# MOLECULAR AND ENZYMATIC STUDIES OF CYCLODEXTRIN GLUCANOTRANSFERASE GENE FROM *Bacillus* sp. TS1-1

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Dedicated to my

appa, amma, esapa, esama, ravimama, lecthu, karthik, renu, rathiya, kathiresh, rubi, ranjini and muhila.

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# ABSTRAK

Jujukan nukleotida 16S rRNA *Bacillus* sp. TS1-1 telah menunjukkan peratus persamaan yang tertinggi (99%) dengan *Bacillus* sp. NER dan dikenalpasti sebagai spesies *Bacillus*. Gen siklodekstrin glukanotransferase (CGTase) dari *Bacillus* sp. TS1-1 telah diisolasi dan jujukan nukleotidanya telah ditentukan. Analisis jujukan nukleotida ini menunjukkan satu rangka bacaan terbuka sepanjang 2163 bp, dengan TTG sebagai kodon permulaan. 46 asid amino di permulaan gen ini dikenalpasti sebagai jujukan peptida isyarat dan diikuti dengan jujukan peptida CGTase sebanyak 674 asid amino. Jujukan asid amino CGTase daripada *Bacillus* sp. TS1-1 menunjukkan 98.7% homologi dan 96% identiti dengan CGTase daripada alkalofilik *Bacillus* sp. 1-1. Gen CGTase dari *Bacillus* sp. TS1-1 ini telah diekspres di dalam *E.coli*. Penulenan enzim CGTase rekombinan telah dilakukan melalui pemendakan ammonium sulfat dan diikuti pula dengan kromatografi afiniti pada kolum α-CD (eposi)-*Sepharose* 6B. Aktiviti spesifik CGTase telah meningkat sebanyak 280 kali ganda, daripada 36.69 U/mg protein kepada 10289.23 U/mg protein. SDS-PAGE menunjukkan CGTase yang ditulenkan itu adalah homogen dan mempunyai berat molekul kira-kira 75 kDa. Pencirian CGTase yang ditulenkan ini menunjukkan suhu optimum pada 60$^{o}$C dan pH optimum pada 6.0. CGTase ini stabil di antara pH 7.0 hingga pH 9.0 dan pada suhu sehingga 70$^{o}$C. Nilai $K_m$ dan $V_{max}$ bagi enzim yang ditulenkan ini adalah masing-masing 0.52 mg/ml dan 54.35 mg β-siklodekstrin/ml/min . Kanji sago didapati sebagai substrat terbaik untuk penghasilan siklodekstrin (CD) jika dibandinggkan dengan kanji terlarut, jagung, sago dan beras. CGTase ini menghasilkan γ- dan β-CD dengan 86% daripada penghasilannya adalah β-CD daripada 10 g/l kanji sago selepas 24 jam pengeraman pada 60$^{o}$C tanpa penambahan sebarang agen pemilih. Jumlah β-CD yang dihasilkan adalah 3.65 g/l.

## ABSTRACT

The 16s rRNA gene sequence from *Bacillus* sp. TS1-1 exhibited the closest match with *Bacillus* sp. NER (99%) and was identified as *Bacillus* sp. A cyclodextrin glucanotransferase (CGTase) gene of *Bacillus* sp. TS1-1 was isolated and cloned into *Escherichia coli*. Beginning from the TTG codon, there was an open reading frame composed of 2163bp (721 amino acids). The $NH_2$ terminal position encoded a 46-amino acids signal peptide and followed by the mature enzyme of 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 specific activity of the CGTase increased by approximately 280 fold, from 36.69 U/mg of proteins to 10289.23 U/mg of proteins. The purified CGTase enzymes exhibited a single band with molecular weight of 75kDa 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 to pH 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 respectively. Sago starch was found to be the best substrate for cyclodextrin (CD) production among other starch sources (corn, rice, soluble and tapioca starch). Only β- and γ-CD were detected during the production of CDs. The CGTase produced about 86% of β-CD from the total CDs production, using sago starch as substrate after 24 hours of incubation at $60^oC$, without adding any selective agent. The total β-CD produced under the conditions mentioned above was 3.65 g/l.

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## LIST OF SYMBOLS/ABBREVIATIONS

| | | |
|---|---|---|
| µg | - | Microgram |
| µm | - | Micrometer |
| Å | - | Angstrom |
| °C | - | Degree Celcius |
| % | - | Percentage |
| A | - | Absorbance |
| Ala | - | alanine |
| Amp | - | Ampicillin |
| Arg | - | arginine |
| Asn | - | asparagine |
| Asp | - | aspartic acid |
| blast | - | Basic local alignment search tool |
| BSA | - | bovine serum albumin |
| bp | - | base pairs |
| Cys | - | cysteine |
| CD | - | Cyclodextrin |
| CGTase | - | Cyclodextrin glucanotransferase |
| DNA | - | deoxyribonucleic acid |
| DNase | - | deoxyribonuclease |
| DMSO | - | dimethyl sulphoxide |
| *E.coli* | - | *Escherichia coli* |
| EC | - | Enzyme Commission |
| EDTA | - | ethylene diamine tetraactic acid |
| g | - | Gram |
| Gln | - | glutamine |

| | | |
|---|---|---|
| Glu | - | glutamic acid |
| Gly | - | glycine |
| HCl | - | hydrochloric acid |
| His | - | histidine |
| HPLC | - | high performance liquid chromatography |
| Ile | - | isoleusine |
| IPTG | - | isopropyl-β-D-thiogalactopyranoside |
| kb | - | kilo base |
| kDa | - | kilo Dalton |
| L | - | Liter |
| LB | - | Luria broth |
| Leu | - | leusine |
| Lys | - | lysine |
| M | - | Molar |
| MBS | - | maltose binding site |
| Met | - | methionine |
| mg | - | Miligram |
| min | - | minute(s) |
| ml | - | Milliliter |
| MW | - | molecular weight |
| NaCl | - | sodium chloride |
| NaOH | - | sodium hydroxide |
| NCBI | - | National Center for Biotechnology Information |
| ng | - | Nanogram |
| ORF | - | Open reading frame |
| OD | - | optical density |
| pmol | - | picolmole |
| PAGE | - | polyacrylamide gel electrophoresis |
| PCR | - | polymerase chain reaction |

| | | |
|---|---|---|
| Phe | - | phenylalanine |
| Pro | - | proline |
| RNase | - | ribonuclease |
| rpm | - | revolution per minute |
| rRNA | - | ribosomal RNA |
| RNase | - | ribonuclease |
| SDS | - | sodium dodecyl sulphate |
| Ser | - | serine |
| TEMED | - | *N,N,N',N'*-tetramethylene-ethylenediamine |
| Thr | - | threonine |
| $T_m$ | - | melting point |
| Tris | - | 2-hydroxymethyl-2-methyl-1,3-propanediol |
| Trp | - | tryptophan |
| U | - | unit enzyme |
| UV | - | ultraviolet |
| Val | - | valine |
| V | - | Volts |
| v/v | - | Volume per volume |
| w/v | - | Weight per volume |
| X-Gal | - | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

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, α-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, besides cyclization it also display intermolecular transglycosylation (coupling, disproportionation) and hydrolytic activity on starch and CDs (Nakamura *et al*., 1993).

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, 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*, *Klebsiella*, *Pseudomonas*, *Brevibacterium*, *Thermoanaerobacterium*, *Corynebacterium*, *Micrococcus*, and *Clostridium* (Gawande *et al.,* 1999). 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 (van der Veen *et al.*, 2000). CGTase is classified into three

different types, α-CGTase, β-CGTase and γ-CGTase according to the major CD produced.

CD molecules have a unique structure with a hydrophobic cavity and hydrophilic outer surface and therefore can form inclusion complexes with a wide variety of hydrophobic guest molecules. Their three dimensional form and size provides an important parameter for complex formation with hydrophobic compounds. Thus, specific (α-, β- and γ-) CD s 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 CDs in analytical chemistry (Armstrong, 1988; Luong *et al*., 1995), agriculture (Saenger, 1980), biotechnology (Allegre and Deratani, 1994; Szejtli, 1994), pharmacy (Albers and Muller, 1995; Thompson, 1997), food (Allegre and Deratani, 1994; Bicchi *et al*., 1999), and cosmetics (Allegre and Deratani, 1994).

A major disadvantage of CD production by CGTase is that, all known wild type CGTase enzyme produce a mixture α-, β- and γ- CD and are subject to inhibition by these cyclic products (van der Veen *et al.*, 2000). Two different industrial approaches are used to purify the specific produced CD : selective crystallization of β- CD (which is relatively poorly water soluble) and selective complexation with organic solvent. Both of this process makes the production of CD too costly for many applications and the usage of organic solvents limits the application involving human consumption (Pedersen *et al*., 1995). This clearly shows that the availability of CGTase enzymes capable of producing an increased ratio of one particular type of CD and with reduced product inhibition would help to avoid the described expensive and harmful procedures involving organic solvents.

Besides that, for industrial and biochemical studies, it is desirable to develop a novel CGTase that is better in production of CD from starch. This situation has strongly simulated genetic engineering techniques to provide a better CGTase. It was reported that the production of CGTase increased as much as several thousand-fold in protein expression of cloned CGTase protein with the use of genetic

expression promoters (van der Veen *et al.*, 2000). CGTase producing bacteria; alkalophilic *Bacillus* sp. TS1-1 has been successfully isolated by our research group.

The enzyme from this bacterium mainly produces β-CD under the usual reaction condition. Therefore, *Bacillus* sp. TS1-1 can be considered as a good model enzyme for further studies for β-CD production.

## 1.2 Objective

The objective of this research is to isolate and clone a cyclodextrin glucanotransferase (CGTase) gene from *Bacillus* sp. TS1-1 and to characterize the recombinant enzyme.

## 1.3 Scope of Research

The scope of this research includes:

a) Bacterial 16S rRNA Identification.
b) Isolation and cloning of CGTase gene from *Bacillus* sp. TS1-1.
c) Sequencing and analysis of the CGTase gene.
d) Expression of CGTase in *E.coli*.
e) To purify the CGTase by using affinity chromatography method.
f) To characterize the purified enzyme.

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