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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