MOLECULAR CLONING OF CYCLODEXTRIN GLUCANOTRANSFERASE GENE FROM *Bacillus* sp. G1

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Dedicated to my beloved parents, siblings and brothers
and sisters in Christ for their love and encouragement

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ABSTRACT

The cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) gene from *Bacillus* sp. G1 was successfully isolated and sequenced. The 16s rRNA gene sequence of *Bacillus* sp. G1 was compared with 18 closet neighbours and it exhibited the closest match with that from *Bacillus* sp. NER (99%). Based on this finding, *Bacillus* sp. G1 are considered to be a member of the *Bacillus* rRNA group 5 and identified as *Bacillus* sp. Determination of the nucleotide sequence revealed the presence of an open reading frame of 2610 bp beginning with a TTG initiation codon which encodes a typical signal peptide of 29 amino acid residues followed by the mature enzyme of 674 amino acid residues. The mature CGTase corresponds to a calculated molecular weight of 75389 Da which is very close to the molecular weight of the wild type *Bacillus* sp G1 (75kDa) estimated through SDS-Page. A putative Shine-Dalgarno sequence AAGG was located 5 bp upstream of the TTG codon. The deduced amino acid sequence of the mature gene *Bacillus* sp G1 showed the highest homology of 98.3%, with 95% identity to alkalophilic *Bacillus* sp 1-1. The three catalytic residues Asp221, Glu249 and Asp320 in conserved regions II, III and IV respectively was found in CGTase from *Bacillus* sp. G1. 11 strictly conserved residues of the raw-starch binding motif were also found in Domain E. Some of the key residues and regions to product specificity are identified which are Tyr188, His47, Phe252, ^{38}ETNPNY^{44} and ^{82}HP---SGY^{85}. The recombinant CGTase was expressed in the same pUC19 vector in *E.coli* and partially purified with ammonium sulphate precipitation. The optimum pH and temperature of the partially purified recombinant CGTase were 6.0 and 60^{o}C. The pH stability was from pH 7.0 to pH 9.0 and the activity was retained up to 50^{o}C after 30 minutes incubation at pH 6.0 in 0.1 M phosphate buffer without any substrate. The partially purified recombinant CGTase was able to prolong its thermal stability up to 60^{o}C in the presence of 20 mM Ca^{2+}. The CGTase was strongly inhibited by Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+}. The CGTase produced γ- and β-CD in a ratio of 0.11: 0.89 to total CD produced from 50 g/l tapioca starch after 24 hours incubation at 60^{o}C, without adding any selective agents. The amount of β-CD produced was 3.79 g/l. The partially purified CGTase from the recombinant *E.coli* retained properties quite similar to those of the wild type CGTase *Bacillus* sp. G1 in terms of molecular mass, reaction conditions, stability and the production of cyclodextrins.

ABSTRAK

Skop penyelidikan ini termasuk mengenalpasti 16S rRNA bacteria dari *Bacillus* sp. G1, memencilkan dan mengklon gen siklodekstrin glukanotransferase (CGTase, EC 2.4.1.19) dari *Bacillus* sp. G1, menganalisa jujukan nukleotida dan jujukan asid amino gen CGTase, mengekpres CGTase rekombinan di dalam *E.coli* dan pencirian CGTase rekombinan separa tulen. Jujukan nukleotida 16S rRNA *Bacillus* sp. G1 telah dibandingkan dengan 18 jujukan nukleotide yang hampir sama dan *Bacillus* sp. NER menunjukkan peratus persamaan yang tertinggi (99%). Berdasarkan ini, *Bacillus* sp. G1 dikategorikan dalam kumpulan 5 *Bacillus* rRNA and dikenalpasti sebagai *Bacillus* sp. Gen CGTase dari *Bacillus* sp. G1 telah dipencilkan dan jujukan nukleotidanya telah ditentukan. Analisis jujukan nukleotida ini menunjukkan satu rangka bacaan terbuka sepanjang 2160 bp, dengan TTG sebagai kodon permulaan dan peptida isyarat yang mempunyai 46 asid amino diikuti oleh jujukan peptida CGTase sebanyak 674 asid amino. Satu jujukan nukleotida Shine-Dalgarno, AAGG telah dikenalpasti kira-kira 5 bp sebelum kodon TTG. Jujukan asid amino CGTase daripada *Bacillus* sp. G1 menunjukkan 98.3% homologi dan 95% identiti dengan CGTase daripada alkalofilik *Bacillus* sp. 1-1. Tiga asid amino katalitik iaitu Asp221, Glu249 dan Asp320 di kawasan II, III dan IV telah dikenalpasti wujud di dalam CGTase *Bacillus* sp. G1. Siklodekstrin glukanotransferase rekombinan telah diekspres di dalam *E.coli* dan penulenan separa telah dilakukan melalui pemendakan ammonium sulfat. Enzim separa tulen siklodekstrin glukanotransferase menunjukkan pH optimum 6.0 dan suhu optimum 60ºC. Enzim ini stabil dari pH 6.0 ke 10.0 dan mengekalkan aktivitinya apabila di eram sehingga suhu 50ºC selama 30 minit dalam penimbal fosfat pH 6.0 tanpa kanji. Kestabilan suhu telah meningkat dengan penambahan 20mM Ca^{2+} tetapi aktiviti direncatkan dengan penambahan Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+} dan Fe^{3+}. Siklodekstrin glukanotransferase ini menghasilkan γ-CD dan β-CD dalam nisbah 0.15:0.85 dari jumlah CD yang dihasilkan apabila ditindakbalas dengan 70 g/l kanji ubi kayu selama 24 jam pada suhu 60ºC, tanpa panambahan sebarang agen selektif. Jumlah β-CD yang terhasil adalah 16.47 g/l. Gen CGTase dari *Bacillus* sp. G1 alkalophilik telah diklonkan dengan berjaya dan telah diekpres ke luar medium kultur.

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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 tetraacetic 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 Bertani
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 (EC 2.4.1.19) is an industrially important enzyme that produces α-, β- and γ-cyclodextrins (CDs) from starch through an intramoleculer transglycosylation reaction. The α-, β- and γ-CD have closed ring structures with six, seven and eight glucose units joined by α- 1,4-glucosidic bonds respectively. The exteriors of CDs are hydrophilic while the interiors are hydrophobic, enabling it to easily form inclusion complexes with either organic or inorganic molecules. The encapsulated guest undergoes advantageous physical and chemical changes, such as improved stability and water solubility in a variety of fine organic and inorganic compounds, sharply reducing volatility, chemical, thermal and light reactivity of guest molecules, stabilization and protection of sensitive hosts such as flavors, odor and aroma. As a result, CDs and its derivatives have wide application in today's industries such as food, cosmetics, chemicals, environmental, agricultural, cosmetics, pharmaceuticals and toiletries.

The CGTase enzyme is generally found in bacteria and 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* etc (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. CGTase is classified into three different types, α-CGTase, β-CGTase and γ-CGTase according to the major CD produced. As a result, industrial production process of CDs and the subsequent separation process are rather elaborate and costly as expensive purification procedures are needed. Furthermore, there is the consideration of solvent toxicity, flammability and the need for a solvent recovery process which is an added disadvantage. Besides, the complete removal of solvent from the CDs is expensive, limiting the use of CDs in the pharmaceutical and food industries (Biwer *et al.,* 2002).

Therefore, the availability of CGTase enzymes that are capable of producing an increased ratio of one particular type of CD and also with reduced product inhibition would help to avoid using expensive and environmentally harmful procedures involving organic solvents. On top of that, it is also desirable to develop a novel CGTase that is better to produce CD in high proportion from starch for industrial and biochemical studies. In order to achieve this, genetic engineering or molecular biotechnology technique will facilitate the process of obtaining better enzymes.

The attractive feature of CGTase from *Bacillus* sp. G1 is that it predominantly produced β-CD (89%) from tapioca starch and this high yield can be increased to 100% yield of β-CD with the addition of 4% (v/v) Triton X-100 (Ho *et al*., 2005). Therefore, CGTase from *Bacillus* sp. G1 can be considered as a good model enzyme for further studies of β-CD production. It is also a potential candidate for commercialization and industrial production of β-CD due to its stability, high specificity in β-CD production, versatility and ease of handling.

1.2 Objectives of the Research

The main objective of this research is to clone a cyclodextrin glucanotransferase gene (CGTase) from *Bacillus* sp. G1 and to express it.

1.3 Scope of Research

There are five scopes in this research:

(a) Bacterial 16S rRNA Identification
(b) Isolation and cloning of CGTase gene from *Bacillus* sp. G1
(c) Analysis of nucleotide sequence of CGTase gene and its deduced amino acid sequence
(d) Expression of CGTase in *E.coli*
(e) Characterization of the partially purified recombinant CGTase

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