratio of one particular type of cyclodextrin and with reduced product inhibition is important. This situation has strongly simulated genetic engineering techniques to provide a better CGTase. A CGTase producing bacteria; alkalophilic *Bacillus* sp. TS1-1 has been successfully isolated by our research group. This paper describe the isolation and cloning of the CGTase gene isolated from the bacterium. Characterization of the recombinant enzyme is also presented.

2. Materials and methods

2.1. Bacterial strain and plasmids

An alkalophilic bacterium, *Bacillus* sp. TS1-1 was isolated from the soil [13]. *Escherichia coli* JM109 [*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k-, mk+), *relA1*, *sup*E44, Δ (*lac-pro*AB), F′ (*tra* D36, *pro* AB, *lacIqZ* Δ M15)] from Promega was used as the host strain. Plasmid pUC19 from Promega was used as the cloning vector.

2.2. Medium and culture conditions

Bacillus sp. TS1-1 was grown overnight at 37 °C, 200 rpm in Horikoshi Broth [14] which contained 1.0 g/1 of KH_2PO_4, 0.2 g/1 of $MgSO_4$, 5.0 g/1 of peptone, 5.0 g/1 of yeast extract and 10.0 g/1 of Na_2CO_3 (autoclave separately). *E. coli* used as a cloning host was cultured in Luria-Bertani (LB) broth at 37 °C while ampicillin (100 μg/ml) was added to the medium to allow the growth of the plasmid-carrying strain.

2.3. DNA manipulation and cloning procedure

The genomic DNA of *Bacillus* sp. TS1-1 was prepared according to the Ish-Horowitz, method [15]. DNA manipulations were performed according to standard methods as described by Sambrook et al. [16]. *Bacillus* sp. TS1-1 genomic DNA was partially digested with *Hin*dIII. The cloning vector, pUC19 was also cleaved with *Hin*dIII and dephosphorylated with shrimp alkaline phosphate (SAP). Genomic DNA fragments were then ligated with the dephosphorylated plasmid pUC19. The ligation products were used to transform into *E. coli* JM 109. The *E. coli* transformants were plated on LB-ampicillin (100 μg/ml) plates, which contained 1% soluble starch. After growth at 37 °C for 24 h, the halo zones that appeared around the colonies after exposure to a KI–I_2 indicator solution suggested the possibility of starch being degraded by the hydrolytic activity of the β-CGTase, and the diameter of the halos indicated the amount of enzyme produced.

2.4. Assay of enzyme activity

The CGTase activity was measured by the method established by Kaneko et al. with modification [17]. The reaction mixture containing 1 ml of 0.04 g starch in 0.1 M phosphate buffer (pH 6.0) and 0.1 ml enzyme solution was used. The mixture was incubated at 60 °C for 10 min in a waterbath. The reaction was stopped by adding 3.5 ml of 0.03 M NaOH solution. 0.5 ml of 0.02% (w/v) phenolphthalein in 0.005 M Na_2CO_3 then was added to the reaction mixture. After 15 min, the decrease in colour intensity was measured at 550 nm. The percentage of reduction in the original colour intensity was interpreted with a standard curve (% OD reduction versus β-CD in mg produced) for the calculation of CGTase activity. One unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol of β-CD from soluble starch in 1 min.

2.5. Nucleotide and protein sequence analysis

The nucleotides and deduced amino acid sequence of *Bacillus* sp. TS1-1 CGTase gene was compared to those available at the GenBank and was aligned by using DNAsis/CLUSTAL X program. The nucleotide sequence reported in this work has been deposited in the GenBank database under the Accession Number AY770576.

2.6. Purification of CGTase

E. coli culture harboring CGTase gene was incubated for 24 h at 37 °C, 200 rpm in an incubator shaker. The cells were separated from supernatant by centrifugation at 8000 rpm for 10 min at 4 °C. Purification steps were carried out at 4 °C. The recombinant CGTase, was precipitated by the addition of solid ammonium sulfate $(NH_4)_2SO_4$ to give a 70% saturation. The mixture were stirred slowly and gently in order to obtain a better dissolution rate of ammonium sulfate and promoting the salting out effect. The mixture was set to stand overnight at 4 °C to enhance the precipitation and stabilization of the enzyme. The resulting precipitate was separated from the supernatant by centrifugation at 3400 × *g* for 20 min at 4 °C. The precipitate was resuspended in 800 ml of 0.01 M acetate buffer, pH 5.5. The mixture was subjected to subsequent purification procedures. Then the mixture was spun at 8000 rpm for 10 min at 4 °C to remove any remaining insoluble material before loaded onto α-cyclodextrin-bound-epoxy-activated Sepharose 6B affinity column. Twenty millilitres of supernatant containing 0.4 mg/ml of protein was subjected to a 15 mm × 100 mm affinity column, which previously had been equilibrated with 0.01 M acetate buffer (pH 5.5), at a flow rate of 21 ml/h. The column was successively washed with the same buffer for 4 h. After the unbound protein was eluted, the elution of the desired bound enzyme was carried out with the same buffer supplemented with 1% α-CD at a flow rate of 19.2 ml/h. Three millilitres of fractions were collected and each one was assayed for CGTase activity and protein content. The fractions that showed CGTase activity were pooled and dialyzed overnight against 0.01 M acetate buffer (pH 5.5), in a regenerated cellulose dialysis tubing (PIERCE, 10,000 MWCO) at 4 °C with three changes of buffer.

2.7. Molecular weight determination

The molecular weight of the purified enzyme was determined by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [18] on a vertical slab gel using 150 V for 3 h at 25 °C. The gel was stained with 1% Coomassie Brilliant Blue R-250.

2.8. Effect of pH on purified CGTase enzyme

Optimum pH for the purified enzyme was measured by reacting the enzyme with soluble starch dissolved in different buffers with varying pH. The buffers are sodium acetate buffer, 0.1 M (pH 4–5), potassium phosphate buffer, 0.1 M (pH 6–8) and glycine–NaOH buffer 0.1 M (pH 9–10). The reaction was carried out using the CGTase assay procedure mentioned before. A pH profile of the relative activity versus pH was drawn by taking the enzyme activity at optimum pH as 100%.

2.9. Effect of temperature on purified CGTase enzyme

Optimum temperature for the purified CGTase was determined by reacting the enzyme with soluble starch in 0.1 M phosphate buffer pH 6.0 at different

temperatures, ranging from 40 to 90 °C for 10 min. Then, the reaction was done according to the method of CGTase assay described previously. A temperature profile of the relative activity versus temperature was drawn by taking the enzyme activity of optimum temperature as 100%.

2.10. Effect of pH on the stability of purified CGTase

The pH stability of the enzyme was measured by incubating 0.1 ml of pure enzyme with 0.2 ml of 0.1 M sodium acetate buffer (pH 4–5), 0.1 M potassium phosphate buffer (pH 6–8) and glycine–NaOH buffer (pH 9–10), respectively at 60 °C, without substrate for 30 min. Then, the residual activity of the enzyme was assayed after reacting 0.1 ml of the enzyme mixture with soluble starch in 0.1 M phosphate buffer (pH 6.0) and incubated at 60 °C for 10 min. A pH stability profile of the residual activity versus pH was drawn by taking the residual activity of the untreated sample as a control (100% activity).

2.11. Effect of temperature on the stability of purified CGTase

The pH stability of the enzyme was measured by incubating 0.1 ml of pure enzyme 0.1 ml of pure CGTase enzyme was diluted with 0.2 ml of 0.1 M phosphate buffer (pH 6.0) without substrate and incubated at different temperatures, ranging from 40 to 90 °C for 30 min. Then, the residual activity of the enzyme was assayed after reacting 0.1 ml of the enzyme mixture with soluble starch in 0.1 M phosphate buffer (pH 6.0) and incubated at 60 °C for 10 min. A temperature stability profile of the residual activity versus temperature was drawn by taking the residual activity of the untreated sample as a control (100% activity).

2.12. Kinetic parameters of purified CGTase

The K_m and V_{max} values for the pure enzyme were determined by incubating 0.1 ml of purified CGTase (8.3U) in 1 ml 0.1 M phosphate buffer, pH 6.0, at various concentrations of soluble starch solution, ranging from 0.4 to 6.0 mg/ml at 60 °C for 10 min. The values of K_m and V_{max} were then determined using Hanes–Woolf plot.

2.13. Analysis of cyclodextrin by HPLC

For all the analyses that involved a high performance liquid chromatography (HPLC), the conditions were set as followed. The column employed was Asahipak amino NH2P-504E from Phenomenex with a length of 300 mm and 7.8 mm internal diameter. The mobile phase of the system was water/acetonitrile 30:70 and the flow rate of the mobile phase was set at 1.0 ml/min. The column temperature was maintained at 30 °C. The eluent from the column was monitored by a refractive index detector (Waters 410) and the data was recorded by the integrated computer system attached to the HPLC (Waters corp.).

3. Results and discussion

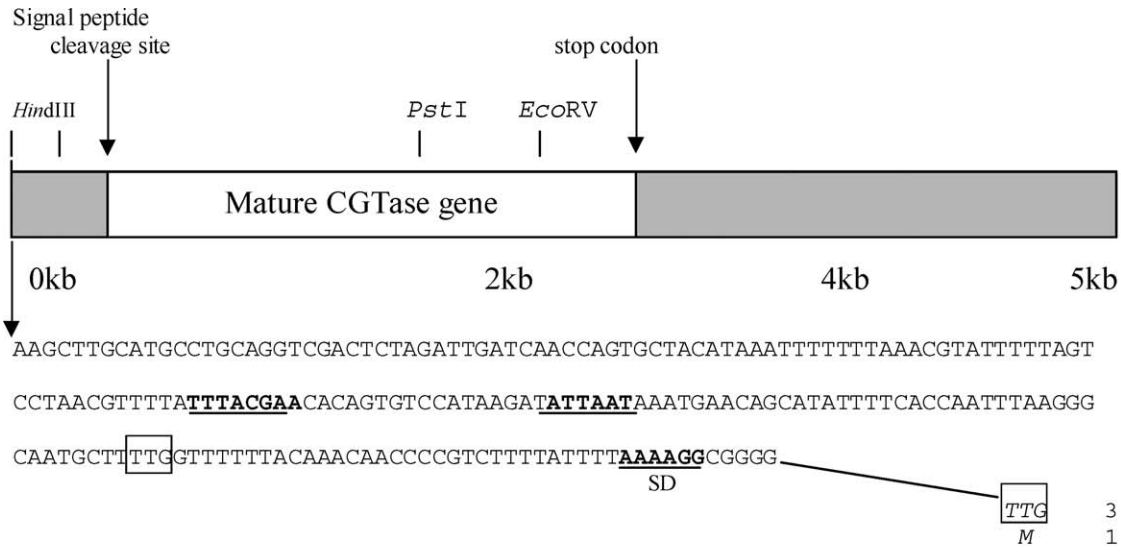
3.1. Molecular cloning of a gene encoding CGTase activity

The partially digested chromosomal DNA of *Bacillus* sp. TS1-1 with *Hin*dIII were inserted into *Hin*dIII site of pUC19, and then transformed into competent cells of *E. coli* JM109. Twenty thousand colonies of the transformants were screened and only one colony showed hydrolytic activity on LB-ampicillin containing 1% soluble starch agar plate. The 4.8 kb insert in the recombinant plasmid was sequenced using the universal M13 reverse sequencing primer. Primer walking was used to sequence the rest of the insert in both directions. Approximately, 2.4 kb nucleotide sequence of the 4.8 kb fragment was determined (Fig. 1). The nucleotide sequence analysis revealed a single ORF of 2163 bp encoding 721 amino acid residues. The initiation codon for this unique ORF was not the ATG translational initiation codon but the initiation codon appeared to be a TTG codon near the beginning of a possible signal peptide region. There were two TTG codons (boxed in Fig. 1) near the beginning of the possible signal peptide region. Since TTG is known to function as the initiation codon in *E.* coli and other prokaryotes [19], either TTG is suspected to function as the translational initiation codon. Moreover, a TTG start codon was also observed in CGTase gene from *Bacillus* sp. A2-5a [20], *Bacillus* sp. E1 [21] and *Bacillus ohbensis* [22].

However, a typical sequence for the ribosome-binding site (AAGG) was located 5 bp upstream from the second TTG codon, which is between nucleotides −6 and −9 relative to the start codon based on the Shine-Dalgarno Hypothesis. This reveals that the second TTG codon is most likely the true initiation codon. There was a putative Pribnow–Schaller box (TTACGA and ATTAAT) upstream of the mature gene starting codon (Fig. 1). Downstream of the open reading frame, a long inverted repeated sequences were found, which can form a stable stem and loop structure. It is known to be a ρ-independent transcriptional termination signal. The amino acid sequence from 1 to 46 was predicted to be a signal peptide, which is involved in secretion of the protein. The amino acid sequence of this signal peptide consists several positively charged amino acids followed by a run of hydrophobic amino acid core and a COOH-terminal alanine residue. This is consistent with the characteristic of signal peptides from other Gram-positive bacteria [23,24]. Moreover, for a probable cleavage site, the residue in position −1 must be small (either Ala, Ser, Gly, Cys, Thr or Gin) and the residue in position −3 must not be aromatic (Phe, His, Tyr, Tip), charged (Asp, Glu, Lys, Arg) or large and polar (Asn, Gin). It was also suggested that Pro must be absent from positions −3 through +1 (Fig. 1). This is the (−3, −l)-rule [25]. The nucleotide sequence reported in this work has been deposited in the GenBank database under the Accession Number AY770575.

3.2. Amino acid sequence analysis

The deduced amino acid sequence of the CGTase from *Bacillus* sp. TS1-1 was compared with other CGTases sequences using the BLAST program. The highest similarity was observed with CGTase from alkalophilic *Bacillus* sp. 1-1 (P31746), with 98.8% homology and 97.5% identity. There were a few other CGTases such as CGTase from *Bacillus* sp. KC201 (BAA02380), *Bacillus* sp. A2-5a (BAA31539), *Brevibacillus* CD162 (AAB65420) and *Bacillus ohbensis* (P27036) that has a high homology (more than 70%) with amino acid sequence of CGTase from *Bacillus* sp. TS1-1. From the multiple sequence alignment of various CGTase, six highly conserved regions (labeled I–VI) and five domains (domains A, B, C, D and E) could be identified in the sequence of the enzymes (Fig. 2). Whenever referred to in the following text, the residues position are relative to the *Bacillus circulans* strain 251 numbering [26], followed by the respective position in *Bacillus* sp. TS1-1 CGTase in brackets. Three catalytic residues, Asp-229 (222), Glu-257 (250) and Asp-328 (321) were found at the active site of recombinant CGTase from *Bacillus* sp. TS1-1. The catalytic functions of these three residues are conserved in all known CGTase. It was suggested

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TTT TGT TTT ATA TAC GTT TTA TCA CAA TTT GTT GAA GGA GGA ATA GAG TTG AAC   57
 F   C   F   I   Y   V   L   S   Q   F   V   E   G   G   I   E   L   N   19

GAT TTA AAT GAT TTT TTG AAA ACG ATT TCA TTA AGC TTT ATC TTT TTC TTG CTT  111
 D   L   N   D   F   L   K   T   I   S   L   S   F   I   F   F   L   L   37

CTT TCT TTA CCT ACT GTT GCG GAG GCT GAC GTA ACA AAC AAA GTC AAT TAC TCA  165
 L   S   L   P   T   V   A   E   A   D   V   T   N   K   V   N   Y   S   55

AAA GAT GTG ATT TAC CAG GTT GTT ACC GAT CGA TTC TCT GAC GGG AAT CCT GGC  219
 K   D   V   I   Y   Q   V   V   T   D   R   F   S   D   G   N   P   G   73

AAC AAT CCT TCA GGC GCT ATC TTT AGT CAA AAC TGT ATA GAT CTT CAT AAG TAT  273
 N   N   P   S   G   A   I   F   S   Q   N   C   I   D   L   H   K   Y   91

TGT GGT GGG GGC TGG CAA GGG ATT ATA GAC AAA ATC AAT GAC GGT TAC TTA ACT  327
 C   G   G   G   W   Q   G   I   I   D   K   I   N   D   G   Y   L   T  109

GAT TTA GGC ATT ACG GCA CTA TGG ATT TCT CAG CCA GTC GAA AAC GTT TAT GCC  381
 D   L   G   I   T   A   L   W   I   S   Q   P   V   E   N   V   Y   A  127

CTA CAC CCA AGC GGC TAT ACC TCC TAC CAT GGA TAT TGG GCT CGA GAT TAC AAA  435
 L   H   P   S   G   Y   T   S   Y   H   G   Y   W   A   R   D   Y   K  145

AAG ACA AAC CCT TAC TAT GGG AAT TTT GAT GAC TTT GAT CGT TTA ATG AGT ACC  489
 K   T   N   P   Y   Y   G   N   F   D   D   F   D   R   L   M   S   T  163

GCA CAT AGC AAT GGG ATA AAG GTA ATC ATG GAT TTC ACG CCA AAT CAT TCA TCA  543
 A   H   S   N   G   I   K   V   I   M   D   F   T   P   N   H   S   S  181

CCG GCA CTT GAA ACG AAC CCT AAC TAT GTT GAA AAT GGG GCG ATA TAT GAT AAT  597
 P   A   L   E   T   N   P   N   Y   V   E   N   G   A   I   Y   D   N  199

GGC ACA TTA TTA GGT AAC TAT TCA AAT GAT CAA CAA AAC CTC TTT CAC CAC AAT  651
 G   T   L   L   G   N   Y   S   N   D   Q   Q   N   L   F   H   H   N  217

GGC GGA ACA GAT TTC TCT TCA TAT GAA GAT AGC ATT TAC AGA AAC TTA TAT GAT  705
 G   G   T   D   F   S   S   Y   E   D   S   I   Y   R   N   L   Y   D  235
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Fig. 1. Nucleotide sequence of the cyclodextrin glucanotransferase (CGTase) gene from *Bacillus* sp. TS1-1. The flanking regions of the nucleotide sequence of the CGTase gene are shown. The signal peptide sequence is shown in italic. The possible promoter region sequences (TTACGA and ATTAAT) and the possible ribosome-binding site, SD, are underlined. Inverted repeats downstream of the stop codon TAA is shown by horizontal arrows below the sequence. A vertical arrow indicates the possible signal peptide cleavage site. This sequence was submitted to the Gen Bank with the Accession Number AY770575.

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CTG GCA GAC TAT GAT TTA AAC AAC ACA GTC ATG GAT CAA TAT TTA AAA GAG TCG  759
 L   A   D   Y   D   L   N   N   T   V   M   D   Q   Y   L   K   E   S   253

ATT AAG TTC TGG TTA GAT AAA GGG ATT GAT GGC ATT CGA GTA GAT GCC GTT AAG  813
 I   K   F   W   L   D   K   G   I   D   G   I   R   V   D   A   V   K   271

CAT ATG TCA GAA GGG TGG CAA ACC TCT TTA ATG AGC GAA ATC TAT TCG CAT AAA  867
 H   M   S   E   G   W   Q   T   S   L   M   S   E   I   Y   S   H   K   289

CCT GTT TTC ACA TTT GGA GAA TGG TTT TTA GGA TCA GGA GAA GTT GAT CCC CAA  921
 P   V   F   T   F   G   E   W   F   L   G   S   G   E   V   D   P   Q   307

AAT CAT CAC TTC GCT AAT GAA AGT GGT ATG AGT TTA TTA GAT TTC CAA TTC GGT  975
 N   H   H   F   A   N   E   S   G   M   S   L   L   D   F   Q   F   G   325

CAA ACC ATT CGT AAC GTC TTA AAA GAT CGC ACA AGC AAC TGG TAT GAT TTT AAT 1029
 Q   T   I   R   N   V   L   K   D   R   T   S   N   W   Y   D   F   N   343

GAA ATG ATT ACC AGT ACA GAA AAA GAG TAT AAC GAG GTC ATT GAT CAA GTA ACC 1083
 E   M   I   T   S   T   E   K   E   Y   N   E   V   I   D   Q   V   T   361

TTT ATT GAT AAT CAC GAC ATG AGT CGT TTT TCG GTA GGA TCA TCT TCA AAC CGT 1137
 F   I   D   N   H   D   M   S   R   F   S   V   G   S   S   S   N   R   379

CAG ACA GAT ATG GCC CTA GCT GTC TTG CTT ACT TCT CGT GGT GTA CCA ACG ATT 1191
 Q   T   D   M   A   L   A   V   L   L   T   S   R   G   V   P   T   I   397

TAC TAC GGG ACA GAG CAG TAT GTA ACA GGT GGC AAC GAC CCT GAA AAT CGC AAA 1245
 Y   Y   G   T   E   Q   Y   V   T   G   G   N   D   P   E   N   R   K   415

CCA TTG AAA ACA TTT GAT CGG TCT ACC AAC TCC TAT CAA ATC ATC AGT AAA CTT 1299
 P   L   K   T   F   D   R   S   T   N   S   Y   Q   I   I   S   K   L   433

GCT TCA CTA CGC CAA ACA AAT TCC GCC TTA GGC TAT GGC ACT ACA ACT GAA CGT 1353
 A   S   L   R   Q   T   N   S   A   L   G   Y   G   T   T   T   E   R   451

TGG CTG AAC GAA GAC ATT TAT ATT TAT GAA AGA ACG TTT GGC AAT AGT ATT GTA 1407
 W   L   N   E   D   I   Y   I   Y   E   R   T   F   G   N   S   I   V   469

TTA ACT GCT GTA AAT AGC AGT AAT AGT AAC CAG ACG ATC ACT AAT TTA AAC ACC 1461
 L   T   A   V   N   S   S   N   S   N   Q   T   I   T   N   L   N   T   487

TCT TTA CCT CAA GGG AAC TAT ACA GAT GAA CTA CAG CAA CGT TTA GAT GGA AAC 1515
 S   L   P   Q   G   N   Y   T   D   E   L   Q   Q   R   L   D   G   N   505

ACG ATT ACT GTT AAC GCC AAT GGA GCC GTA AAT TCC TTT CAA TTA CGA GCA AAT 1569
 T   I   T   V   N   A   N   G   A   V   N   S   F   Q   L   R   A   N   523

AGC GTA GCG GTT TGG CAA GTA AGC AAC CCC TCT ACG TCT CCT CTA ATC GGC CAA 1623
 S   V   A   V   W   Q   V   S   N   P   S   T   S   P   L   I   G   Q   541

GTG GGT CCT ATG ATG GGT AAG TCC GGG AAT ACC ATA ACA GTA AGC GGT GAA GGA 1677
 V   G   P   M   M   G   K   S   G   N   T   I   T   V   S   G   E   G   559

TTT GGT GAT GAG AGA GGA AGC GTT CTC TTT GAT TCA ACC TCT TCT GAA ATT ATT 1731
 F   G   D   E   R   G   S   V   L   F   D   S   T   S   S   E   I   I   577

TCT TGG TCA AAT ACA GAA ATA AGC GTA AAG GTG CCT AAT GTA GCA GGC GGT TAT 1785
 S   W   S   N   T   E   I   S   V   K   V   P   N   V   A   G   G   Y   595
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Fig. 1. (*Continued*)

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TAT GAT CTA TCC GTC GTA ACT GCA GCA AAC TTA AAA AGC CCT ACT TAC AAA GAG 1839
 Y   D   L   S   V   V   T   A   A   N   L   K   S   P   T   Y   K   E   613

TTT GAA GTA TTG TCA GGC AAT CAA GTC CGT GTC CGC TTT GGT GTT AAC AAT GCC 1893
 F   E   V   L   S   G   N   Q   V   R   V   R   F   G   V   N   N   A   631

ACA ACG AGC CCA GGA ACC AAT TTA TAT ATC GTT GGG AAT GTG AGC GAG CTG GGG 1947
 T   T   S   P   G   T   N   L   Y   I   V   G   N   V   S   E   L   G   649

AAT TGG TAT GCT GAT AAA GCA ATT GGA CCT ATG TTT AAC CAA TTG ATG TTC CAA 2001
 N   W   Y   A   D   K   A   I   G   P   M   F   N   Q   L   M   F   Q   667

TAC CCA ACG TGG TAC TAT GAT ATT AGC GTT CCT GCC GGA AAA AAC CTT GAA TAC 2055
 Y   P   T   W   Y   Y   D   I   S   V   P   A   G   K   N   L   E   Y   685

AAA TAC ATT AAA AAA GAT CAG AAC GGC AAC GTT GTC TGG CAA AGT GGC AAT AAT 2109
 K   Y   I   K   K   D   Q   N   G   N   V   V   W   Q   S   G   N   N   703

CGA ACC TAT ACG TCA CCT ACT ACC GGA ACA GAT ACG GTT ATG ATT AAT TTG TAA 2163
 R   T   Y   T   S   P   T   T   G   T   D   T   V   M   I   N   W   *   721

CGAAAGAGTAGATTACCCCCATTTTCAATTGTAGAAAATGGGGTTGATTGATAGAGAAAATCCTATAATACTTTCCTTT
                  ---------->                <----------
ATCAAACCATTTTATTGAGTTGCGTAGATGTTGATAAATAGTTCATTTTGCCAAAACCAATTGAAGGAAAATAATAGTACG
TTACAATAATAAGTAGCAATTAGAGACTCTCATAAAAGAGGCTGGGACATAACGAAAAGTAGTATTTGAAAAGACGAATAG
TCTAAAATATGCTGAGTAGCACCGCTACAGGAGAATCCTTCGCTTTCCAGGGACACGGCCTCAGCCTCCTCCGTGGAAAAC
CGCCACTTCAGAGTCTTCAGACACGTGCTGATCCCCCAGGAGTCTTGGATTCTCCTTCCGTTAATGTTCATATAAAATAAA
CGACGAACGTTCCTATTTTCAGGAATGTTCGTCATTTTGGTCTGTCTATCTATTTTTGTCCCAGCCTCTTTTAAAGATTGT
TATTAACTAATAATCGTTCAATTATGCGATAAAGGATCGCACGTTCATTCTTACTAAATTTTATAAATAGTTACTGATTAA
ATGCAAATTATATAATTAGCCGAATCAGATTGCTAATACTTCAAGGTATAGTTACTTAGTTAAAAAC
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Fig. 1. (*Continued*).

that these three residues play an important role in the enzymatic reaction catalyzed by CGTase [27]. Glu-257 (250) involved in catalysis of CGTase and is the proton donor in the first step of the cyclization reaction. Asp-229 (222) acts as the general base or nucleophile while Asp-328 (321) is involved in substrate binding.

The specific CGTase residues in Domain A1, which are unique and completely conserved, were also found in CGTase from *Bacillus* sp. TS1-1. These include, Asp27 (23), Asn32 (28), Asn33 (29) and Asp53 (49), which are ligands of a calcium binding site observed in CGTases but not in α-amylases [28]. In Domain B of *Bacillus* sp. TS1-1, more unique CGTase residues are found which is Phel36 (129), Glul53 (146), Glyl65 (158), Tyrl67 (160), Phel75 (168), Hisl77 (170), Glyl80 (173), Ilel90 (183), Tyr191 (184) and the stretch ^{185}K(R),N.L.Y.D^{189}. Apart from that, several unique residues are found in Domain A2 of CGTase *fromBacillus* sp. TS1-1 which is Tyr210 (203), Trp218 (211), Ile226 (219) and Trp258 (251). Eleven strictly conserved residues of the raw-starch binding motif were found in this region of Domain E in CGTase from *Bacillus* sp. TS1-1. Four of the 11 conserved residues which are Trp616 (604), Lys651 (644), Trp662 (652) and Asn667 (657) are a part of maltose binding site one (MB SI) while another three; Thr598 (587), Gly601 (590) and Trp636 (625) are a part of MBS2. The remaining four strictly conserved residues in CGTase from *Bacillus* sp. TS1-1; Gly608 (597), Leu613 (602), Gly614 (603) and Pro634 (623) are probably required for structural support of the raw starch-binding domain. Sequence comparisons of different CGTases from different sources also suggest that amino acid residue at position 47 (*Bacillus circulans* 251 CGTase numbering) affects cyclodextrin product specificities [29]. In position 47, Arg or Lys is found to be in CGTases producing mainly α- and/or β-CD while His is found in CGTases with no α-CD produced. His can be found at the same position in CGTase from *Bacillus* sp. TS1-1 and this implies why *Bacillus* sp. TS1-1 did not produce any α-CD.

3.3. Enzymatic properties of the purified CGTase

The recombinant CGTase was expressed in *E. coli* using pUC19 as a cloning vector. About 17.80 U/ml CGTase activity was observed secreted by the recombinant clone into the culture medium. However this value is lower compared to the parental strain, which exhibited 49.25 U/ml CGTase activity. The recombinant enzyme was successfully purified to homogeneity in two steps by ammonium sulfate precipitation, followed by α-cyclodextrin-bound-epoxy-activated Sepharose 6B affinity chromatography. The purified enzyme showed a single band of molecular weight of 75 kDa on SDS-PAGE (Fig. 3). This is in agreement from deduced molecular weight from amino acid sequence of CGTase from *Bacillus* sp. TS1-1, which is calculated to be 75,139.92 Da. The enzyme eluted from the α-cyclodextrin-bound-epoxy activated Sepharose 6B column resulted in 280-fold purification with a 5.23% yield.

The enzyme displayed optimum activity at 60 °C and were stable up to 70 °C, retaining 90% of the original activity after heat treatment at 60 °C for 30 min. The effect of pH on the CGTase activity and stability were also determined. The enzyme exhibited an optimum activity at pH 6.0 and was stable from pH 7.0 to 9.0 with a gradual loss of activity at higher and

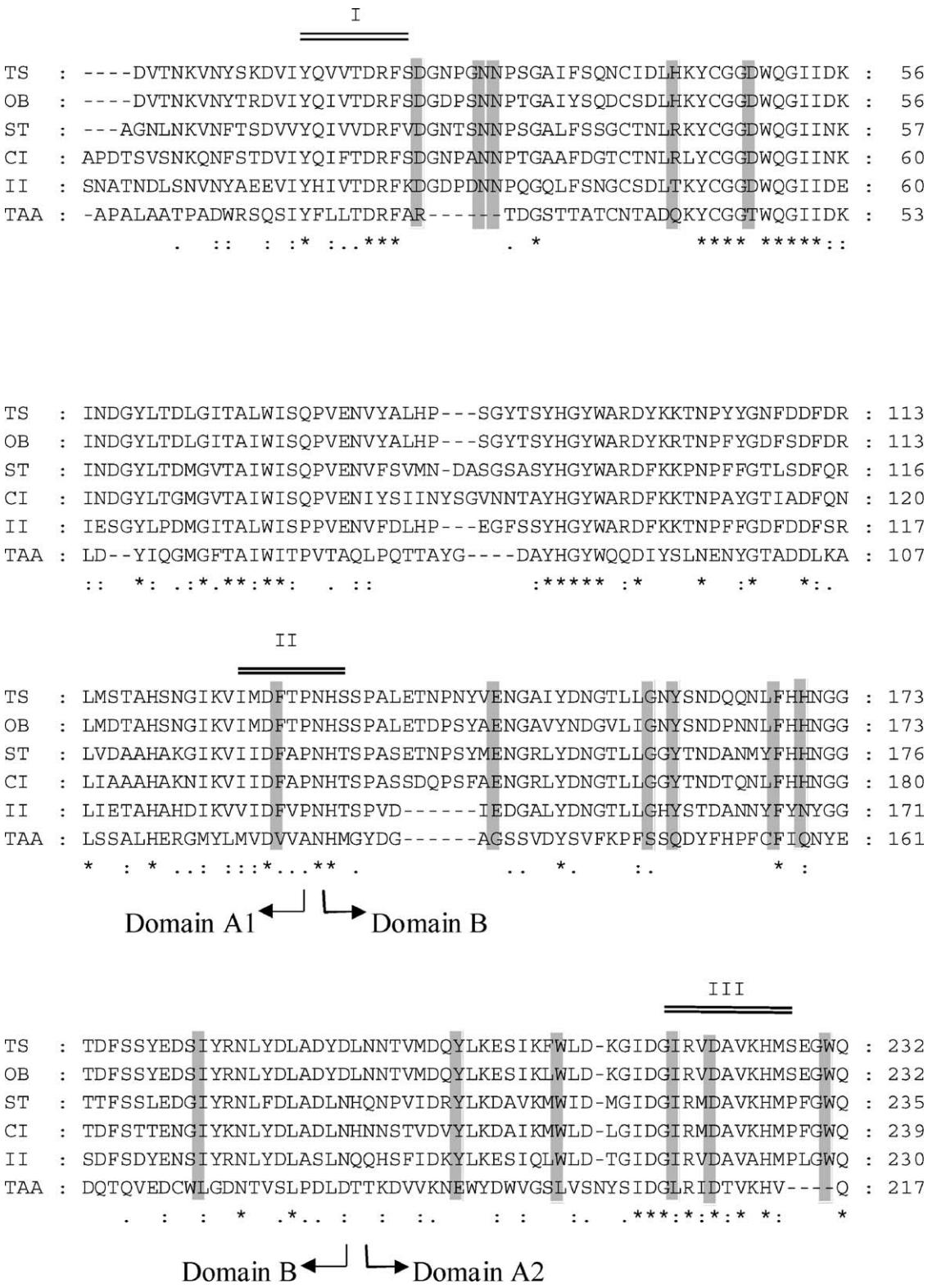

Fig. 2. Comparison of the deduced amino acid sequence *of Bacillus* sp. TS1-1 with other typical α-, β-, γ-CGTases and α-amylase. The six highly conserved regions in different CGTases are overlined. The important amino acids are shaded. The numbering starts after the respective signal sequence, with identity (*), strongly similar (:) and weakly similar (·). TS: CGTase from *Bacillus* sp. TS1-1 (β/γ-CGTase); OB: CGTase from *Bacillus ohbensis* (β/γ-CGTase); ST: CGTase from *Bacillus stearothermophilus* (α/β-CGTase); CI: CGTase from *Bacillus circulans* strain 251(β-CGTase); II: CGTase from *Bacillus clarkii* 7364 (γ-CGTase); TAA: α-amylase from *Taka-amylase* A.

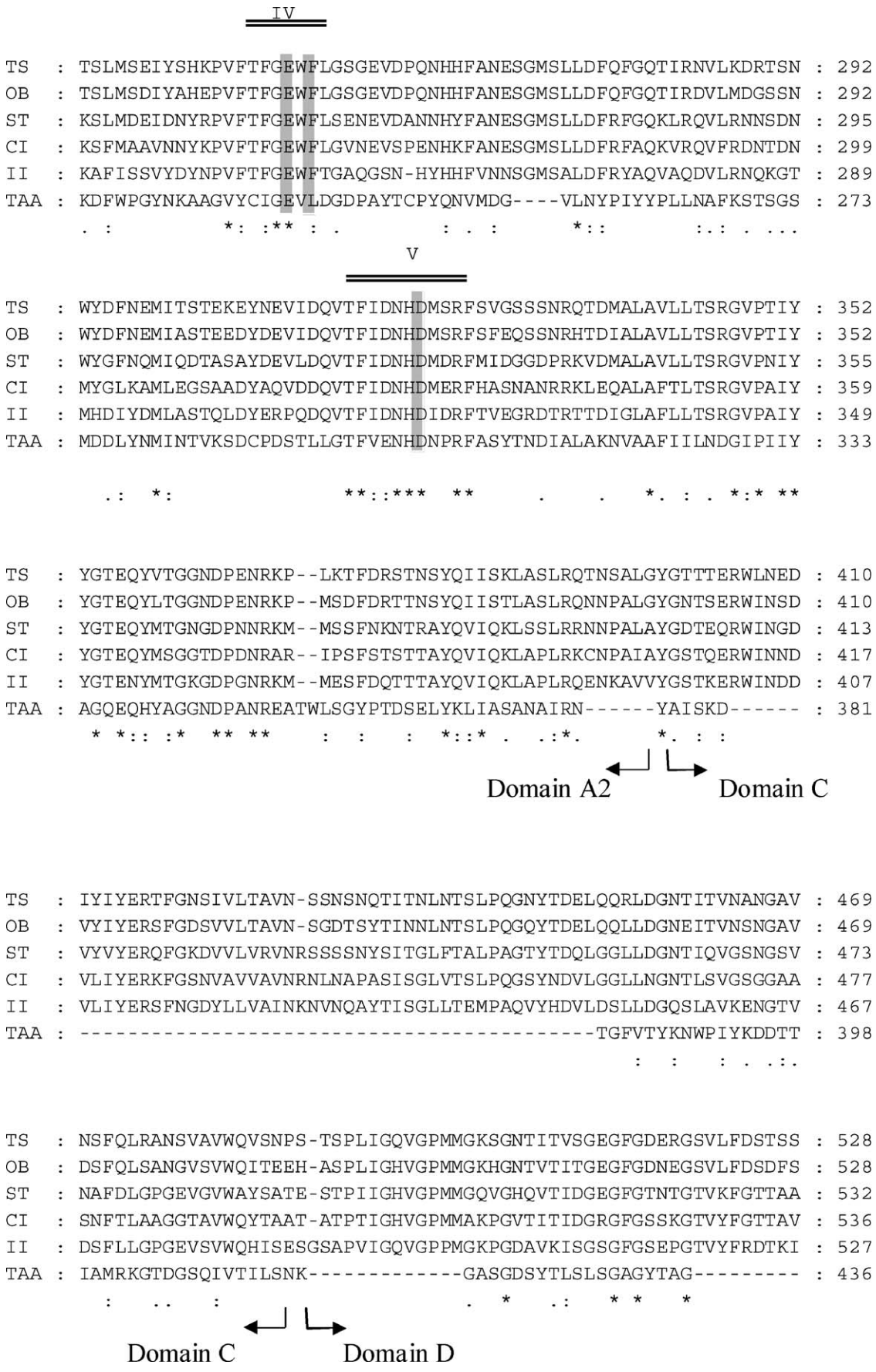

Fig. 2. (*Continued*)

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TS  : E---IISWSNTEISVKVPNVAGGYYDLSVVTAANLKSPTYKEFEVLSGNQVSVRFGVNNA : 585
OB  : D---VLSWSDTKIEVSVPDVTAGHYDISVVNAGDSQSPTYDKFEVLTGDQVSIRFAVNNA : 585
ST  : N---VVSWSNNQIVVAVPNVSPGKYNITVQSSSGQTSAAYDNFEVLTNDQVSVRFVVNNA : 589
CI  : SGADITSWEDTQIKVKIPAVAGGNYNIKVANAAGTASNVYDNFEVLSGDQVSVRFVVNNA : 596
II  : D---VLTWDDETIVITLPETLGGKAQISVTNSDGVTSNGYD-FQLLTGKQESVRFVVDNA : 583
TAA : -------QQLTEVIGCTTVTVGSDGNVPVPMAGGLPRVLYPTEKLAGSKICSSS------ : 483
                .   :    . .  .  :: *  : .     *   ::  ..  *
                                          Domain D      Domain E

TS  : TTSPGTNLYIVGNVSELGNWDADKAIGPMFNQVMYQYPTWYYDISVPAGKNLEYKYIKKD : 645
OB  : TTSLGTNLYMVGNVNELGNWDPDQAIGPMFNQVMYQYPTWYYDISVPAEENLEYKFIKKD : 645
ST  : TTNLGQNIYIVGNVYELGNWDTSKAIGPMFNQVVYSYPTWYIDVSVPEGKTIEFKFIKKD : 649
CI  : TTALGQNVYLTGSVSELGNWDPAKAIGPMYNQVVYQYPNWYYDVSVPAGKTIEFKFLKKQ : 656
II  : HTNYGENVYLVGNVPELGNWNPADAIGPMFNQVVYSYPTWYYDVSVPADTALEFKFIIVD : 643
TAA : ------------------------------------------------------------ :   -

                VI
TS  : QNGNVVWQSGNNRTYTSPTTGTDTVMINW-- : 674
OB  : SSGNVVWESGNNHTYTTPATGTDTVLVDWQ- : 675
ST  : SQGNVTWESGSNHVYTTPTNTTGKIIVDWQN : 680
CI  : GS-TVTWEGGSNHTFTAPSSGTATINVNWQP : 686
II  : GNGNVTWESGGNHNYRVTSGSTDTVRVSFRR : 674
TAA : ------------------------------ :   -
```

Fig. 2. (*Continued*).

lower pH values (Fig. 4). The result suggests that the CGTase required a slightly acidic pH or near-neutral pH range to perform its reaction optimally. Extreme pH values were not suitable for the enzyme to carry out cyclization activity. Most of the reported CGTase exhibited optimum pH ranging from 5.0 to 8.0 [30].

The initial reaction rate of the purified CGTase was measured at various concentrations of soluble starch and the initial reaction rate was analyzed using Hanes–Woolf plot. The K_m and V_{max} values calculated were 0.52 mg/ml and 54.35 mg β-cyclodextrin/ml/min (8603 mg β-cyclodextrin/mg protein/min), respectively. K_m values for several CGTases have been reported. CGTase from *Bacillus circulans* E 192 [31] exhibited a K_m value of 5.7 mg/ml while CGTase from *Bacillusfirmus* [32] have a K_m value of 1.21 mg/ml. On the other hand, Martins and Hatti-Kaul [33] found that CGTase from *Bacillus agaradhaerens* had a K_m value of 21.2 mg/ml. All the values shown above, obtained by different researchers, were based on soluble starch as the substrate. The values obtained from this study suggested that CGTase produced by *Bacillus* sp. TS1-1 was comparatively more active compared to the reported CGTases, since it needed a much lower concentration of substrate (soluble starch) to achieve half of the V_{max}. This is because enzyme with small value of K_m has a high affinity for the substrate.

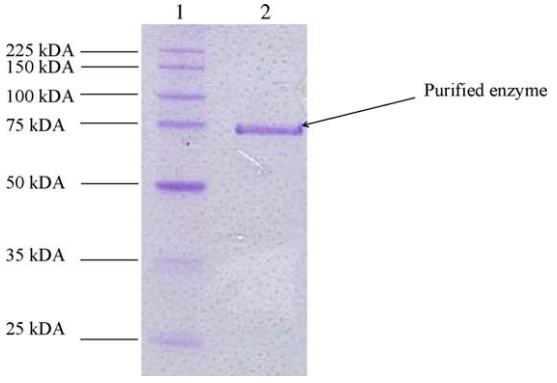

Fig. 3. SDS-PAGE analysis of purified CGTase from the recombinant *E. coli*. *Lane 1*: broad range protein molecular weight markers (from Promega), *Lane 2*: purified CGTase enzyme.

The product yield from the reaction of purified CGTase on soluble starch was investigated by using HPLC.The ratio of γ- and β-CD, in this study, was 0.14:0.86, respectively, after 24 h of incubation at 60 °C in 0.1 M phosphate buffer pH 6.0 (Fig. 5). It can be easily noticed that the production of CDs, particularly β-CD, accelerated rapidly in the first 6 h of reaction. Nonetheless, the production rate seemed to increase steadily when it reached the 12 h of reaction and still managed to increase slowly until the 24 h. From the data, there was no α-cyclodextrin as discussed in Section 3.2.

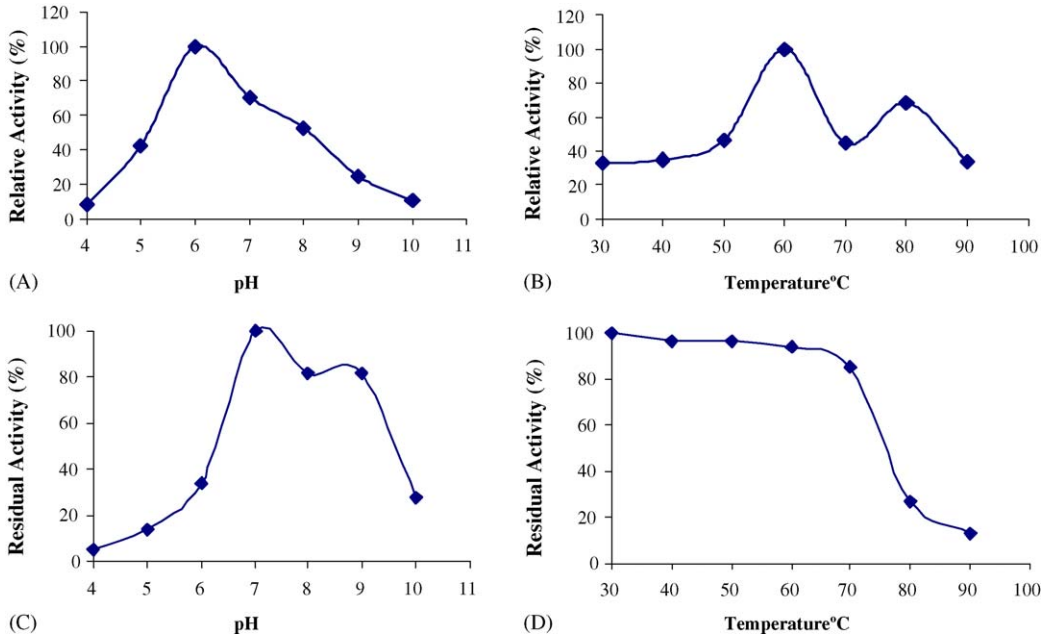

Fig. 4. Properties of the purified recombinant CGTase from *E. coli*. (A) Optimum pH, (B) optimum temperature, (C) pH stability, (D) thermal stability.

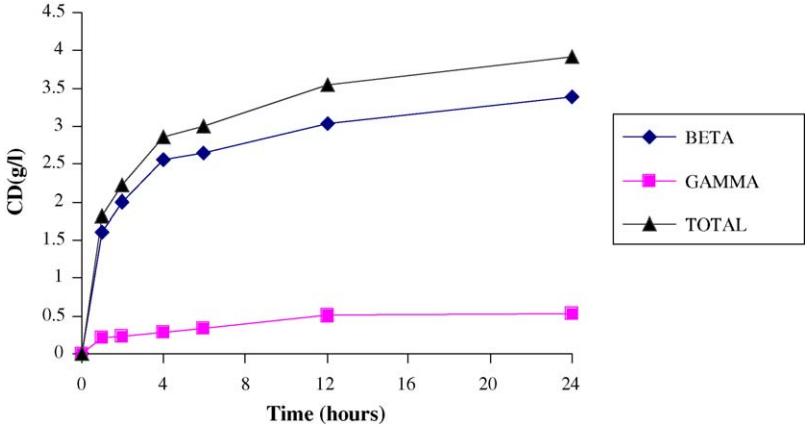

Fig. 5. Time course of CDs production by purified recombinant CGTase from *E. coli* using 10 g/1 of soluble starch as substrate. The reaction was carried out at 60 °C in 100 mM phosphate buffer, pH 6.0.

4. Conclusions

A cyclodextrin glucanotransferase (CGTase) gene from *Bacillus* sp. TS1-1 was isolated, sequenced and then cloned into *E. coli* using pUC19 as a cloning vector. The deduced amino acid sequence of the mature CGTase exhibited 98.7% homology and 96% identity to the CGTase sequence from alkalophilic *Bacillus* sp. 1-1. The purified recombinant CGTase exhibited a single band of molecular weight 75 kDa on SDS-PAGE; optimum temperature for activity of 60 °C; thermal stability up to 70 °C; the optimum pH for activity is pH 6.0 and the pH stability is between 7 and 9. The K_m and V_{max} values calculated were 0.52 mg/ml and 54.35 mg β-cyclodextrin/ml/min, respectively, and the ratio of γ- and β-CD in this study was 0.14:0.86, after 24 h of incubation at 60 °C in 0.1 M phosphate buffer pH 6.0 using soluble starch as substrate.

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