UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF THERMOSTABLE GLYCOGEN BRANCHING ENZYME FROM *GEOBACILLUS* SP. GEO5 BY GENOME MINING

NUR SYAZWANI BINTI MOHTAR

FS 2013 15

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NUR SYAZWANI BINTI MOHTAR

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2013

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By

NUR SYAZWANI BINTI MOHTAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

July 2013

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

IDENTIFICATION OF THERMOSTABLE GLYCOGEN BRANCHING ENZYME FROM *GEOBACILLUS* SP. GEO5 BY GENOME MINING

By

NUR SYAZWANI BINTI MOHTAR

July 2013

Chairman: Professor Mohd Basyarudin Bin Abdul Rahman, PhD

Faculty: Science

Glycogen branching enzyme (EC 2.4.1.18) has increasing demand from food and beverages processing industries. This enzyme, which catalyses the formation of α-1,6-glycosidic branch points in glycogen structure, is used to enhance nutritional value and quality of food and beverages. To be applicable in industries, enzymes that are stable and active at high temperature are much desired. A thermophilic bacterium, *Geobacillus* sp. Geo5, was isolated from Sungai Klah Hot Springs at 97°C and therefore it was postulated that this bacterium species would produce thermostable glycogen branching enzyme that is active at high temperature. The objectives of this research are to identify the branching enzyme gene (*glgB*) of *Geobacillus* sp. Geo5, to produce the enzyme using *Escherichia coli* and to characterise the biochemical properties of the enzyme. Using genome data mining, the nucleotide sequence of *glgB* was fished out from *Geobacillus* sp. Geo5 genome sequence provided by Malaysia Genome Institute. The size of the gene is 2013 bp and the theoretical molecular weight of the protein is 78.43 kDa. The gene sequence was then used to predict the three dimensional structure of the enzyme using an online software, I-TASSER. The percentage sequence identity of the template

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(*Mycobacterium tuberculosis* H37RV; PDB ID: 3K1D) in the threading aligned region with the *Geobacillus* sp. Geo5 sequence was only 45%. Subsequently, *glgB* from *Geobacillus* sp. Geo5 was isolated using polymerase chain reaction (PCR). To study the enzyme, the gene was cloned into pET102/D-TOPO® vector by PCR cloning and overexpressed in BL21 Star ™ (DE3) *E. coli.* The expression of active enzyme was the highest when the expression was induced with 0.75 mM of IPTG, at 30°C for 8 hours. The recombinant protein was also expressed together with bacteriocin release protein to secrete the protein into *E. coli* culture medium. The study shown that induction with 5 ng/mL of mitomycin C for 8 hour was enough to secrete the recombinant protein to extracellular environment (34.1 U/mL) although not entirely since 43.0 U/mL of the activity was still in the cell. Therefore, the intracellular expression system was chosen for further studies on the enzyme. The recombinant protein from intracellular expression was then purified by affinity chromatography using HisTrap HP column with the recovery of 84%. The purified enzyme was used to study the effect of temperature and pH on enzyme activity and stability, and the inhibitory effect by metal ion on enzyme activity. This thermostable glycogen branching enzyme was found to be most active at 55°C and the half-life at 60°C and 70°C was 24 hours and 5 hours, respectively. The enzyme was stable at pH 5 to pH 9 and the optimum pH for enzyme activity was at pH 6. Metal ions, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Ca^{2+} seem to inhibit the activity of this enzyme. Mg^{2+} however does not affect the enzyme activity. From this research, a thermostable glycogen branching enzyme was successfully isolated from *Geobacillus* sp. Geo5 by genome mining together with molecular biology technique. The stability of this enzyme would be very practical for industrial applications especially in carbohydrates processing such as nutraceutical, food and beverages industries.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

IDENTIFIKASI ENZIM PENCABANG GLYCOGEN TERMOSTABIL DARIPADA *GEOBACILLUS* SP. GEO5 MELALUI PERLOMBONGAN GENOM

Oleh

NUR SYAZWANI BINTI MOHTAR

Julai 2013

Pengerusi: Professor Mohd Basyarudin Bin Abdul Rahman, PhD

Fakulti: Sains

Enzim pencabang glikogen (EC 2.4.1.18) mendapat permintaan yang meningkat daripada industri pemprosesan makanan dan minuman. Enzim ini yang memangkin pembentukan titik cabangan α-1,6-glikosida dalam struktur glikogen, digunakan untuk meningkatkan nilai pemakanan dan kualiti makanan dan minuman. Untuk diguna pakai dalam industri, enzim yang stabil dan aktif pada suhu tinggi sangat diingini. Satu bakteria termofilik, *Geobacillus* sp. Geo5, telah dipencilkan dari Kolam Air Panas Sungai Klah pada 97°C dan oleh itu ia diandaikan bahawa spesis bakteria ini akan menghasilkan enzim pencabang glikogen yang termostabil and aktif pada suhu tinggi. Objektif kajian ini adalah untuk mengenal pasti gen enzim pencabang (*glgB*) daripada *Geobacillus* sp. Geo5, menghasilkan enzim tersebut menggunakan *Escherichia coli* dan mencirikan sifat-sifat biokimia enzim tersebut. Menggunakan kaedah perlombongan data genom, jujukan nukleotida gen enzim pencabang (*glgB*) dipancing keluar daripada genom *Geobacillus* sp. Geo5 yang telah dijujuk oleh Institut Genom Malaysia. Saiz gen adalah 2013 bp dan berat molekul teori protein adalah 78.43 kDa. Jujukan gen kemudiannya digunakan untuk meramal struktur tiga dimensi enzim mengunakan perisian dalam talian, I-TASSER. Peratusan identiti jujukan bagi templat (*Mycobacterium tuberculosis* H37RV; PDB ID: 3K1D)

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di kawasan yang sejajar dengan jujukan *Geobacillus* sp. Geo5 adalah hanya 45%. Seterusnya, *glgB* daripada *Geobacillus* sp. Geo5 telah dipencilkan menggunakan tindak balas berantai polimerase (PCR). Untuk mengkaji enzim tersebut, gen diklon ke dalam vektor pET102/D-TOPO® melalui pengklonan PCR dan diekspres di dalam *E. coli* BL21 Star TM (DE3). Penghasilan enzim yang aktif adalah paling banyak apabila ekspresi diaruh dengan 0.75 mM IPTG, pada 30°C selama 8 jam. Protein rekombinan juga diekspres bersama-sama dengan protein pelepasan bakteriosin untuk merembeskan protein tersebut ke dalam media kultur *E. coli*. Kajian ini menunjukkan bahawa induksi dengan 5 ng/mL mitomycin C cukup untuk merembeskan protein rekombinan ke persekitaran ekstrasel (34.1 U/mL) walaupun tidak keseluruhannya memandangkan aktiviti sebanyak 43.0 U/mL masih di dalam sel. Disebabkan itu, sistem pengekspresan intrasel dipilih untuk kajian lanjut ke atas enzim. Protein rekombinan daripada pengekspresas intrasel seterusnya ditulenkan melalui kromatografi afiniti menggunakan turus HisTrap HP dengan pemulihan sebanyak 84%. Enzim yang telah ditulenkan digunakan untuk mengkaji kesan suhu dan pH ke atas aktiviti dan kestabilan enzim, dan kesan rencatan oleh ion logam pada aktiviti enzim. Enzim pencabang glikogen didapati paling aktif pada 55°C dan separuh hayat pada 60°C dan 70°C masing-masing adalah 24 jam dan 5 jam. Enzim stabil pada pH 5 hingga pH 9 dan pH optimum bagi aktiviti enzim adalah pada pH 6. Ion-ion logam, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} dan Ca^{2+} didapati telah merencat aktiviti enzim ini. Mg^{2+} bagaimanapun tidak menjejaskan aktiviti enzim. Daripada kajian ini, enzim pencabang glikogen yang termostabil telah berjaya dipencil daripada *Geobacillus* sp. Geo5 melalui kaedah perlombongan genom bersama-sama dengan teknik biologi molekul. Kestabilan enzim ini akan menjadi sangat praktikal untuk aplikasi perindustrian terutamanya dalam pemprosesan karbohidrat seperti industri nutraseutikal, makanan dan minuman.

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ACKNOWLEDGEMENTS

Alhamdulillah, all praises and thanks to Allah, The Most Gracious and The Most Merciful. I would like to express my gratitude to MGI and their personnel who were generous enough provide this research with the *Geobacillus* sp. Geo5 isolate and its genome sequence. I would also like to express my utmost appreciation to my supervisors Professor Dr. Mohd Basyaruddin Abdul Rahman, Professor Dr. Raja Noor Zaliha Raja Abdul Rahman and Professor Dato' Dr. Abu Bakar Salleh, for their dedication, guidance and encouragement through the course of this project. Dr Adam Leow Thean Chor and Dr. Mohd. Syukuri, I really appreciate their generous ideas and guidance.

I would like to give special thanks to my friends from Enzyme Technology Lab, IBS and 140 Lab, Faculty of Biotechnology and Biomolecular Sciences who have been together with me throughout the course. To all who has helped me and gave me the possibility to complete this thesis, thank you very much. I also thank National Science Fellowship for sponsoring my Master degree.

Most importantly, I am forever grateful to family for their endless love and support, they always believe in me and stay by my side. May we always be in His blessings.

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I certify that a Thesis Examination Committee has met on 12 July 2013 to conduct the final examination of Nur Syazwani Binti Mohtar on her thesis entitled "Identification of thermostable glycogen branching enzyme from *Geobacillus* sp. Geo5 by genome mining" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Emilia Binti Abdul Malik, PhD
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Janna Ong Binti Abdullah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Rosimah Binti Nulit, PhD
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Abdul Munir Bin Abdul Murad, PhD
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
Malaysia
(External Examiner)

NORITAH OMAR, PhD
Assoc. Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 August 2013

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Mohd Basyaruddin Bin Abdul Rahman, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Raja Noor Zaliha Binti Raja Abdul Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

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BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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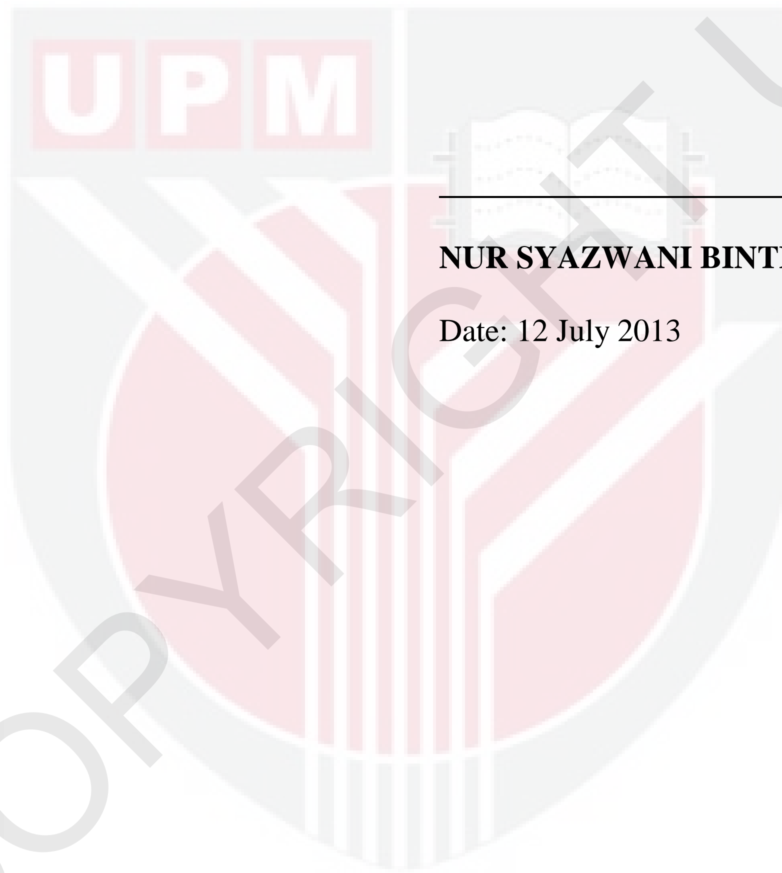
I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institutions.



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NUR SYAZWANI BINTI MOHTAR

Date: 12 July 2013

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temperatures

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LIST OF ABBREVIATIONS

3D	Three-dimensional
A_{260}	Absorbance at 260 nm
$A_{260/280}$	Ratio of absorbance at 260 nm and 280 nm
A_{600}	Absorbance at 600 nm
ADP	Adenosine diphosphate
bp	Base pair
BRP	Bacteriocin release protein
CASP	Critical Assessment of Structure Prediction
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	Enzyme classification
G5GBE	Recombinant GBE from *Geobacillus* sp. Geo5
GBE	Glycogen branching enzyme
GH	Glycoside hydrolase
HCl	Hydrochloric acid
I_2	Iodine
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilo base pair
kDa	Kilo Dalton
KI	Potassium iodide
LB	Luria-Bertani
M	Molar
MALDI TOF/TOF	Matrix-assisted laser desorption ionization tandem time-of-flight
mg	Milligram
MGI	Malaysia Genome Institute

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mL	Millilitre
mL/min	Millilitre per minute
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
ng/mL	Nanogram per millilitre
ORF	Open reading frame
PCA	Polymerase chain assembly
PCR	Polymerase chain reaction
PDB	Protein Data Bank
pET/GBE	Recombinant plasmid; *glgB* of *Geobacillus* sp. Geo5 in pET102
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
× g	Gravitational force
U	Unit
U/mg	Unit per milligram
U/mL	Unit per millilitre
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
µg/mL	Microgram per millilitre
µL	Microlitre
µM	Micromolar
µmol	Micromole

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CHAPTER 1

INTRODUCTION

Enzymes are amazing catalysts that are naturally occurring in all living organisms, efficiently catalyse from simple to complex reactions by accepting selectively a wide range of molecules as substrates. As an alternative to conventional chemical catalysts, enzymatic reactions produce fewer by-products that are mostly harmless, hence making enzymes as an environmentally friendly choice (Schmid *et al.*, 2001). Efficient selective catalysis is essential and therefore has enormous potential especially in the industrial processing and synthesis of fine chemicals, food and pharmaceuticals (Burton *et al.*, 2002). BCC Research reported that in 2010, the value of global market for industrial enzymes was US$3.6 billion and the largest segment of the industrial enzymes industry comes from food and beverage enzymes with revenues of nearly US$1.2 billion (BCC Research, 2012). These enzymes are used either to increase the nutritional value or to make the food and drinks more appealing. Therefore enzymes like amylase, lipase and protease have gained a lot of interest in the research field. Other than aforementioned enzymes, one of the enzymes that have a rising demand in food and beverages industries is branching enzyme (van der Maarel, 2009).

Branching enzyme (EC 2.4.1.18) is a type of transferase that is found naturally in plants, animals and microorganisms. This enzyme carries out the transglycosylation reaction of starch and glycogen. The transglycosylation reaction transfers

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an α-1,4-glycosidic linkage to α-1,6 making the starch and glycogen structure branched out. These branches in starch and glycogen structures help the digestion of the polysaccharides as they are more soluble and easier to be absorbed by the cells (Abad *et al.*, 2002). As glycogen branching enzyme produces more branches than starch branching enzymes, many studies are focusing on commercialisation of glycogen branching enzymes for applications in beverage, food processing and nutraceutical industries. In order to be practical in industries, enzymes that can withstand high temperature would be very sought after. One of the major concerns is to find enzymes that are naturally active and stable in high temperature. For that reason, researchers have been isolating thermostable glycogen branching enzymes from thermophilic microorganisms. However, the production of this enzyme in its thermophilic host is very low. Therefore recombinant DNA technology, such as *Escherichia coli* cloning and expression systems, were often utilised in order to maximise enzyme production to supply for industries and studies on the enzyme. *E. coli* system often preferred as this system is easy to manipulate, capable to produce enzyme rapidly and reasonably cheap.

Studies on branching enzymes have made the gene sequence of the enzyme accessible (van der Maarel *et al.*, 2002). On top of that, the venture in genome sequencing is contributing vast information on new genetic sequences and therefore abundance of novel enzymes and proteins remain to be explored. When put together, that knowledge can be exploited in the search for novel biocatalysts by a technique called genome mining. The term ‘genome mining’ brings the meaning of a post genome analysis where the desired gene is fished out of a genome sequence using bioinformatics tools and databases. However, genetic sequence alone cannot be used

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to predict the exact catalytic function of putative enzymes unless their structural information is analysed and then verified through molecular biology experimentation. Nevertheless, this genetic codes can be use to generate 3D structures of the proteins through computational prediction by protein threading and homology modelling methods (Zhang and Skolnick, 2005; Bowie *et al.*, 1991). From the structural information, the enzyme characteristics such as enzyme stability, substrate preference and mechanism of the enzyme can be predicted. From there, further studies can be done to improve the enzyme. Although about 90% of protein structures deposited in Protein Databank (PDB) were elucidated by X-ray crystallography, this technique takes time and requires a big budget. Therefore researchers often use protein structure prediction as a foundation to study the characteristic and structure of the desired protein.

In this research, genome mining was used to isolate glycogen branching enzyme gene (*glgB*) from *Geobacillus* sp. Geo5 genome, which was sequenced by Malaysia Genome Institute (MGI) under their Whole Genome Sequencing Project. This *Geobacillus* sp. Geo5 was isolated from a 97°C hot spring in Sungai Klah, Perak and therefore the enzyme isolated is likely to be thermostable and active at high temperature. Thus, this research is conducted with the following objectives:

1. To identify thermostable glycogen branching enzyme gene from *Geobacillus* sp. Geo5 genome sequence.
2. To produce thermostable recombinant glycogen branching enzyme in *Escherichia coli* expression system.
3. To characterise the biochemical properties of the purified recombinant glycogen branching enzyme.

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CHAPTER 2

LITERATURE REVIEW

2.1 Glycogen

Glycogen is a type of polysaccharide made up of glucose residues linked by α-1,4-glycosodic linkages and is branched by α-1,6-glycosidic linkages. For animals, glycogen is the major form of stored carbohydrate and can be found in the muscle cells. Microorganisms, especially bacteria, utilise glycogen as a storage compound that would provide carbon and energy source. Bacteria accumulate glycogen when growth condition is limited and carbon source is in excess. Therefore, glycogen synthesis usually happens during the slow growth and stationary phase (Preiss, 1984). The synthesis of glycogen in bacteria is mainly catalysed by three enzymes. Glycogen synthase (EC 2.4.1.21) and adenosine diphosphate (ADP) glucose pyrophosphorylase (EC 2.7.7.27) catalyses the reaction where a glucosyl unit, ADP-glucose, is linked to a pre-existing α-1,4-glucan by an α-1,4-glycosidic linkage. The product of these reactions will produce a rather linear type of glycogen. Then, branching enzyme (EC 2.4.1.18) will branch out the linear form of glycogen by transferring the α-1,4-glycosidic linkage to an α-1,6-glycosidic linkage. The positions of the linkages and the branching point are depicted in Figure 1. In *E. coli*, glycogen synthase, branching enzyme and ADP-glucose pyrophosphorylase are encoded by genes named *glgA*, *glgB* and *glgC*, respectively (Romeo *et al.*, 1988).

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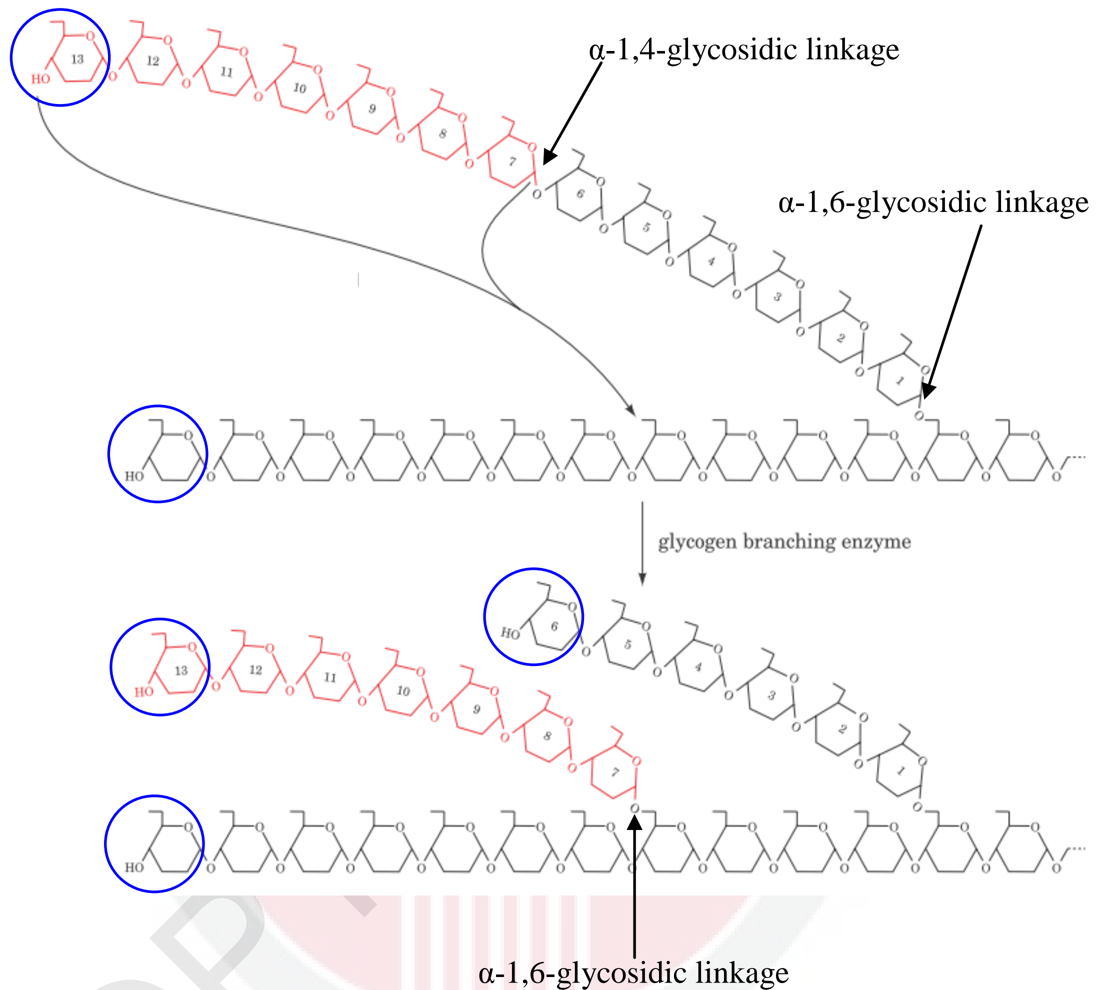

Figure 1. Structural formula of glycogen. Branching enzyme transfers an α-1,4-glycosidic linkage to an α-1,6-glycosidic linkage in every 5 to 16 glucans in chain. Circled in blue are the non-reducing ends. (Adapted from Voet and Voet, 2011)

2.2 Glycogen branching enzyme

This research is mainly focused on branching enzyme, particularly glycogen branching enzyme (GBE) produced in bacteria. Studies that are related to GBE mostly highlighted on the properties and applications of the enzyme (Takata *et al.*, 2010; Terada *et al.*, 1997; Takata *et al.*, 1994). Only until recently, researchers are venturing on the structural and mechanism studies of the enzyme (Palomo, *et al.*, 2011; Pal *et al.*, 2010). Therefore, there are still a lot of potential researches can be conducted on GBE in many areas, for example to study the enzyme on its catalytic and substrate promiscuity and to widen the application of this enzyme into pharmaceutical industry other than agricultural and food industries.

Branching enzyme is classified under α-amylase family (Kuriki and Imanaka, 1999). There are two types of branching enzymes classified into this family, glycogen branching enzyme (GBE) and starch branching enzyme (SBE). α-amylases are predominantly found in plants, bacteria and fungi (Reddy *et al.*, 2003). These enzymes shared three common properties. Firstly, their activities involve in hydrolysis and formation of α-1,4 and α-1,6 glycosidic linkages in the α-conformation. Secondly, they have a similar structure of their active sites that is the α/β-barrel structure. Finally, they share four conserved regions in their active sites (Table 1). These conserved regions involve in the catalysis and stabilised the α/β-barrel structure (Reddy *et al.*, 2003).

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Table 1. Four conserved regions of amino acid sequence of α-amylase family enzymes.

	I β2	II β4	III β5	IV β7
Amylomaltase	EALGIRIIGDMPIFVAED	LFHLVRIDHFRG	VPVLAEDLGVI	VVYTGTHDNDT
Amylosucrase	HEAGISAVVDFIFNHTSN	GVDILRMDAVAF	VFFKSEAIVHP	VNYVRSHDDIG
CGTase	HAKNIKVIIDFAPNHTSP	GIDGIRMDAVKH	VFTFGEWFLGV	VTFIDNHDMER
CMDase	HDNGIKVIFDAVFNHCGY	DIDGWRLDVANE	AIIVGEVWHDA	FNLIGSHDTER
BE	HQAGIGVILDWVPGHFCK	HVDGFRVDAVAN	ILMIAEDSTDW	FILPFSHDEVV
Isoamylase	HNAGIKVYMDVVYNHTAE	GVDGFRFDLASV	LDLFAEPWAIG	INFIDVHDGMT
M.amylase	HQKAIRVMLDAVFNHSGY	DIDGWRLDVANE	AYILGEIWHDA	FNLLGSHDTPR
Pullulanase	HAHGVRVILDGVFNHTGR	GVDGWRLDVPNE	AYIVGEIWEEA	MNLLTSHDTPR
Sucrose Pase	LGECSHLMFDFVCNHMSA	GAEYVRLDAVGF	TVIITETNVPH	FNFLASHDGIG
BLamylase	HERGMYLMVDVVANHMGY	SIDGLRIDTVKH	VYCIGEVLDGD	GTFVENHDNPR

Note: Highlighted are the four conserved regions and the corresponding β-sheets found in the amino acid sequence of α-amylase family enzymes. (Source: van der Maarel *et al.*, 2002)

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Under classification of Henrissat (1991), most of α-amylases fall under glycoside hydrolase family 13 (GH-13 family). This includes both GBE and SBE. Henrissat compared and classified 301 of glycoside hydrolases and related enzymes according to their similarity in amino acid sequences. Among those enzymes, about 20 other enzymes are also classified under GH-13 family (Table 2). These enzymes catalyse hydrolysis and transglycosylation of α-1,4 and α-1,6 glycosidic linkages using various substrates as mentioned in Table 2 (Reddy *et al.*, 2003).

However, a novel branching enzyme that has a different structure from other reported branching enzymes was discovered. This novel enzyme, which was isolated from *Thermococcus kodakaraensis* KOD1, is classified in the GH-57 family (Murakami *et al.*, 2006). Compared to GH-13 family, the structure of this novel branching enzyme has longer N-terminal region and two copies of helix-hairpin-helix motif at the C-terminal. Although the structure is totally different from GH-13 family branching enzyme, the function remains the same. According to Murakami *et al.* (2006), this enzyme is optimum at 70°C, pH 7.0 and stable up to 90°C. This enzyme has 44% identity with GH-57 branching enzyme ortholog, TT1467 protein, which was isolated from *Thermus thermophilus* HB8. The crystal structure of TT1467 (PDB: 1UFA) was therefore used as the template for homology modelling to predict the 3D structure of the *T. kodakaraensis* KOD1 branching enzyme. The results showed that five residues (His^{10}, His^{145}, Trp^{270}, Trp^{407} and Trp^{416}) near the catalytic site were conserved between the two models (Murakami *et al.*, 2006).

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Table 2. Glycoside hydrolase family 13 enzymes.

Enzyme	EC number	Main substrate
Amylosucrase	EC: 2.4.1.4	Sucrose
Sucrose phosphorylase	EC: 2.4.1.7	Sucrose
Glucan branching enzyme	EC: 2.4.1.18	Starch, glycogen
Cyclomaltodextrin glycosyltransferase	EC: 2.4.1.19	Starch
Amylomaltase	EC: 2.4.1.25	Starch, glycogen
Maltopentaose-forming alpha-amylase	EC: 3.2.1.-	Starch
Alpha-amylase	EC: 3.2.1.1	Starch
Oligo-1,6-glucosidase	EC: 3.2.1.10	1,6-alpha-D-glucosidic linkages in some oligosaccharides
Alpha-glucosidase	EC: 3.2.1.20	Starch
Amylopullulanase	EC: 3.2.1.41	Pullulan
Cyclomaltodextrinase	EC: 3.2.1.54	Linear and cyclomaltodextrin
Isopullulanase	EC: 3.2.1.57	Pullulan
Isoamylase	EC: 3.2.1.68	Amylopectin
Maltotetraose-forming alpha-amylase	EC: 3.2.1.60	Starch
Glucodextranase	EC: 3.2.1.70	Starch
Trehalose-6-phosphate hydrolase	EC: 3.2.1.93	Trehalose
Maltohexaose-forming alpha-amylase	EC: 3.2.1.98	Starch
Maltogenic amylase	EC: 3.2.1.133	Starch
Neopullulanase	EC: 3.2.1.135	Pullulan
Malto-oligosyl trehalase hydrolase	EC: 3.2.1.141	Trehalose
Malto-oligosyl trehalose synthase	EC: 5.4.99.15	Maltose

(Source: Reddy *et al.*, 2003)

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2.2.1 Mechanism of action of glycogen branching enzyme

Branching enzyme catalyses the reaction of transglycosylation where it transfers an α-1,4 glycosidic linkage to α-1,6-glycosidic branch point during the glycogen and starch synthesis. Pal *et al.* (2010) proposed that the glycogen branching enzyme reaction is a two-step mechanism, namely, an amylase reaction and isomerisation. In the amylase reaction, α-1,4 glycosidic linkage was broken and followed by isomerisation reaction where a new α-1,6 glycosidic linkage was then formed (Figure 2). Both of the reactions are believed to occur in the same catalytic pocket. As shown in Figure 2, Glu^{464} acts as a general acid catalyst to protonate approximately the 7th glycosidic oxygen of an α-1,4 glycosidic linkage from a non-reducing end. This happens during the amylase reaction. Following that, Asp^{411}, which acts as the attacking nucleophile, forms a bond with C1 to creating a β-glucosyl-enzyme intermediate. The substrate is then transferred to the hydroxyl group at C6 of a glucose unit on the same or another chain to form the α-1,6 glycosidic linkage (Pal *et al.*, 2010).

Although made up of the same monomers, glycogen and starch molecules differ slightly in their molecular structure. One of the reasons is believed due to the differences between GBE and SBE in their substrate preferences primarily due to the specificity for the substrate chain length (Guan *et al.*, 1997). Substrate chain length is the minimum length of glucan chain used by branching enzymes for the transglycosylation process. GBE favoured short chain length (5 to 16 glucans) to form the branches. As for SBE, there are two types, SBEI and SBEII. SBEI mainly transfer long chains (larger than 10 glucans) and takes up amylose as the substrate.

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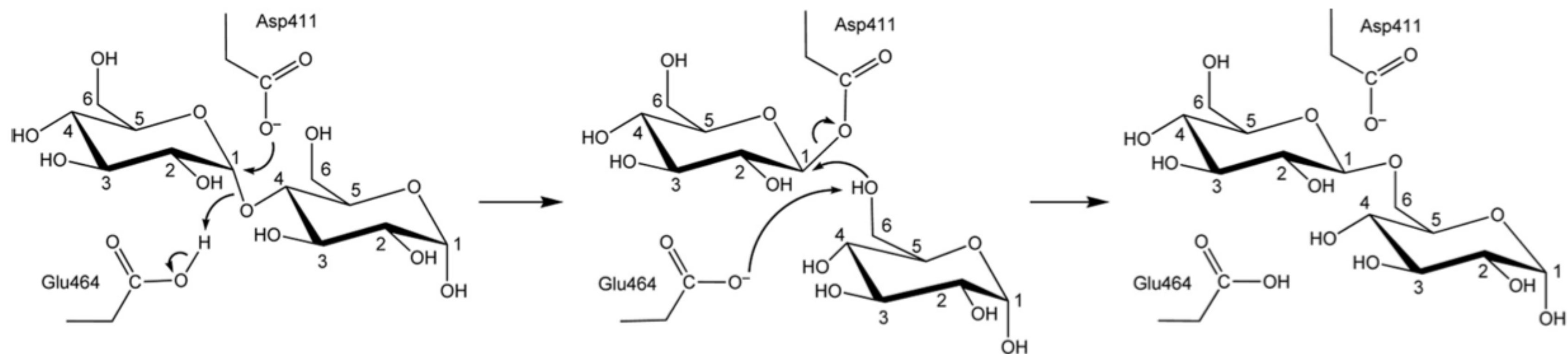

Figure 2. Reaction mechanism of glycogen branching enzyme. Glu[464] is the general acid catalyst and Asp[411] is the attacking nucleophile. (Source: Pal *et al.*, 2010)

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On the other hand, SBEII predominantly transfers short chains (three to nine glucans) and therefore using amylopectin as the substrate (Guan *et al.*, 1997). The substrate chain length preference by those enzymes causes glycogen to have more branches than starch (Sasangka *et al.*, 2002). That is why branching enzyme is essential in determining the structure of glycogen and starch. The significance of those branches is that they increase the number of non-reducing ends, which make the branched polysaccharides more soluble and easier to be absorbed by the cells and thus accelerate the synthesis and digestion of the polysaccharides (Abad *et al.*, 2002).

2.2.2 Thermostable glycogen branching enzyme

Microorganisms live almost anywhere on earth and that includes even the most extreme places that no other organism can survive. Those microorganisms that tolerate extreme environment are called extremophiles. These organisms can adapt in various milieus where temperature, pH, salinity and pressure are extreme (van den Burg, 2003). One of the reasons these organisms are able to adapt harsh conditions is because their proteins are stable and functional in those conditions (Demirjian *et al.*, 2001). Therefore, extremophiles can be a valuable source for novel enzymes that can withstand the extreme reaction conditions in industrial processing (van den Burg, 2003).

Among all extremophiles, research society is mostly interested in thermophiles (van den Burg, 2003; Fujiwara, 2002). Thermophiles are microorganisms that live in high temperature. Thermophiles normally grow between 65°C to 80°C while moderate thermophiles can grow in temperature between 45°C to 65°C, and microorganisms

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that can live in temperature beyond 80°C are considered as hyperthermophiles (Demirjian *et al.*, 2001; Madigan and Marrs, 1997). These thermophiles are mostly isolated from hot springs and hydrothermal vents (Madigan and Marrs, 1997). Enzymes produced by thermophiles would tolerate high temperature and thus are referred to as thermostable enzymes. Thermostable enzymes that are active at high temperature are very useful in industrial applications because reactions at high temperature would help with the mixing process and substrate solubilisation, with less risk of reaction contamination (Turner *et al.*, 2007). In addition, they have long shelf life in room temperature. Thermostable enzymes are widely applied in industries like chemical, food, beverages, pharmaceutical, paper and textile (Fujiwara, 2002). Thermostable enzymes that are used in these industries are, for example, amylases, glycosidases, lipases, xylanases, proteases and DNA polymerases (Fujiwara, 2002; Schiraldi and De Rosa, 2002; Demirjian *et al.*, 2001). In the eyes of consumers, enzymes usage is more preferred than chemical catalysts as enzymes are nature products and so is believed to be less harmful (Turner *et al.*, 2007; van der Maarel *et al.*, 2002).

Quite a number of studies have been done to discover thermostable branching enzyme. Takata *et al.* (1994) claimed to be the first to purify a thermostable branching enzyme, which is from *Geobacillus stearothermophilus* that have the optimum temperature at 50°C. GBE that is stable at highest temperature was isolated form *Aquifex aeolicus* (van der Maarel *et al.*, 2003). This bacteria, which was found near underwater volcanoes have the optimum temperature for activity at 80°C and stable up to 90°C (van der Maarel *et al.*, 2003). Other than bacteria, thermostable GBE was also isolated from hyperthermophilic archaeon, which was from

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T. kodakaraensis KOD1 (Murakami *et al.*, 2006). This enzyme showed an optimal temperature of 70°C and also stable up to 90°C (Murakami *et al.*, 2006). Some other potential candidates with high enzyme optimum temperatures that researchers have focused on were *Anaerobranca gottschalkii* (Thiemann *et al.*, 2006) and *Rhodothermus obamensis* (Shinohara *et al.*, 2001). The optimum temperatures for the GBE activity from those microorganisms are at 50°C and 65°C (Thiemann *et al.*, 2006; Shinohara *et al.*, 2001).

For this research, GBE was isolated form *Geobacillus* sp. Geo5 genome. As in the name, "Geobacillus" means earth or soil bacillus (Nazina *et al.*, 2001). *Geobacillus* sp., which is also known as thermophilic bacilli is an obligate thermophile that grows in temperature range between 37°C to 75°C and the optimum temperature for growth is around 55°C to 65°C (Nazina *et al.*, 2001; Zeigler, 2001). Since the bacteria form endospores, therefore, the bacteria are still able to survive in temperature out of that range and in adverse conditions, although not rapidly (Marchant and Banat, 2010; Marchant, *et al.*, 2008). The vegetative cells are rod-shaped that occur either singly or in short chains and the cell wall is generally Gram-positive (Nazina *et al.*, 2001). *Geobacillus* sp. Geo5 used in this research was sampled from Sungai Klah Hot Springs, Sungkai, Perak. The bacterium was sampled at 97°C, therefore the GBE isolated was postulated to be thermostable and active at high temperature.

2.2.3 Applications of glycogen branching enzyme

Glycogen branching enzyme is mainly applied in food and beverages processing industries and also in nutraceutical industries. Studies have been done to utilise this

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enzyme either *in vivo* or *in vitro* in order to boost up the quality of starchy food by increasing the branches in starch molecules (Lee *et al.*, 2008; Kim *et al.*, 2005; Kawabata *et al.*, 2002; Kortstee *et al.*, 1996).

Plants, which use starch as the main storage of carbohydrate, would have starch branching enzyme. However, to further increase the branching in the starch structure, GBE gene from bacteria was introduced into the plants. Starch producing crops, such as potato and rice, were targeted for the research to construct transgenic plants as an approach to enhance our food source. For example, Kortstee *et al.* (1996) has studied the expression of *E. coli* glycogen branching enzyme in potatoes. A decade after that, Kim *et al.* (2005) modified rice plant by inserting multiple copies of GBE gene from *E. coli* so that the rice starch would have elevated degree of branching. GBE would increase the degree of branching in starch by producing many short branches that made up of 5 to 16 glucans. Compared to the wild-type plants, the genetically engineered plants showed lower gelatinise temperature, thus minimised the chances of retrogradation and therefore hold a potential to boost up nutritional value in those crops (Kim *et al.*, 2005). Retrogradation happens when gelatinised starch is cooled down and so the molecules rearrange themselves into a more crystalline structure forming a stiff gel as water is expelled from the structure. Retrograded starch, which often causes faulty food products like bread staling and loss of viscosity in sauces and soups, is hard to digest by our system (van der Maarel *et al.*, 2002). As Malaysia is a rice producer, therefore this kind of research would gain the interest of local researchers as the research and technology of transgenic crops may be more develop in this country in near future.

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Rather than doing starch modification *in vivo* by producing transgenic crop that expressed GBE, highly branched starch was also produced by treating the starch with GBE. One example is the treatment of waxy corn starch with GBE isolated from a type of mould, *Neurospora crassa* (Kawabata *et al.*, 2002). The result showed that GBE changed the molecular structure of the corn starch and the highly branched starched produced showed low tendency of retrogradation (Kawabata *et al.*, 2002). A similar research to this was done by Lee *et al.* (2008) where rice starch was modified to be highly branched by enzymatic reactions using GBE in addition to another enzyme, maltogenic amylase.

Another application of branching enzyme in industry is the manufacturing of a compound called highly branched cyclodextrin. Cyclodexterin is another name for cyclic α-1,4-glucans, a compound results mainly from the reaction of cyclodextrin glucanotransferase (EC2.4.1.19). Terada *et al.* (1997) reported that other than cyclodextrin glucanotransferase, a thermostable GBE from *G. stearothermophilus* that have optimum temperature for enzyme activity of 50°C, can also catalyse the formation of this branched cyclic glucans by introducing intramolecular transglycosylation when amylose and amylopectin were used as the substrate. The properties of cyclodextrin that makes it favourable in starch processing are that it is highly soluble in water, low viscosity and also low tendency for retrogradation (Takata *et al.*, 1996). A Japan company, Glico, used branching enzyme from *Bacillus strearothermophilus* to produce cyclodextrin with a trade name of Cluster Dextrin. This product is used as an ingredient in sports drinks, to enhance the taste of food and also as a spray-drying aid (Takata *et al.*, 2010).

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Other than those mentioned above, there are several other applications of GBE that have been patented. Those patents describe that branching enzymes potentially can be used of in bread as an anti-staling agent, produce low viscosity and high molecular weight starch, use for paper coating and even warp sizing textile fibers to make the fibers stronger (van der Maarel *et al.*, 2001). Studies of GBE is also emerging into therapeutics application, for example, against tuberculosis and glycogen branching enzyme deficiency disease (Pal *et al.*, 2010; Garg *et al.*, 2007; Bruno *et al.*, 1993).

2.3 Genome mining

Genome mining is a term given to a technique that uses basic bioinformatic tools and databases to search for genes with a specific function, such as enzymes, natural products and metabolites, from genome sequences of numerous kinds of organisms (Challis, 2008; Banskota et al., 2006; Ferrer et al., 2005; Berger, 2004). This technique exploits the readily accessible public databases that stores gene and genome sequences, for example, Genbank at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), UCSC Genome Browser (http://genome.ucsc.edu) and Ensembl Genome Browser (http://www.ensembl.org) (Schattner, 2009; Corre and Challis, 2007).

Examples of enzymes that were discovered by genome mining are a thermostable Baeyer–Villiger monooxygenase from the genome of a thermophilic actinomycete, *Thermobifida fusca* and mandelonitrile hydrolase bll6402 from *Bradyrhizobium japonicum* USDA110 (Zhu *et al.*, 2007; Fraaije *et al.*, 2004). Genome mining too has

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been applied to discover novel natural products, for example, the isolation of orfamide A, a novel cyclic lipopeptide that involves in biological control of plant disease from *Pseudomonas fluorescens* Pf-5 and an antifungal agent, ECO-02301 from *Streptomyces aizunensis* (Loper and Gross, 2007; McAlpine *et al.*, 2005). Another example where genome mining was used is the discovery of novel prenylated quinolin-2-one alkaloids, a mebolite that involves in a rare benzodiazepine–quinolinone pathway from *Aspergillus nidulans* (Scherlach and Hertweck, 2007).

In this research, genome mining technique was utilised where GBE was mined from the genome sequence of *Geobacillus* sp. Geo5 elucidated by MGI together with genome sequences from other *Geobacillus* sp. using *glgB* sequences from other bacteria species and bioinformatic tools like ClustalW and BLAST. Other genome sequences of *Geobacillus* sp. that are available to date according to GenBank are from *Geobacillus* sp. Y412MC61 (NC_013411), *Geobacillus* sp. C56-T3 (NC_014206), *Geobacillus* sp. Y412MC52 (NC_014915), *Geobacillus* sp. Y4.1MC1 (NC_014650), *Geobacillus* sp. WCH70 (NC_012793), *Geobacillus thermoglucosidasius* (NC_015660), *Geobacillus kaustophilus* (NC_006510), *Geobacillus thermodenitrificans* NG80-2 (NC_009328) and *Geobacillus thermoleovorans* (NC_016593). The availability of these genome sequences would facilitate the isolation of GBE by genome mining.

One of the main objectives of doing genome mining is to determine the function of the gene of interest, may it be an enzyme, a metabolite or a natural product with drug properties like antifungal, antibacterial or antitumor. Genome mining will provide

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the sequence for the gene of interest. From the sequence, the function of the protein can be predicted by two approaches, namely, sequence alignment and sequence-motif methods using tools like Prosite, Blocks, Prints and Emotif (Skolnick and Fetrow, 2000). However, protein function prediction using only sequence-based methods is insufficient, especially when the proteins have a tendency to be multifunctional. Therefore, functional prediction techniques that use protein sequence in combination with structural information have been successfully developed (Skolnick and Fetrow, 2000).

2.4 Structural studies of glycogen branching enzyme

Structural study of an enzyme is very important. There are a lot of information about the enzyme can be known by studying its structure. From the structure of an enzyme, we can know about the function and the reaction mechanism, the characteristics and also the substrate preference. Using that knowledge as a platform, further studies can be done on the enzyme to improve the enzyme activity and stability and also to explore on substrate promiscuity. These can be achieved by doing enzyme modifications either via rational design that uses site directed mutagenesis technique, or directed evolution by performing random mutagenesis to the enzyme. It can be said that the structural studies of branching enzymes are just emerging. Up until now the crystal structures deposited in Protein Databank (PDB) for branching enzymes come from only four species. Two of the structures are glycogen branching enzymes from GH-13 family, isolated from *Escherichia coli* and *Mycobacterium tuberculosis*, and two other structures are branching enzymes from GH-57 family, which were isolated from *T. thermophiles* and *T. kodakaraensis* KOD1 (Palomo *et al.*, 2011;

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Santos *et al.*, 2011; Pal *et al.*, 2010; Abad *et al.*, 2002). The availability of these crystal structures would rapidly expand the knowledge on this enzyme. These structures would also help to improve the accuracy in structural predictions of GBEs from other sources as they can be use as the template for the structure determination.

2.4.1 Crystal structures of branching enzymes

The first crystal structure of branching enzyme reported was GBE from *E. coli*. The crystal structure that was reported by Hilden *et al.* (2000) however was not deposited in Protein Databank (PDB) because the X-ray diffraction data was not adequate for X-ray structure determination as the resolution achieved was only 4.1 Å. The first X-ray structure of GBE deposited in PDB was by Abad *et al.* (2002), which was also a GBE from *E. coli* (PDB ID: 1M7X). However that crystal structure was not from a full-length enzyme, it was in fact missing the first 113 amino acids (Abad *et al.*, 2002). This is because when compared to a full length enzyme, the truncated enzyme was able to overexpress more amount of soluble protein that are active at protein concentration above 1 mg/mL and therefore has let the diffracting crystals of the enzyme to be produced (Hilden *et al.*, 2000).

The resolution of the branching enzyme structure (PDB ID: 1M7X) is 2.3 Å. It consists of three major domains; the N-terminal, C-terminal and central α/β-barrel domain, which locates the catalytic residues; Asp^{405}, Asp^{526} and Glu^{458} (Figure 3) (Abad *et al.*, 2002). N-terminal domain that consists of 128 amino acid residues is organised into seven stranded β-sandwich fold, while the C-terminal, which is made up of 116 amino acid residues is arranged into seven β-strands (Abad *et al.*, 2002).

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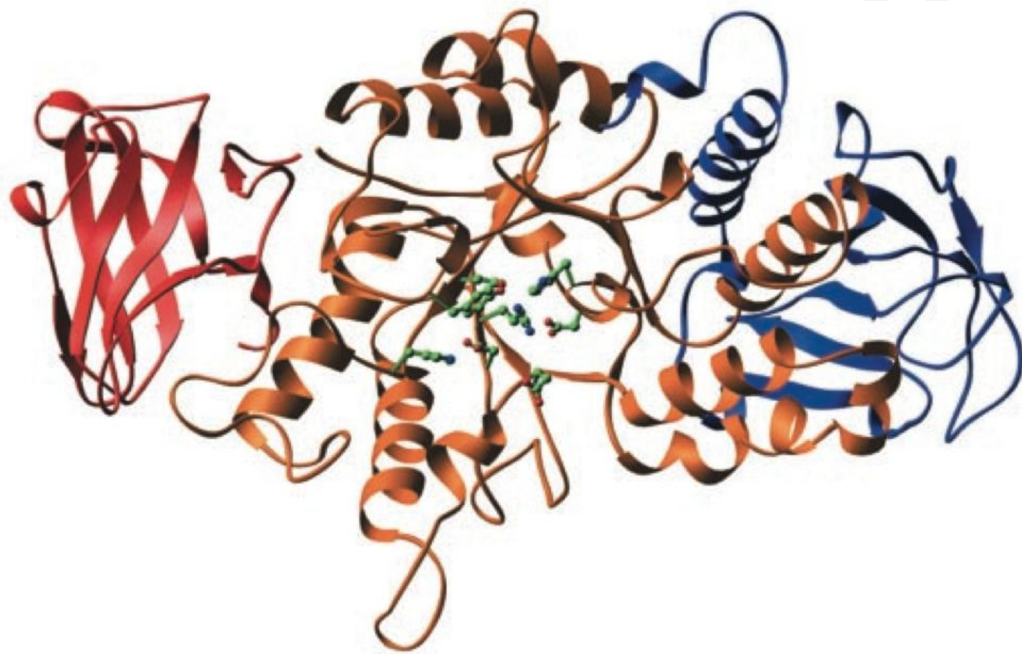

Figure 3. X-ray crystal structure of truncated *Escherichia coli* branching enzyme. PDB ID: 1M7X. The resolution is 2.3 Å. Red indicates the N-terminal domain; orange indicates the central α/β-barrel catalytic domain; and blue indicates the C-terminal domain. (Source: Abad *et al.*, 2002).

Studies on the enzyme with truncated N-terminal domain has proven that this domain determines the length of glucan chain transferred to form a branch, while the C-terminal domain plays a role in substrate preference and specificity (Devillers *et al.*, 2003; Binderup *et al.*, 2002). The central α/β-barrel domain have a dimension of 30.5 x 17.7 x 17.7 Å acts as a cavity to accommodate and bind branched glucose chains during the enzymatic reaction (Abad *et al.*, 2002). This cavity surface is electronegative as it harbours four negatively charged amino acids, Asp^{335}, Asp^{405}, Asp^{526} and Glu^{458}, which are involved in sugar-protein interaction during the substrate binding and catalysis (Abad *et al.*, 2002).

Another structure of GBE from GH-13 family was from *M. tuberculosis.* Pal *et al.* (2010) reported a full-length crystal structure of the enzyme with the resolution of 2.33Å (PDB ID: 3K1D). The structure consists of four domains; the N1 β-sandwich, N2 β-sandwich, a central α/β-barrel domain and a C-terminal β-sandwich (Figure 4), the structure showed good homology when compared with GBE structure from *E. coli* (Pal *et al.*, 2010). Apart from a few insertions and deletions in both *E. coli* and *M. tuberculosis* GBE sequences, the *E. coli* crystal structure does not have the N1 domain in the N-terminal, which involves in substrate specificity, recognition and binding.

T. thermophilus and *T. kodakaraensis* KOD1 produce GH-57 famlily branching enzyme, which has a completely different structure from branching enzyme of GH-13 family. The reported crystal structure from *T. thermophilus* (PDB ID: 3P0B) and *T. kodakaraensis* KOD1 (PDB ID: 3N8T) have a resolution of 1.35 Å and 2.4 Å, respectively (Palomo *et al.*, 2011; Santos *et al.*, 2011). The structure shows

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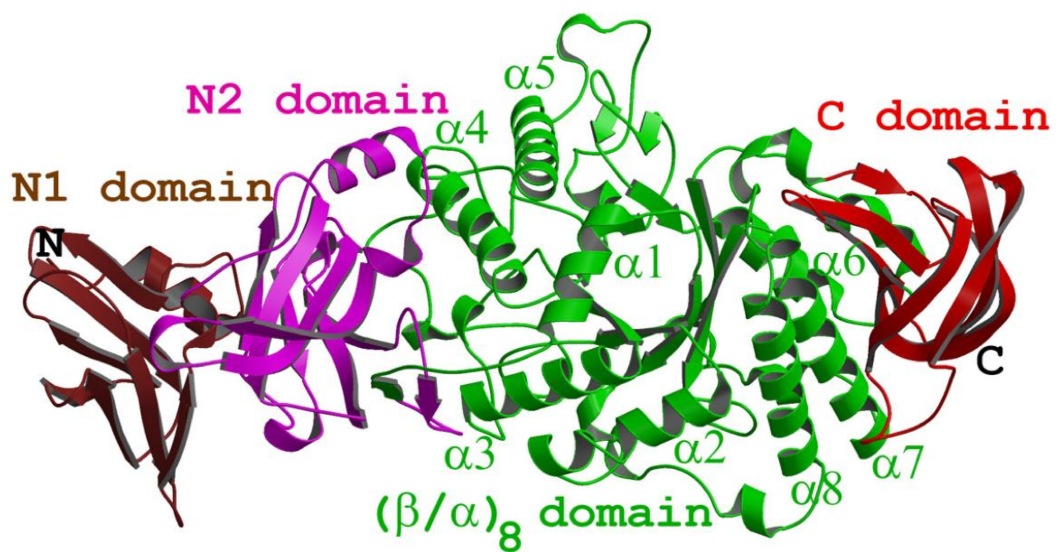

Figure 4. X-ray crystal structure of *Mycobacterium tuberculosis* branching enzyme. PDB ID: 3K1D. The four domains, N1 β-sandwich, N2 β-sandwich, α/β-barrel and C-terminal β-sandwich, were labelled. (Source: Pal *et al.*, 2010)

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that N-terminal domain of the enzyme has a distorted α/β-barrel domain A with an inserted domain B between β2 and α5 while the C-terminal domain made up of five α-helices (Figure 5) (Palomo *et al.*, 2011; Santos *et al.*, 2011). The active site is made up of three loops, which are located in the N-terminal domain and next to the C-terminal domain, which creates the entrance to the active site. The C-terminal has shown to be necessary for substrate binding and catalysis. Even though the GH-57 GBE has the same catalytic mechanism as GH-13 GBE, the differences are that GH-57 GBE produces less branching points and makes shorter branches, therefore the structure of the product is slightly different (Palomo *et al.*, 2011).

2.4.2 Protein structure prediction

Protein structure prediction now becomes a very useful method to discover information on protein of interest. This technique has been used to study proteins since two decades ago. Some of the examples where structural prediction has been applied are as follows. Structural prediction has been used to test theories on the function of the protein by designing mutants. Wu *et al.*, (1999) did site-directed mutagenesis to change one particular amino acid selected based on the predicted structure in order to convert lactate dehydrogenase from *Trichomonas vaginalis* to malate dehydrogenase. Structural prediction has also been used to identify active sites and to study on substrate specificity of many proteins including branching enzyme and α-amylase (Kuriki and Imanaka, 1999; Kuriki *et al.*, 1996; Jespersen *et al.*, 1993). The study done by Ring *et al.* (1993) used structural prediction to determine the binding site of ligands for serine and cysteine proteases, where they identified the previously unknown inhibitors from protease family enzymes to

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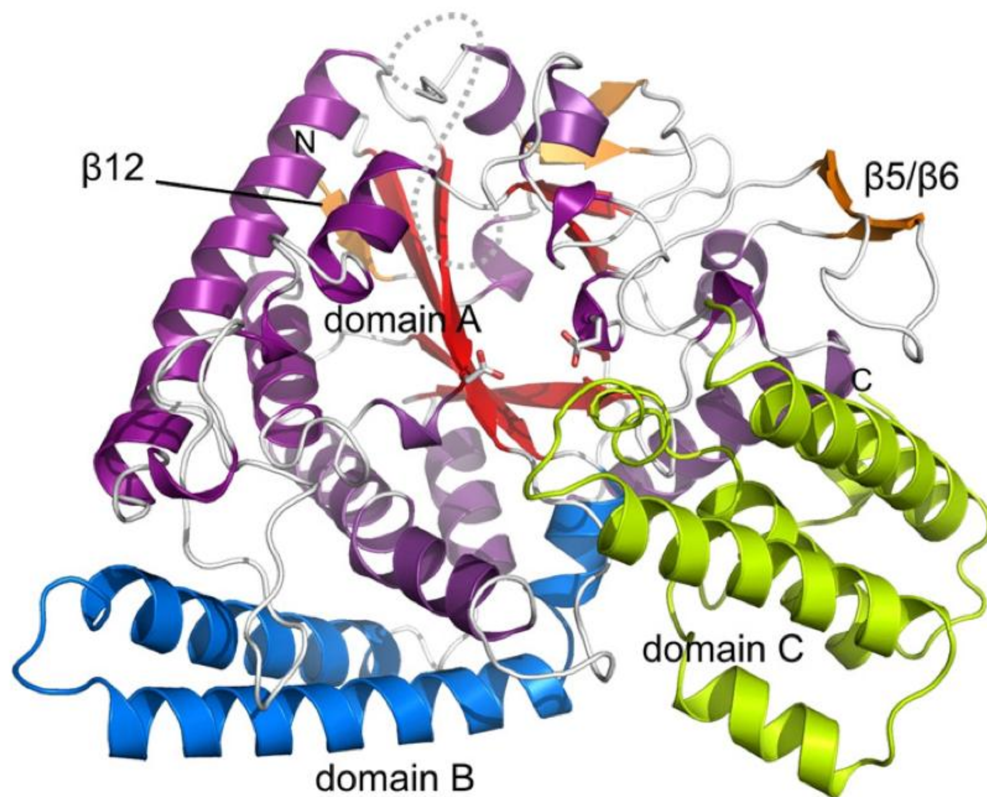

Figure 5. X-ray crystal structure of *Thermus thermophilus* branching enzyme. The catalytic residues are shown in stick representation. Domain A: purple with core β-strands in red and additional β-strands in orange; domain B: blue; domain C: green. (Source: Palomo *et al*., 2011)

develop antiparasitic agents for drug-designing experiment. Also, structural prediction could assist in molecular replacement in X-ray structure and to justify known experimental observations, as in the experiment done on the determination of δ' subunit of the clamp-loader complex of *E. coli* DNA polymerase III (Marti-Renom *et al.*, 2000; Guenther *et al.*, 1997).

Protein structure prediction is very suitable for those who cannot afford to generate protein crystals due to the limitations on expertise or expenses (Baker and Sali, 2001). Although the possibility of inter-atomic interactions of polypeptide chains of a protein are millions, the basis of protein folding may not be as complex as expected and therefore new methods and advances in computer algorithms have shown great promise in predicting protein folding mechanisms, 3D structures and protein functions (Roy *et al.*, 2010; Baker, 2000). There are three methods to do computational modelling of protein structure; comparative modelling, threading methods and *ab initio* modelling. The method to be used is selected based on the availability of known protein structures that are related to the protein of interest in the PDB library. Four basic steps are use to model a protein structure from its amino acid sequence. The steps are finding templates, aligning the sequence with the templates, constructing a model and finally assessing the model (Baker and Sali, 2001).

The accuracy of a protein model built is correlated to the percentage of sequence identity between template and query (Figure 6) (Baker and Sali, 2001). Comparative modelling is used when evolutionary related homologous protein template is accessible and this method often generates high-resolution models (Roy *et al.*, 2010;

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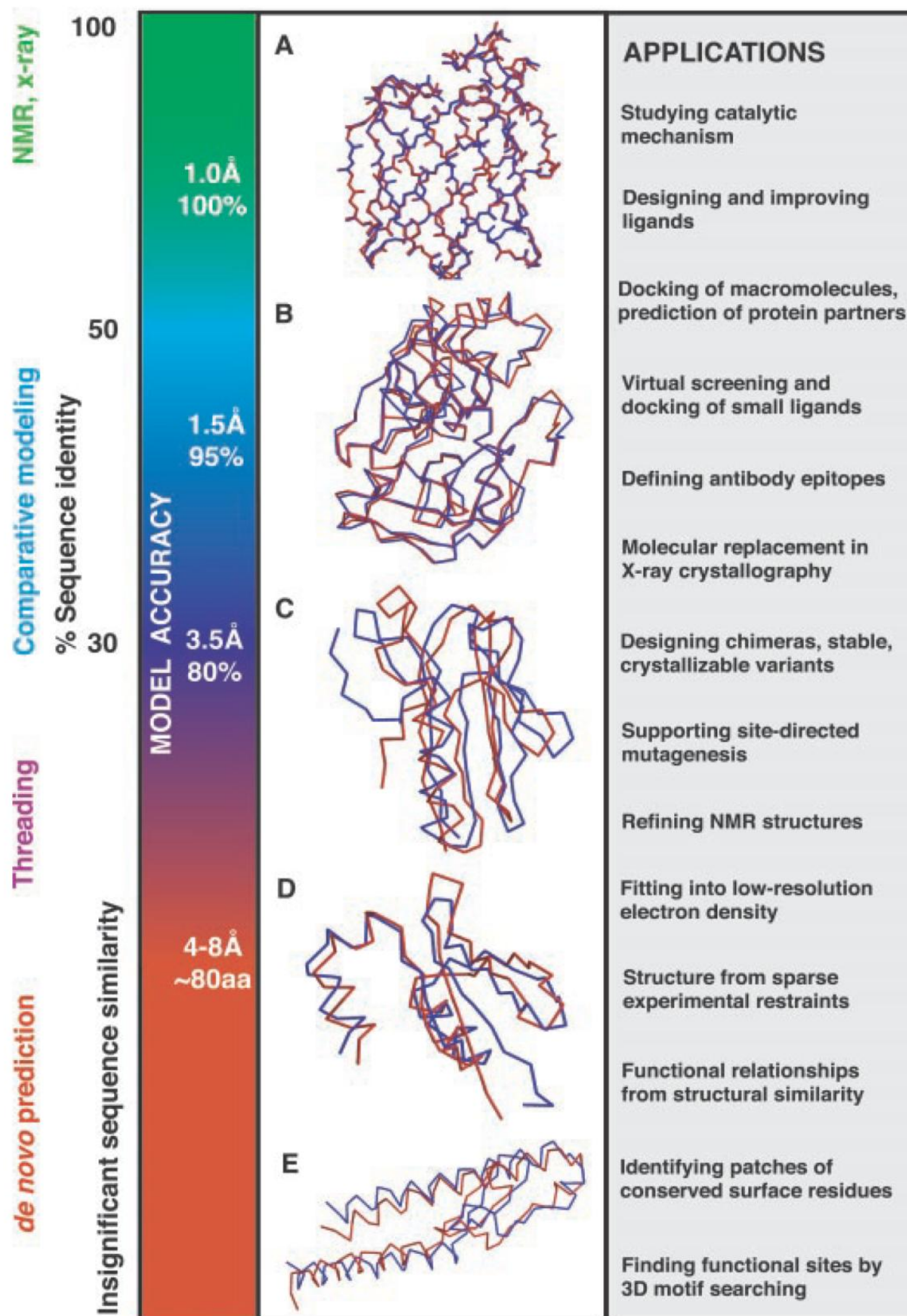

Figure 6. Accuracy and applications of protein models. Shown are the methods to produce protein structures, the corresponding accuracy of protein structure models and their applications. Predicted structures are in red and actual structures are in blue. The sequence identity of samples to their template structures are about 60% for A, 40% for B, and 30% for C. D and E are examples of Rosetta *de novo* structure predictions for the CASP4 structure prediction experiment. (Source: Baker and Sali, 2001)

Marti-Renom *et al.*, 2000). If the protein of interest comes from a different evolutionary origin, threading method will generate protein models by matching the query protein sequence with similar protein folds of other proteins that are in the PDB library (Roy *et al.*, 2010; Zhang, 2008). For proteins that are not structurally related to any of solved protein structures in PDB library, the model has to be built from scratch and this is called *de novo* or *ab initio* modelling (Wu *et al.*, 2007).

According to the 7th Critical Assessment of Structure Prediction (CASP), the designed experiments that assess the latest method of protein structure prediction, Iterative Threading Assembly Refinement (I-TASSER) was ranked as the best automated structure prediction among other servers (Zhang, 2008). This server, which is available online at http://zhang.bioinformatics.ku.edu/I-TASSER, is very suitable for non-experts to construct structural models of their protein of interest. I-TASSER server provides free service to generate 3D structure of a protein from an amino acid sequence provided by the user using multiple threading alignments and iterative structural assembly simulations (Roy *et al.*, 2010; Zhang, 2009; Zhang, 2008). It is highly recommended that the results from structural prediction are evaluated and verified using softwares like PROCHECK, Verify3D and PROSAII to ensure the accuracy and reliability of the predicted structures (Laskowski *et al.*, 1993; Sippl, 1993; Luthy *et al.*, 1992). Other than providing 3D atomic models of a protein, I-TASSER also does functional prediction and provides amino acid sequence that involves in the binding site for that particular protein.

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2.5 Lab-scale production of GBE in *Escherichia coli* expression system

Application in industries requires the enzyme to be produce in abundance. Mass production of enzyme can be achieved by optimizing the lab-scale fermentation and then scaled up the production to industrial size. Almost all lab-scale productions of enzymes use recombinant DNA technology and heterologous protein expression (Makrides, 1996; Hanahan *et al.*, 1991; Hanahan, 1983). In order to use recombinant DNA, the gene of the enzyme has first to be identified and then isolated from the chromosomal DNA by polymerase chain reaction (PCR). The isolated gene is then ligated to a plasmid vector and transformed into a microbial host to express the gene. There are various options of plasmid vectors and hosts can be used depending on the protein expression strategies. Those vectors and hosts are commercially available although some researchers preferred to construct those themselves for novelty especially when they require extra or special features (Hanahan *et al.*, 1991; Hanahan, 1983).

It is safe to say that bacterial system, especially *E. coli*, is the most favoured cloning and expression host for recombinant DNA for heterologous protein expression. The reasons for this are due to the ability of *E. coli* to grow rapidly on economical substrates and they are able to host and express foreign DNA even of complex eukaryotic proteins. On top of that, having a small genome, *E. coli* genetic is so well characterised than any other microorganisms and therefore a variation of cloning vectors and mutant host strains are largely available (Baneyx, 1999). The current knowledge of transcription, translation and protein folding mechanisms of *E. coli*,

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together with new and improved genetic tools and databases are making this bacterium more precious than ever.

E. coli has been used to clone and express GBE from many other organisms. One of many examples is the GBE isolated from *G. stearothermophilus*, which had an optimum temperature for enzyme activity of 50°C (Takata *et al.*, 1994). Research done by Murakami *et al.* (2006) discovered a novel branching enzyme from GH-57 family in the hyperthermophilic archaeon *T. kodakaraensis* KOD1. In order to study the enzyme, they cloned and expressed the enzyme in *E. coli* DH5α and *E. coli* BL21-CodonPlus (DE3), respectively. Another example is the cloning and expression of GBE from *M. tuberculosis* in *E. coli* DH5α and *E. coli* BL21 (DE3), respectively (Garg *et al.*, 2007).

Although *E. coli* system was generally used to express GBE, other bacterial systems were also used to express GBE. For example, Thiemann *et al.* (2006) expressed a novel branching enzyme from the thermoalkaliphilic anaerobic bacterium *A. gottschalkii* in *Staphylococus caenosus.* Another example is the expression of branching enzyme from cyanobacterium *Synechococcus* sp. PCC7942 in *Bacillus subtilis* (Kiel, 1990). On top of all, there is no limitation or a standard procedure for choosing the cloning and expression vectors and hosts. It all depends on the strategy and creativity of the researchers to produce the expected outcome.

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2.5.1 Cloning of PCR product

To clone a gene into bacterial system, the gene sequence has first to be isolated from the chromosomal genome using PCR. PCR is a technique used to generate thousands to millions copies of a specific DNA sequence. This technique was developed by Kary Mulkis in 1983 (Bartlett and Stirling, 2003). This PCR product, which encodes the gene of interest, will then be ligated into a cloning vector, which is a plasmid DNA. Depending on the DNA polymerase used, the PCR product will have either blunt ends or with A overhangs. These features are utilised in the cloning of the PCR product into the plasmid vector like in blunt end cloning, TA cloning, directional cloning or cloning using restriction enzymes. The PCR product will be integrated into cloning vector by using either DNA ligase or DNA topoisomerase I (Cheng and Shuman, 2000).

2.5.2 Transformation of *Escherichia coli* with plasmid

Transformation is a process where the bacteria take up and establish exogenous DNA stably in their system. In the context of recombinant DNA, it is the uptake of recombinant plasmid by the cloning and expression host, *E. coli*. The efficiency of the transformation depends on two major parameters, the method used to induce the competency of the host cells and the genetic constitution of the host strain. Competence is defined as the ability to identify and transfer DNA material into cells. The cells competency can be induced in two ways, chemical induction and electroporation, depends on the characteristic of the cells being transformed and the purpose of the transformation (Hanahan *et al.*, 1991). The transformation efficiency

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is not affected by the presence of multiple plasmids or the conformation of the plasmids, either relaxed or supercoiled. However, the efficiency of transformation declines when size of plasmid increases (Hanahan, 1983).

2.5.3 Expression of recombinant protein

Gene cloning is done mainly to express the gene of interest, which will produce the recombinant protein. Exogenous gene expression in the expression host is not always a success. Although *E. coli* is known to give high level expression of recombinant protein, this organism does not promise that every recombinant proteins can be expressed efficiently. Expression level is very much affected by cell growth characteristics of the host, composition of the gene sequence, post-translational modifications, protein folding, proteases produced by the host, major differences in codon usage and the effect of toxic recombinant protein on host (Makrides, 1996). Therefore, the selection of expression system is very critical in order to ensure high expression level of the recombinant protein.

Apart of expression level, solubility of the expressed protein must also be considered. When the recombinant protein is over-expressed, the host would triggers stress response, which often cause the protein produced to aggregates as inclusion bodies. This phenomenon is not desired as when the protein is in the form of inclusion bodies, the folding of the protein in not precise, hence the protein is not functional (Villaverde and Cario, 2003). When the recombinant protein is expressed in *E. coli*, the parameters that affect the protein solubility are the aeration of the culture media, culture temperature, *E. coli* strains and the timing of induction

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(Berrow *et al.*, 2006). That is why the researchers have to optimise those parameters in order to achieve not only high level of expression but also high level of soluble proteins.

2.5.4 Secretory expression of recombinant protein

The secretion of proteins into the culture medium has advantages especially when heterologous recombinant proteins have to be isolated from Gram-negative bacteria like *E. coli*. Secretion of target protein into culture medium would facilitate protein purification as there would be less of contaminating bacterial proteins in the medium. Other than that, proteolytic activity is lesser as the proteinases presence is fewer in the culture medium, which provides a better folding environment for the protein (Ni and Chen, 2009).

One way to release heterelogous proteins from *E. coli* periplasm and cytoplasm is by using bacteriocin release protein (BRP). BRP is a small lipoprotein made up of only 28 amino acids, synthesised with a signal peptide to transport bacteriocin across the cell. The presence of BRP together with phospholipase A will cause the release of bacteriocin, a toxin that kills bacteria by increasing the permeability of inner and outer membrane of the bacteria (van der Wal *et al.*, 1995). Utilising the ability to increase cell permeability, studies has shown that BRP expression is capable to promote the leakage of heterologous protein from both *E. coli* periplasm and cytoplasm into the culture medium (Lloubes *et al.*, 1993).

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BRP-mediated protein release is done by co-transforming the plasmid that encodes for BRP into host cells that are harbouring the recombinant plasmid, which encodes for the protein of interest. Both BRP plasmid and recombinant plasmid can be expressed in the same host by using either the same inducer or different inducers, depending on the promoter of those plasmids. Nonetheless, high expression of BRP will lyse the cells. This effect, quasi-lysis and lethality, can be seen in liquid cultures and in agar plates as well (van der Wal *et al.*, 1998). Therefore the amount of BPR produced has to be controlled wisely.

2.6 Purification of recombinant protein

There are various ways to isolate the expressed recombinant protein from other proteins that are naturally produced by the host cells. To purely obtain the recombinant protein, affinity-tagged fusion protein expression systems are often chose because these systems offer a very simple and convenient method to purify the recombinant protein by using affinity chromatography. Some of the advantages of these affinity-tagged systems include adsorption purification from just a single step, have insignificant effects on tertiary structure as well as biological activity, easy and precise removal of the tag to get the native protein, simple and accurate assay for the fusion recombinant protein detection and finally adaptable by different types of proteins (Terpe, 2003). There are many tags to choose from like HIS, CBP, CYD, FLAG, Strep II and MBP. The yield and purity achieved using this tags varies depend on the characteristics of the tags, such as, the binding capacity and specificity, and the source of the protein to be purified (Arnau *et al.*, 2005; Litchi *et al.*, 2005).

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Mainly, protein purification consists of a primary isolation process followed by clarification or cleaning up. Isolation of intracellular protein involves harvesting, breaking the cell pellet, removing cell debris and finally applying the enzymes to the chromatography systems (Amersham Pharmacia Biotech, 1999). Co-expression of BRP secretes out recombinant protein into the culture medium and therefore, the protein harvested is in the supernatant instead of the cell pellet. The protein has first to be concentrated by ammonium sulphate precipitation, ultrafiltration or extraction using organic solvents (Saxena *et al.*, 2003). The objectives of this isolation step are to rapidly remove proteases that might degrade the protein, to remove impurities and particles that may affect the following chromatographic purification steps and also to concentrate the protein sample (Jonasson *et al.*, 2002).

However, if higher protein purity is required, for example, in pharmaceuticals (>99%) and protein crystallization (95-99%), a single chromatography step is often not sufficient. Following the first affinity chromatography, an intermediate purification step and polishing step have to be done so that the remaining impurities are totally removed. Frequently used methods for these steps are ion-exchange chromatography, hydrophobic interaction chromatography, size-exclusion chromatography and reversed phase chromatography (Jonasson *et al.*, 2002).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The chemicals and solutions used in this project are listed down in Appendix A.

3.2 Genome mining

The genome sequence of *Geobacillus* sp. Geo5 used in this research was contributed by Malaysia Genome Institute (MGI). MGI has started the Whole Genome Sequencing Project of *Geobacillus* sp. Geo5 in the year 2007. The genome sequence consists of contiguous sequences that were not yet assembled and annotated. These sequences were then used to create the database for local BLAST using BioEdit software. Known *glgB* nucleotide sequences from other *Geobacillus* sp. were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank/) and were used in sequence alignment softwares, local BLAST and ClustalW, to locate the position of the open reading frame (ORF) of *glgB* in the *Geobacillus* sp. Geo5 genome. *glgB* sequences of *Geobacillus* sp. obtained from GenBank that were used are as follows:

- *Bacillus* sp. NBRC 15315 (AB294568)
- *Geobacillus stearothermophilus* (M35089)
- *Geobacillus* sp. Y412MC10 (YP003251719)
- *Geobacillus* sp. Y412MC61 (CP001794)
- *Geobacillus thermodenitrificans* NG80-2 (NC_009328)

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3.3 Enzyme structure prediction

Once the nucleotide sequence of the gene was obtained, the nucleotide sequence was translated into amino acid sequence using BioEdit and confirmed with ExPaSy Translate tool (http://expasy.org/tools/dna.html). The amino acid sequence of the enzyme was then submitted to I-TASSER server at http://zhanglab.ccmb.med.umich.edu/I-TASSER/ to predict the 3D structure of the enzyme. I-TASSER built 3D models based on multiple-threading alignments of secondary structure called Profile-Profile Threading Alignment (PPA) and iterative implementation of the Threading Assembly Refinement (TASSER) program (Zhang, 2008). Other than structural prediction, I-TASSER also predicted Enzyme Classification (EC) number, the amino acids in the binding site of the enzyme and the function of the protein by matching the predicted models with protein function databases. The predicted models of the enzyme were then evaluated using PROCHECK to determine the stereochemistry of the structures and results are presented in Ramachandran plots (Laskowski *et al.,* 1993). Using visualisation tool, PyMOL, the position of conserved amino acids that involve in the catalysis were located in the predicted model.

3.4 Culturing *Geobacillus* sp. Geo5

The glycerol stock of *Geobacillus* sp. Geo5 was acquired from MGI. From the glycerol stock, a loop was streaked onto thermus agar plate and nutrient agar plate. The plates were incubated at 70°C for 18 hours. To avoid the plates from drying out when incubated at such high temperature, the inoculated plates were sealed in a

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plastic bag during the incubation. After incubation, an isolated single colony was picked and inoculated into 10 mL thermus broth and nutrient broth. The culture was incubated at 60°C with 250 rpm shaking, for 18 hours, in INFORS HP (Ecotron) incubator shaker. Although *Geobacillus* sp. Geo5 was isolated at 97°C, the experiment done by MGI showed that the optimum temperature for growing this bacterium in laboratory was at 60°C.

3.4.1 Thermus broth preparation

Thermus broth comprised of 0.4% yeast extract, 0.8% peptone and 0.2% NaCl. Broth was aliquoted (10 mL) into universal bottles and autoclaved at 121°C for 20 minutes to sterilise (Yurieva *et al*., 1997).

3.4.2 Thermus agar preparation

Thermus agar comprised of 0.4% yeast extract, 0.8% peptone and 0.2% NaCl and 3% bacteriological agar (Yurieva *et al*., 1997). The agar mixture was autoclaved at 121°C for 20 minutes. Sterilised agar was poured into sterile petri dishes.

3.5 Genomic DNA extraction of *Geobacillus* sp. Geo5

A single colony of the bacterium was inoculated into 10 mL of thermus broth in universal bottle and incubated at 60°C with 250 rpm shaking, for 18 hours, in INFORS HP (Ecotron) incubator shaker. The cell culture (3mL) was centrifuged at 10,000 × g for 10 minutes. The cell pellet was subjected to purification procedure.

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Genomic DNA from *Geobacillus* sp. Geo5 was extracted using Qiagen DNeasy® Blood and Tissue Kit according to the manufacturer's instructions. The DNA concentration was determined by measuring the optical density at A_{260}. The purified DNA solution was kept at -20°C.

3.6 Polymerase chain reaction of *glgB*

glgB from *Geobacillus* sp. Geo5 were amplified using PCR. PCR primers, F1 (5'–ATG CGA TCC AGC TTG ATT GC– 3') and R1 (5'–TCA ATG ATC CGG TAC TTC CC– 3'), were designed starting from the start codon until the stop codon of the open reading frame (ORF) based on the result from genome mining.

Amplification process was carried out in a reaction mixture containing 20-50 ng DNA template, 0.2 µM forward and reverse primers, 0.2 mM dNTP mix, 1.2 U *Pfu* DNA polymerase and 1× *Pfu* Buffer with $MgSO_4$ (Roux, 1995). The genes were amplified using a thermocycler (MyCycler™, BioRad) with the temperature program of predenaturation at 95°C for 5 minutes; 35 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 57°C and 4 minutes extension at 72°C; followed by final elongation step at 72°C for 7 minutes and hold at 10°C. Detection of PCR product was carried out by gel electrophoresis, where 3 µL of PCR product was run in agarose gel electrophoresis (1% (w/v) agarose gel stained with GelRed™ (Biotium), run at 100 volts for 35 minutes) and visualised under UV radiation (302 nm).

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3.7 Polymerase chain assembly

Polymerase chain assembly (PCA) was done to amplify *glgB* from *Geobacillus* sp. Geo5 without the integrated transposase (Wu *et al.*, 2006; TerMaat *et al.*, 2009; Marchand and Piccoud, 2012). Three steps of PCR were done using four different primers (Figure 7). Step 1 was to amplify the gene upstream to the transposase using these primers:

Forward primer, F1: 5'–ATG CGA TCC AGC TTG ATT GC– 3'

Reverse primer, R2: 5'–TTC CGA ATA CGC ATA AAG GAG GGA
AAA ACT GAC TTG GTT ATG GGC GTA TT– 3'

Step 2 was to amplify the gene downstream to the transposase using these primers:

Forward primer, F2: 5'–CTC CTT TAT GCG TAT TCG GAA– 3'

Reverse primer, R1: 5'–TCA ATG ATC CGG TAC TTC CC– 3'

Primer R2 and F2 were designed in such that they were complementary and overlapping each other (Figure 7) by at least 20 bp to ensure that the PCR products from both steps are able to be assembled. In step 1 and step 2 PCR, *Geobacillus* sp. Geo5 genomic DNA was used as the DNA template.

Finally, step 3 was the PCA. This is where the PCR products from the previous two steps were assembled to construct the whole gene using primer F1 and R1. In this step, the purified PCR products from the previous two steps were used as the DNA template instead of the genomic DNA. All the three steps of amplification process were carried out in a reaction mixture containing 20-50 ng DNA template,

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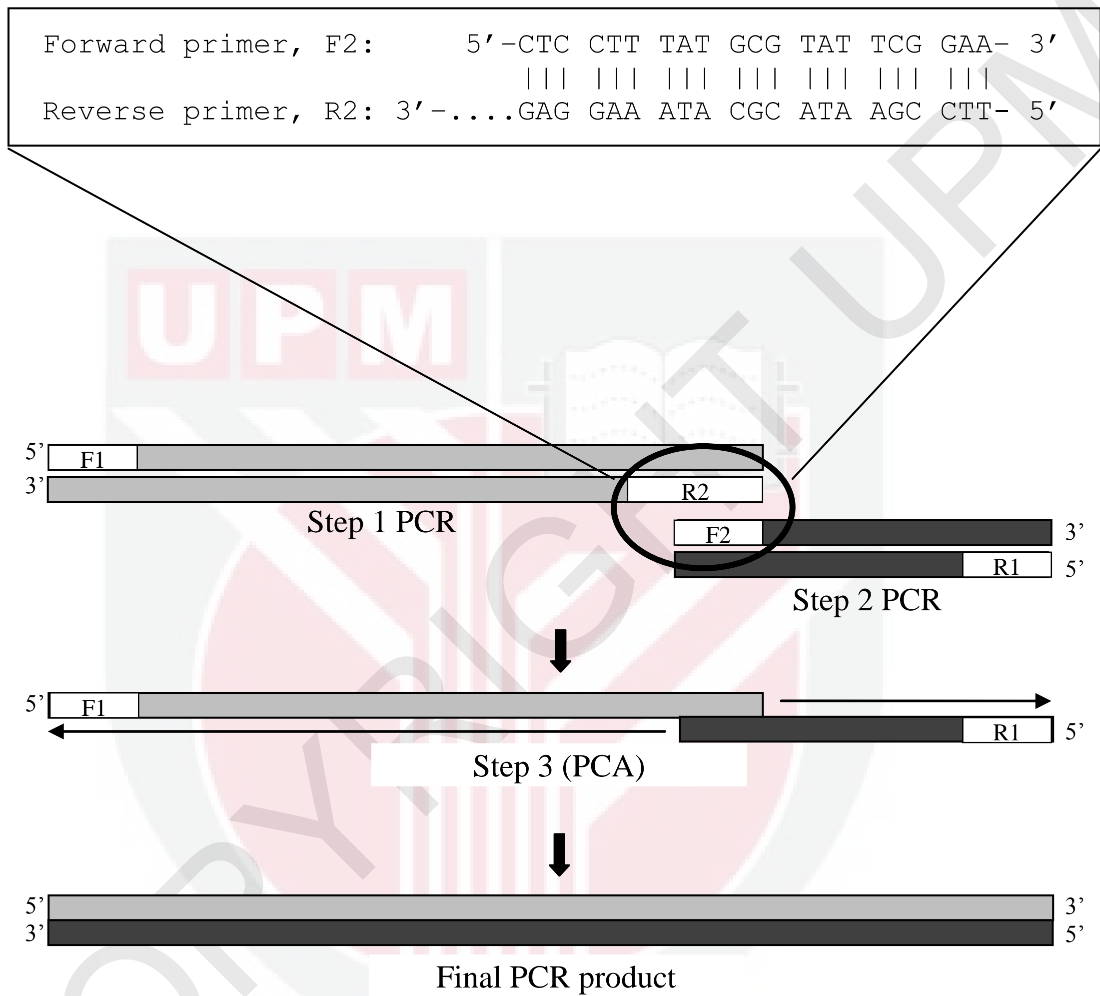

Figure 7. PCR and PCA. F1 and F2 are forward primers. R1 and R2 are reverse primers. (→) represents the direction of DNA synthesis by DNA polymerase; (■) and (■) represent DNA strands.

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0.2 µM forward and reverse primers, 0.2 mM dNTP mix, 1.2 U *Pfu* DNA polymerase and 1× *Pfu* Buffer with $MgSO_4$. The reactions were amplified using a thermocycler (MyCycler™, BioRad) with the temperature program of predenaturation at 95°C for 5 minutes; 35 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 57°C and 4 minutes extension at 72°C; followed by final elongation step at 72°C for 7 minutes and hold at 10°C. Detection of PCR products was carried out by gel electrophoresis, where 3µL of PCR products was run in agarose gel electrophoresis (1% (w/v) agarose gel stained with GelRed™ (Biotium), run at 100 volts for 35 minutes) and visualised under UV radiation (302 nm).

3.8 PCR purification by gel extraction

PCR product (150 µL) was electrophorised on 1% agarose gel electrophoresis at 100 volts for 35 minutes and then stained with GelRed™ (Biotium). The desired band was excised with a sterile scalpel. The DNA was extracted using QIAquick Gel Extraction Kit according to the manufacturer's instructions. The DNA concentration of the purified PCR product was determined by measuring the optical density at A_{260} and was verified by DNA sequencing. For DNA sequencing, the samples were sent to First BASE Laboratories Sdn Bhd.

3.9 Cloning of PCR products

To prepare the insert for cloning reaction, the forward primer used in PCR was primer F3, instead of primer F1. This primer has additional four bases (CACC) at the 5' end as the requirement for cloning into pET102/D-TOPO® vector. Fresh PCR

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products of *glgB* from *Geobacillus* sp. Geo5 was cloned into pET102/D-TOPO® vector from Champion™ pET Directional TOPO® Expression Kit (Appendix B). Cloning reactions were prepared and transformed into One Shot® TOP10 chemically competent *E. coli* according to manufacturer's instructions. The gene cloned in pET102/D-TOPO® vector will be expressed as a fusion protein to His-Patch thioredoxin. His-Patch thioredoxin, which is a mutated thioredoxin protein, have metal binding site and therefore it has high affinity towards divalent cations (Lu *et al.*, 1996). This characteristic can be used to purify the fusion protein using metal chelating resins.

3.10 Recombinant plasmid purification

An isolated colony harbouring the recombinant plasmid, pET/GBE, was picked and cultured in 10 mL LB broth containing 100 µg/mL of ampicillin at 37°C with 250 rpm shaking, for 16 hours in INFORS HP (Ecotron) incubator shaker. The recombinant plasmid was purified from 5 mL culture using QIAprep® Spin Miniprep Kit according to the manufacturer's instructions.

3.11 Analysing transformed recombinant plasmids

The plasmids were analysed to ensure that the gene was successfully integrated into the vector and to confirm that the gene was in frame with the His-Patch thioredoxin at the N-terminal of the pET102/D-TOPO® vector.

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3.11.1 PCR of recombinant plasmid DNA

Amplification process was carried out in a reaction mixture containing 1-10 ng plasmid DNA, 0.2 μM forward and reverse primers (set 1: F1 and R1, set 2: TrxFus forward and T7 reverse) 0.2 mM dNTP mix, 1.2 U *Taq* DNA polymerase and 1× *Taq* Buffer with $MgSO_4$. For TrxFus forward and T7 reverse priming sites, refer to Appendix C. The genes were amplified using a thermocycler (MyCycler™, BioRad) with the temperature program of predenaturation at 95°C for 5 minutes; 35 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 57°C and 2 minutes extension at 72°C; followed by final elongation step at 72°C for 7 minutes and hold at 10°C. Detection of PCR product was carried out by gel electrophoresis, where 3 μL of PCR product was run in agarose gel electrophoresis (1% (w/v) agarose gel stained with GelRed™ (Biotium), run at 100 volts for 35 minutes) and visualised under UV radiation (302 nm). If the gene would be successfully integrated into the vector, both sets of PCR would be successful and if the gene integrated was not in frame, PCR using set 2 primers would fail.

3.11.2 *Pst*I and *Bgl*II digestion

The digestion reaction contains 50-100 ng of plasmid DNA, 0.2 μL of restriction enzyme (FastDigest® *Bgl*II and, or *Pst*I) and 1× FastDigest® Buffer in a 10 μL reaction. The reaction components were incubated at 37°C for 20 minutes. Then, the digestion product was run in agarose gel electrophoresis (1% (w/v) agarose gel stained with GelRed™ (Biotium), run at 100 volts for 35 minutes) and visualised under UV radiation (302 nm). The expected sizes for a single digestion of empty

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pET102/D-TOPO® vector and pET/GBE are 6315 bp and 8328 bp, respectively. Since these sizes are quite close to each other especially when viewed in agarose gel, therefore a double digestion using both *Bgl*II and *Pst*I was performed so that the size difference could be viewed clearly. For double digestion, two fragments were expected. Empty vector would give 1826 bp and 4489 bp fragments, while pET/GBE would give 3839 bp and 4489 bp fragments.

3.11.3 DNA sequencing of recombinant plasmid DNA

The purified plasmids were sent for DNA sequencing to First BASE Laboratories Sdn Bhd. The sequencing was done using PCR primers F1 and R1, TrxFus forward and T7 reverse.

3.12 Intracellular expression of G5GBE

Heterologous expression of GBE from *Geobacillus* sp. Geo5 in *E. coli* was studied. Recombinant plasmid harbouring *glgB* from *Geobacillus* sp. Geo5 was purified as in section 3.10. The purified plasmid (10 ng/µL) was transformed into the expression host, *E. coli* BL21 Star™ (DE3) according to the manufacturer's instructions.

A colony of the positive transformant was picked and inoculated into 10 mL LB broth containing 100 µg/mL ampicillin and grew at 37°C with 250 rpm shaking, for 16 hours in INFORS HP (Ecotron) incubator shaker. The broth (2 mL) was then transferred into 200 mL LB broth containing 100 µg/mL ampicillin in 1 L Schott bottle and incubated at 37°C with 250 rpm shaking in INFORS HP (Ecotron)

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incubator shaker. The expression was induced with IPTG when optical density A_{600} reached 0.5. The expression was done in various induction conditions (IPTG concentration: 0 to 1 mM, induction temperature: 20°C to 37°C and induction time: 0 to 28 hours) in order to achieve the highest expression level.

After induction, 10 mL of culture was centrifuged at 12,000 × g for 20 minutes at 4°C. The cell pellet was resuspended in 10 mL of 50 mM sodium phosphate buffer (pH 7.0), sonicated (Branson Digital Sonifier; 2 minutes with 30 seconds lapse; amplitude: 30%) and cleared by centrifugation (12,000 × g, 20 minutes, 4°C). Cell lysate was subjected to iodine stain assay and SDS-PAGE. The recombinant protein, G5GBE, was expressed as a fusion protein with His-Patch thioredoxin at the N-terminal. The theoretical molecular weight of GBE was 78 kDa and with the addition of His-Patch thioredoxin, the expected size of the fusion protein would be 91 kDa.

3.13 Determination of G5GBE expression level

The expression level of G5GBE was represented by GBE activity. There are two methods used to determine GBE activity quantitatively, namely, iodine stain assay and branching linkage assay (Takata *et al.*, 1994). On top of that, the expression level of G5GBE was determined qualitatively by SDS-PAGE.

3.13.1 Iodine stain assay

Iodine stain assay was done as in the method provided by Takata *et al.* (1994). Enzyme solution in 50 mM sodium phosphate buffer, pH 7.0 (50 µL) was incubated

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with 50 μL of substrate at 50°C for 30 minutes. The substrate was 0.1% amylose from potato (Sigma) dissolved in 50 mM sodium phosphate buffer (pH 7.0) and 10% (v/v) of DMSO. The reaction was terminated by the addition of 1 mL of iodine reagent. Iodine reagent was prepared fresh from 0.5 mL of stock solution (0.26 g of I_2 and 2.6 g of KI in 10 mL of distilled water), 0.5 mL of 1 M HCl and diluted to 130 mL in distilled water. One unit (U) of enzyme activity was defined as the decreased of A_{660} reading by 1% per minute. The decreased of A_{660} reading represents the amylose-iodine complex (Shinohara *et al.*, 2001).

Amylose, which was used as the substrate, is the linear component of starch. Amylose retains iodine molecules within its hydrophobic helical coils, where the reaction of branching enzyme will disrupts the coils by introducing the branches to amylose, converting amylose to amylopectin (van der Maarel *et al.*, 2003). Hence, less iodine molecules held inside the hydrophobic helical coils and consequently decrease the optical reading of amylose-iodine complex at A_{660}.

3.13.2 Branching linkage assay.

This assay quantitated the amount of α-1,6-glycosidic linkages synthesised. As described by Takata *et al.* (1994), the branching enzyme was incubated with reduced amylose as the substrate. The substrate was prepared using the method provided by Takeda *et al.* (1993). The branched product was then debranched by using isoamylase. This enzyme specifically hydrolysed the α-1,6 branch point. Then, the liberated reducing ends were determined by 2,2'-bicinchoninate (BCA) assay (Utsumi *et al.*, 2009; Thiemann *et al.*, 2006). One unit of enzyme activity was

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defined as the quantity of branching enzyme that gives 1 μmol of the branch linkage per minute, where the amount of branch linkage is correlative to the amount of the reducing sugars obtained (Takata *et al.*, 1994).

However, the results of GBE activity shown in this thesis are all based on iodine stain assay, not branching linkage assay. Branching linkage assay is very tedious to perform and would take longer time to give the results compared to iodine stain assay and therefore it was not preferred. Branching linkage assay was done only to confirm the results obtained by iodine stain assay only when necessary.

3.13.3 SDS-PAGE

SDS-PAGE (12% running gel, 6% stacking gel) was done using Laemmli's method (Laemmli, 1970). Sample, 90 μL, was mixed with 10 μL of 10× sample buffer and boiled for 10 minutes. The sample (10 μL) was loaded into the gel and run at 180 volts for 1 hour. The gel was then stained with Coomassie Brilliant Blue R-250 solution (0.1% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol and 5% (v/v) acetic acid). This staining method can detect as little as 0.1 μg of protein in the band (Wang *et al.*, 2007).

3.14 Secretory expression of G5GBE

pSW1 vector (Mobitec, Germany) was used for BRP expression (Appendix D). This vector is resistant towards tetracyclin and use mitomycin C as the inducer for the secretory expression system.

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3.14.1 Co-transformation of pSW1 vector

BRP expression vector, pSW1, was co-transformed into competent cells *E. coli* BL21 Star™ (DE3) (Invitrogen) that harbours pET/GBE. Competent cells preparation and plasmid transformation was done using calcium/manganese-based (CCMB) buffer and transformation method by Hanahan *et al.* (1991).

3.14.2 Analysis of positive transformants

The positive transformants were analysed by digesting the purified plasmid with restriction enzyme *Hin*dIII. The plasmids were purified as in section 3.10. The digestion reaction (50-100 ng plasmid DNA, 0.2 µL FastDigest® *Hin*dIII and 1× FastDigest® Buffer in 10 µL reaction) was incubated at 37°C for 20 minutes. The digested DNA was run in agarose gel electrophoresis (1% (w/v) agarose gel stained with GelRed™ (Biotium), run at 100 volts for 35 minutes) and two bands were expected when gel was visualised under UV radiation (302 nm). The size of pSW1 vector and pET/GBE is 3.9 kb and 8.3 kb, respectively.

3.14.3 Quasi-lysis test

An overnight culture (1 mL) was inoculated into 100 mL of LB broth containing 20 µg/mL of tetracycline and 100 µg/mL of ampicillin in 500 mL Schott bottle. The culture was incubated at 37°C with 250 rpm shaking in INFORS HP (Ecotron) incubator shaker until optical density at A_{600} reached about 0.1. Then, the expression was fully induced with 0.5 µg/mL of mitomycin C. Two controls were done where

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the expression was induced with advised concentration of mitomycin C (20 ng/mL) according to the pSW1 vector mafacturers instructions and the other one was not induced. A_{600} reading was read every one hour for 6 hours.

3.14.4 Effect of mitomycin C concentration on secretory expression

The expression of G5GBE was induced with 0.75 mM of IPTG when optical density at A_{600} reached about 0.5. At the same time point, the co-expressed BRP was induced with various concentrations of mitomycin C, from 0 to 25 ng/mL. A control for expression with the same condition was done with expression host that has BRP plasmid in absent and therefore only contains pET/GBE. The expression with the optimum concentration of mitomycin C was studied for 28 hours in order to achieve the highest expression level.

After induction, 10 mL of culture was centrifuged at 12,000 × g for 20 minutes at 4°C. Both the supernatant and the cell pellet were subjected to iodine stain assay. The cell pellet was resuspended in 10 mL of 50 mM phosphate buffer (pH 7.0), sonicated using Branson Digital Sonifier (2 minutes with 30 seconds lapse; amplitude: 30%) and cleared by centrifugation (12,000 × g, 20 minutes, 4°C) prior to assay.

3.15 Purification of G5GBE

G5GBE expressed in *E. coli* BL21 Star™ (DE3) was purified by affinity chromatography technique using Äkta Explorer (GE Healthcare, Sweden). Cell

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culture (200 mL) was harvested by centrifugation (15,600 × g, 30 minutes, 4°C) and the cell pellet was resuspended in 10 mL of binding buffer (20 mM sodium phosphate, 0.5 m NaCl, 30 mM imidazole, pH 7.4). The cell suspension was sonicated using Branson Digital Sonifier (2 minutes with 30 seconds lapse; amplitude: 30%) and cleared by centrifugation (12,000 × g, 20 minutes, 4°C). The crude protein (10 mL) was loaded into 1 mL HisTrap HP column (GE Healthcare) at flowrate of 1 mL/min. The column was then washed with 20 column volume of binding buffer. Finally, the bound enzyme was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4) by a linear gradient. Protein fractions that were collected were run in SDS-PAGE as in section 3.13.3. The protein content was determined by Quick Start™ Bradford protein assay (Biorad) according to manufacturer's instructions, and enzyme activity was tested out by iodine stain assay as in section 3.13.1.

3.15.1 MALDI TOF/TOF analysis

In order to verify that the purified protein is the protein of interest, GBE, the purified protein was run through SDS-PAGE as in section 3.13.3. The gel band was excised and sent for protein sequencing using a tandem mass spectrometry technique, which is matrix-assisted laser desorption ionization time-of-flight (MALDI TOF/TOF). This process was outsourced to First BASE Laboratories Sdn Bhd.

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3.16 Characterisation of purified GBE

The general biochemical properties of GBE were characterised based on the effect of temperature on the enzyme activity and stability, the effect of pH on enzyme activity and stability, and the effect of metal ions on enzyme activity. These properties were chosen to be characterised since the U. S. Food and Drug Administration (FDA) has listed them in the guideline of enzyme preparations for enzymes to be used in food processing industries (U. S. Food and Drug Administration, 2010). Since GBE is most likely would be applied in food and beverages industries, therefore those characterisation parameters were taken into consideration.

3.16.1 Effect of temperature on the enzyme activity

The effect of temperature on GBE activity was studied at temperatures from 30°C to 80°C with 5°C intervals. GBE activity was assayed using iodine stain assay (refer to section 3.13.1).

3.16.2 Effect of temperature on enzyme stability

The enzyme stability test was done by incubating the enzymes at 40°C to 80°C. Enzyme activity was assayed every 4 hours for 24 hours. After the incubation, the enzyme was immediately cooled in an ice bath prior to iodine stain assay, which was done at 50°C for 30 minutes (refer to section 3.13.1).

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3.16.3 Effect of pH on enzyme activity

The effect of pH on GBE activity was studied at pH 4 to pH 10. GBE activity was assayed in a few buffers with different pHs; 50 mM acetate buffer for pH 4-6, 50 mM potassium phosphate buffer for pH 6-8, 50 mM Tris-Cl buffer for pH 8-9 and 50 mM glycine-NaOH for pH 9-10. The iodine stain assay was done at 50°C for 30 minutes (refer to section 3.13.1).

3.16.4 Effect of pH on enzyme stability

The effect of pH on GBE stability was studied for pH 4 to pH 10. The enzyme was incubated in various buffers with different pHs at 25°C for 1 hour. The buffers used were 50 mM acetate buffer for pH 4-6, 50 mM potassium phosphate buffer for pH 6-8, 50 mM Tris-Cl buffer for pH 8-9 and 50 mM glycine-NaOH for pH 9-10. GBE activity was assayed using iodine stain assay at 50°C, pH 7.0 as in section 3.13.1 straightaway after the treatment.

3.16.5 Effect of metal ions on enzyme activity

Metal ions may have either enhancement or inhibition effect on GBE activity. To see if these effects occur, GBE was treated with 1 mM and 5 mM of metal ions (Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}) for 30 minutes at 25°C. Immediately after the treatment, GBE activity was assayed using iodine stain assay at 50°C as in section 3.13.1.

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CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Genome mining

The sequence of *glgB* was determined by a technique called genome mining where the genome sequence of *Geobacillus* sp. Geo5 was aligned with known nucleotide sequences of *glgB* from other *Geobacillus* spp., which were obtained from GenBank, using local BLAST and ClustalW (Appendix E). As the genome sequence was not yet assembled and annotated, the position of the gene in the *Geobacillus* sp. Geo5 genome was found fragmented into two contiguous sequences. The sequences were joined to make up the gene, which has the size of 2013 bp that codes for 630 amino acids (Figure 8). The theoretical molecular weight is 78.43 kDa, was predicted using the "Compute pI/Mw tool" from ExPASy Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/).

The four conserved regions of α-amylase family enzymes as reported by Abad *et al.* (2002) and van der Maarel *et al.* (2003) were determined by aligning the amino acid sequences of *glgB* from *Geobacillus* sp. Geo5 with five other *Geobacillus* spp., *E. coli* and *M. tuberculosis* using ClustalW (Table 3). Within the four conserved regions, there are seven highly conserved amino acids that have important roles in the catalysis and substrate binding. Three of the conserved residues are the catalytic residues; Asp^{313} in region II, Glu^{356} in region III and Asp^{424} in region IV. These residues corresponded to Asp^{405}, Glu^{458} and Asp^{526} of the *E. coli* branching enzyme

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```
      PCR primer F1
ATGCGATCCAGCTTGATTGCGGCGAATCCGACAGATTTAGAAGTGTATTTGTTTCATGAA
 M  R  S  S  L  I  A  A  N  P  T  D  L  E  V  Y  L  F  H  E

GGCCGTTTGTATCAAAGTTATGAGCTGTTCGGCGCTCATGTCATCCGCGACGGCGGAGCG
 G  R  L  Y  Q  S  Y  E  L  F  G  A  H  V  I  R  D  G  G  A

GTCGGCACTCGCTTTTGCGTGTGGGCGCCCCATGCGCGGGAAGTCCGTCTTGTCGGCAGT
 V  G  T  R  F  C  V  W  A  P  H  A  R  E  V  R  L  V  G  S

TTCAACGATTGGAATGGGGCGAATTCCCCCCTGACGAAGGTGAACGACGAAGGGGTATGG
 F  N  D  W  N  G  A  N  S  P  L  T  K  V  N  D  E  G  V  W

ACGATCGTTGTTCCAGAAAACTTGGAAGGGCATCTCTATAAATATGAGATCATCACACCG
 T  I  V  V  P  E  N  L  E  G  H  L  Y  K  Y  E  I  I  T  P

GATGGCCGTGTTCTGTTGAAAGCCGACCCGTACGCCTTTTACTCCGAATTGCGCCCTCAT
 D  G  R  V  L  L  K  A  D  P  Y  A  F  Y  S  E  L  R  P  H

ACCGCCTCGATTGTCTACGATTTGAAAGGATACGAGTGGAATGATTCATCTTGGCAGCGG
 T  A  S  I  V  Y  D  L  K  G  Y  E  W  N  D  S  S  W  Q  R

AAGAAACGGCGAAAGCGGATTTATGACCAACCGATGGTCATTTATGAACTTCATTTCGGT
 K  K  R  R  K  R  I  Y  D  Q  P  M  V  I  Y  E  L  H  F  G

TCGTGGAAAAAGAAACCGGACGGCCGCTTTTATACGTACCGTGAGATGGCCGACGAACTC
 S  W  K  K  K  P  D  G  R  F  Y  T  Y  R  E  M  A  D  E  L

ATTCCGTACGTGCTGGAGCGCGGATTTACGCACATTGAGCTGCTTCCGCTTGTCGAGCAT
 I  P  Y  V  L  E  R  G  F  T  H  I  E  L  L  P  L  V  E  H
                                                PCR primer F4
CCGCTCGATCGTTCGTGGGGATATCAAGGGACCGGCTATTATTCGGTGACAAGCCGCTAT
 P  L  D  R  S  W  G  Y  Q  G  T  G  Y  Y  S  V  T  S  R  Y

GGCACGCCGCACGATTTCATGTATTTCGTCGACCGCTGCCATCAAGCGGGGCTTGGCGTC
 G  T  P  H  D  F  M  Y  F  V  D  R  C  H  Q  A  G  L  G  V

ATCATCGACTGGGTGCCGGGGCATTTTTGCAAGGACGCCCACGGGCTGTACATGTTTGAC
 I  I  D  W  V  P  G  H  F  C  K  D  A  H  G  L  Y  M  F  D

GGCGCACCGACGTATGAATACGCGAATGAAAAAGACCGAGAAAATTACGTCTGGGGGACG
 G  A  P  T  Y  E  Y  A  N  E  K  D  R  E  N  Y  V  W  G  T

GCGAATTTTGACTTGGGCAAGCCGGAAGTGCGCAGTTTTCTGATCTCCAATGCGTTGTTT
 A  N  F  D  L  G  K  P  E  V  R  S  F  L  I  S  N  A  L  F

TGGCTGGAGTATTACCATGTGGACGGGTTTCGCGTCGATGCGGTCGCCAATATGCTTTAT
 W  L  E  Y  Y  H  V  D  G  F  R  V  D  A  V  A  N  M  L  Y

TGGCCGAACAACGACCGGCTCTATGAAAATCCGTATGCGGTCGAGTTTTTGCGCCAGTTG
 W  P  N  N  D  R  L  Y  E  N  P  Y  A  V  E  F  L  R  Q  L

AATGAGGCGGTGTTTGCCTATGACCCGAACGTCTTGATGATCGCTGAAGATTCGACCGAC
 N  E  A  V  F  A  Y  D  P  N  V  L  M  I  A  E  D  S  T  D

TGGCCTCGGGTGACCGCGCCGACGTACGATGGCGGCCTTGGGTTTAACTACAAGTGGAAC
 W  P  R  V  T  A  P  T  Y  D  G  G  L  G  F  N  Y  K  W  N
```

```
ATGGGCTGGATGAACGACATGCTGAAGTATATGGAAACGCCGCCGCATGAGCGGAAATAC
 M  G  W  M  N  D  M  L  K  Y  M  E  T  P  P  H  E  R  K  Y

                   PCR primer R2
                              PCR primer F2
GCCCATAACCAAGTCAGTTTTTCCCTCCTTTATGCGTATTCGGAAAATTTCATTTTGCCA
 A  H  N  Q  V  S  F  S  L  L  Y  A  Y  S  E  N  F  I  L  P

TTTTCCCATGATGAAGTTGTGCATGGCAAAAAATCGCTGCTCAATAAAATGCCTGGGTCG
 F  S  H  D  E  V  V  H  G  K  K  S  L  L  N  K  M  P  G  S
                                  PCR primer R3
TACGAAGAGAAGTTCGCCCAGCTGCGCCTATTGTATGGCTACATGATGGCCCACCCTGGG
 Y  E  E  K  F  A  Q  L  R  L  L  Y  G  Y  M  M  A  H  P  G

AAAAAGCTGCTGTTTATGGGCAGTGAGTTTGCCCAGTTTGATGAATGGAAGTTTGAGGGA
 K  K  L  L  F  M  G  S  E  F  A  Q  F  D  E  W  K  F  E  G

GAGCTCGACTGGGTGCTGTTCGATTTTGAATTGCACCGGAAAATGGACGAATATGTGAAG
 E  L  D  W  V  L  F  D  F  E  L  H  R  K  M  D  E  Y  V  K

CAGCTGATCGCCTGCTATAAACGGTATAAGCCGTTTTACGAGCTTGATCATGATCCGAGG
 Q  L  I  A  C  Y  K  R  Y  K  P  F  Y  E  L  D  H  D  P  R

GGGTTTGAATGGATTGACGTTCATAATGCCGAGCAAAGCATTTTCTCGTTCGTCCGCCGC
 G  F  E  W  I  D  V  H  N  A  E  Q  S  I  F  S  F  V  R  R

GGGAAAAAAGACGGCGATCTATTGGTAATTGTTTGCAATTTCACAAATCAAGCGTATGAC
 G  K  K  D  G  D  L  L  V  I  V  C  N  F  T  N  Q  A  Y  D

GATTACAAAGTCGGCGTGCCGCTTTTGGCGCCGTACCGCGAAGTGCTTAACAGCGATGCA
 D  Y  K  V  G  V  P  L  L  A  P  Y  R  E  V  L  N  S  D  A

GCGGAGTTTGGCGGTTCGGGACATGTCAACGGGAAGCGGCTTCCCGCTTTCAGTGAGCCG
 A  E  F  G  G  S  G  H  V  N  G  K  R  L  P  A  F  S  E  P

TTTCATGGAAAACCGTACCATGTGCGCATGACGATTCCGCCGTTTGGCATTTCCATTTTG
 F  H  G  K  P  Y  H  V  R  M  T  I  P  P  F  G  I  S  I  L

CGGCCAGTGCAAAAACGAGGGGAGAGAAAGCAGAATGAAGAAGAAGTGCATCGCCATGTT
 R  P  V  Q  K  R  G  E  R  K  Q  N  E  E  E  V  H  R  H  V

ATTGGCCGGCGGGCAAGGAAGCCGGCTTCGCTCGCTGACGAAAAACATCGCGAAACCAGC
 I  G  R  R  A  R  K  P  A  S  L  A  D  E  K  H  R  E  T  S
             PCR primer R1
CGTGCCGTTTGGGGGGAAGTACCGGATCATTGA
 R  A  V  W  G  E  V  P  D  H  -
```

Figure 8. Nucleotide and amino acid sequence of *glgB* from *Geobacillus* sp. Geo5. GenBank accession number: KC951870.

Table 3. Conserved regions in glycogen branching enzyme from *Geobacillus* spp., *Escherichia coli* and *Mycobacterium tuberculosis*.

	Conserved region			
	I	II	III	IV
Geobacillus sp. Geo5 [1]	HQAGLGVIIDWVPGHFCK	HVDGFRVDAVAN	VLMIAEDSTDW	FILPFSHDEVV
Geobacillus sp. Y412MC10 [1]	HQAGIGVLLDWVPAHFAK	HIDGLRVDAVTS	ALMMAEESSAW	FTLPLSHDEVV
Geobacillus sp. Y412MC61 [1]	HQAGLGVIIDWVPGHFCK	HVDGFRVDAVAN	VLMIAEDSTDW	FILPFSHDEVV
Geobacillus sp. NBRC 15315 [1]	HQAGIGVILDWVPGHFCK	HVDGFRVDAVAN	VLMIAEDSTDW	FILPFSHDEVV
Geobacillus stearothermophilus [1]	HQQGIGVILDWVPGHFCK	HVDGFRVDAVAN	ILMIAEDSTDW	FILPFSHDEVV
Geobacillus thermodenitrificans NG80-2 [1]	HQAGIGVIMDWVPGHFCK	HIDGFRVDAVAN	VLMIAEDSTDW	FILPFSHDEVV
Escherichia coli (PDB ID: 1M7X)	HAAGLNVIMDWVPGHFPT	GIDALRVDAVAS	AVTMAEESTDF	FILPFSHDEVV
Mycobacterium tuberculosis (PDB ID: 3K1D1)	HQAGIGVIVDWVPAHFPK	HIDGLRVDAVAS	IVTIAEESTPW	YVLPLSHDEVV

Note: [1] Refer to p. 36 for accession number. The conserved amino acids are highlighted.

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and Asp^{411}, Glu^{464} and Asp^{532} of the *M. tuberculosis* branching enzyme (refer to section 2.3.1) (Pal *et al.*, 2010; Abad *et al.*, 2002). Four other conserved residues; Asp^{243} and His^{248} in region I, Arg^{311} in region II and His^{423} in region IV are responsible for substrate binding (van der Maarel *et al.*, 2003; Abad *et al.*, 2002).

van der Maarel *et al.* (2003) demonstrated that thermostability of an enzyme can be presumed from its primary sequence information. Somehow, there are correlations between the number of aromatic amino acids (phenylalanine, tryptophan and tyrosine), glutamine and asparagine with the thermostability (Vieille *et al.*, 2001; Serrano *et al.*, 1991; Burley and Petsko, 1985). Enzymes with high number of aromatic residues in combination with low number of glutamine and asparagine would show higher temperature stability. The reason behind this is that the hydrophobic interactions between the aromatic groups are responsible for the stability of a thermophilic protein. Also, the deamination of thermolabile amino acids, asparagine and glutamine, resulted the inactivation of enzymes at elevated temperature and therefore, less number of asparagine and glutamine would minimize the chances of enzyme inactivation and so contribute to thermostability (Vieille *et al.*, 2001).

The percentage of aromatic residues in GBE is 15% and the percentage of asparagine and glutamine is 6.3%. Placing those values in Table 4, those percentages are comparable to GBE from *B. stearothermophilus*, which has 16.3% of aromatic residues and 7.7% of asparagine and glutamine. Thus it is postulated that GBE from *Geobacillus* sp. Geo5 would have optimum temperature for enzyme activity at around 50°C and stable at about 60°C.

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Table 4. Percentage of aromatic amino acids, glutamine and asparagine in relation to the temperature optimum for activity and stability of three bacterial and two maize branching enzymes.

Source	%FWY[1]	%QN[2]	Optimum temperature (°C)	Temperature stability (°C)
A. aeolicus	17.4	4.8	80	90
R. obamensis	16.8	5.2	65	80
B. stearothermophilus	16.3	7.7	55	60
E. coli	13.2	7.6	30	nd
Maize I	12.2	6.6	30	nd
Maize II	13.4	6.8	20	nd

Note: [1] F: phenylalanine, W: tryptophan, Y: tyrosine; [2] Q: glutamine, N: asparagine; nd, not determined. (Source: van der Maarel *et al.*, 2003)

4.2 Enzyme structure prediction

Visualisation of the 3D structure of an enzyme is important to understand the enzyme features and mechanism of action. The distinctive construction of the domains can be observed and compared with other similar enzymes. Also, features like disulphide bond formation, the position of the conserved amino acids and the catalytic triad can be localised and evaluated. However, the determination of protein structure does not necessarily reveal its function. This is because proteins with similar folds might always have different functions and therefore it is best to test out the protein functions by biochemical experimentation (Skolnick and Fetrow, 2000).

Amino acid sequence of *glgB* was submitted to I-TASSER server to get the 3D structure of the enzyme predicted. I-TASSER gave five predicted models of the

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enzyme, which were simulated based on the template of GBE from *M. tuberculosis* H37RV (PDB ID: 3K1D) (Figure 9). The percentage of sequence identity of the template in the threading aligned region with the query sequence was 45%, while the percentage sequence identity of the whole template chains with query sequence was 42%. According to Baker and Sali (2001), for 30% to 50% of sequence identity, about 90% of the main-chain modelled would have 1.5 Å RMS error (refer to Figure 6, p. 27). The errors are frequently due to side-chain packing, core distortion and loop modelling; and occasionally due to alignment mistakes. Fortunately for threading and comparative modelling, errors in functional region of the protein are relatively low. This is because active sites are usually very conserved in evolution than the other part of the protein fold (Baker and Sali, 2001).

The quality of the predicted models was shown by the C-score (confidence score) that range from -5 to 2 where higher value shows higher confidence and vice-versa. The highest C-score of the predicted model was only 0.24, which means that the quality of the model was satisfying (Table 5).

Table 5. C-score of the predicted models.

Model	C-score
1	0.24
2	-1.12
3	-1.38
4	-2.10
5	-3.04

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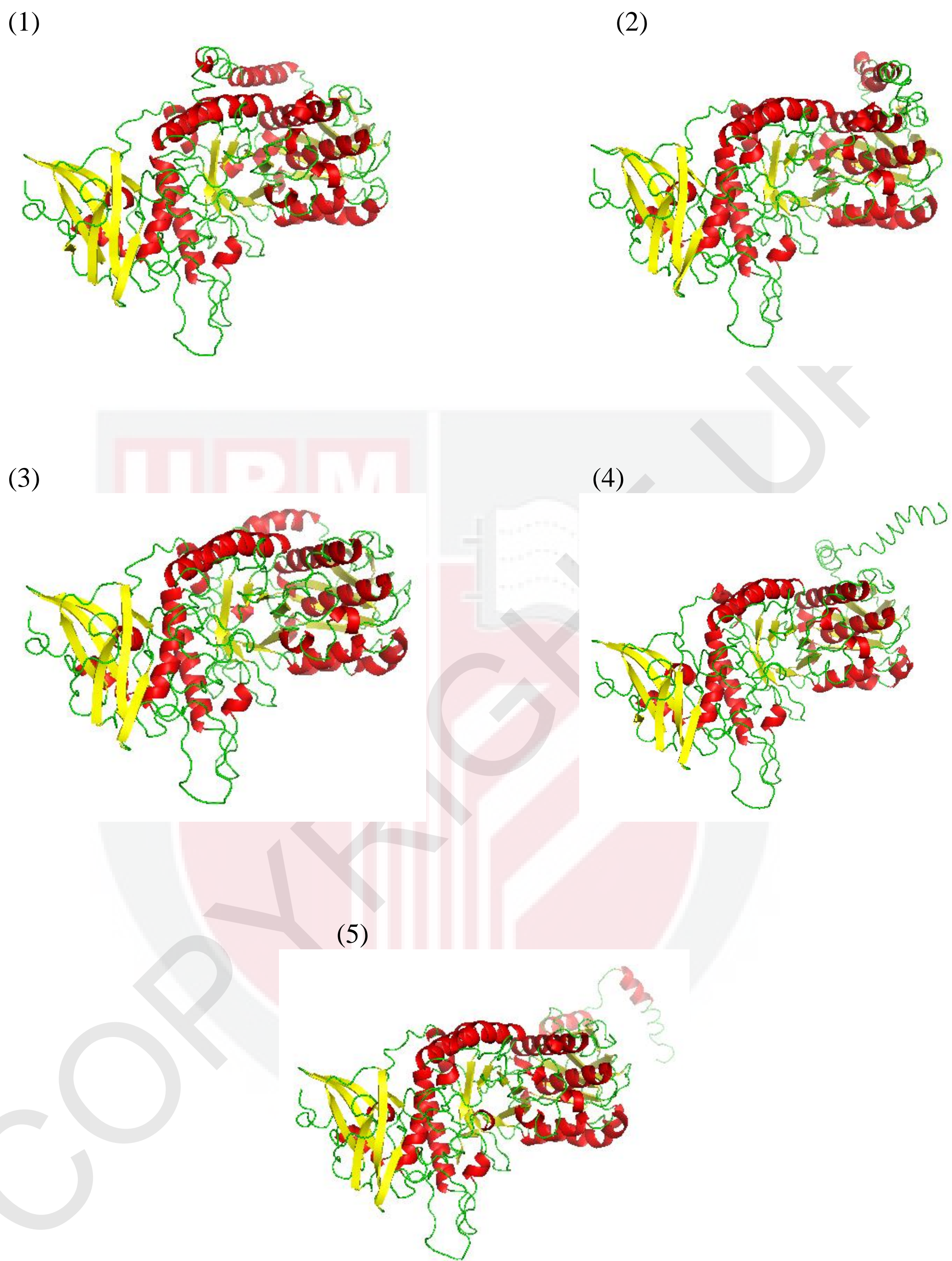

Figure 9. Five predicted structures of GBE from *Geobacillus* sp. Geo5 by I-TASSER. These structures used GBE from *Mycobacterium tuberculosis* as the template. α-helices were represented in red, β-sheets were represented in yellow and loops were represented in green.

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To estimate the accuracy of the model, I-TASSER provided the TM-score, but only for model 1, which is 0.75 ± 0.11. That value indicates that the two protein structures, GBE from *Geobacillus* sp. Geo5 and *M. tuberculosis*, are similar. TM-score, which is short for Template Modelling score, is used to measure similarity between two protein structures by a score range of 0 to 1, where 1 means that both structures are a perfect match (Zhang and Skolnick, 2004). Looking at all five of the predicted structures given, the three distinctive domains of branching enzyme were observed; the central catalytic domain, which is made up of eight α/β helices that forms a barrel, and seven β-strands in both N and C-terminals (Figure 9) (Pal *et al.*, 2010; Abad *et al.*, 2002). There was no disulphide bond predicted for this structure. Both crystal structures of GH-13 family GBE also did not show any disulphide bond formation. I-TASSER also stated that the predicted Enzyme Classification (EC) number to the queried sequence is 2.4.1.18, which is the EC number for 1,4-α-glucan branching enzyme.

The five predicted structures were evaluated further using PROCHECK software (Laskowski *et al.*, 1993). PROCHECK would evaluate the stereochemistry of protein structures using Ramachandran plot by calculating the backbone dihedral angles psi (ψ) against phi (φ) of amino acid residues in a protein structure. The Ramachandran plots for all five of the predicted models by I-TASSER are shown in Figure 10 and the summary of the outcome is displayed in Table 6. Evaluating those data, model 1 is supposed to be the best model although amino acids that lied in the most favoured regions was only 68.6%, lower than model 2, 4 and 5, but the percentage of amino acids in the disallowed region was the lowest. A good quality model however, would be expected to have over 90% of amino acid residues in the most favoured regions.

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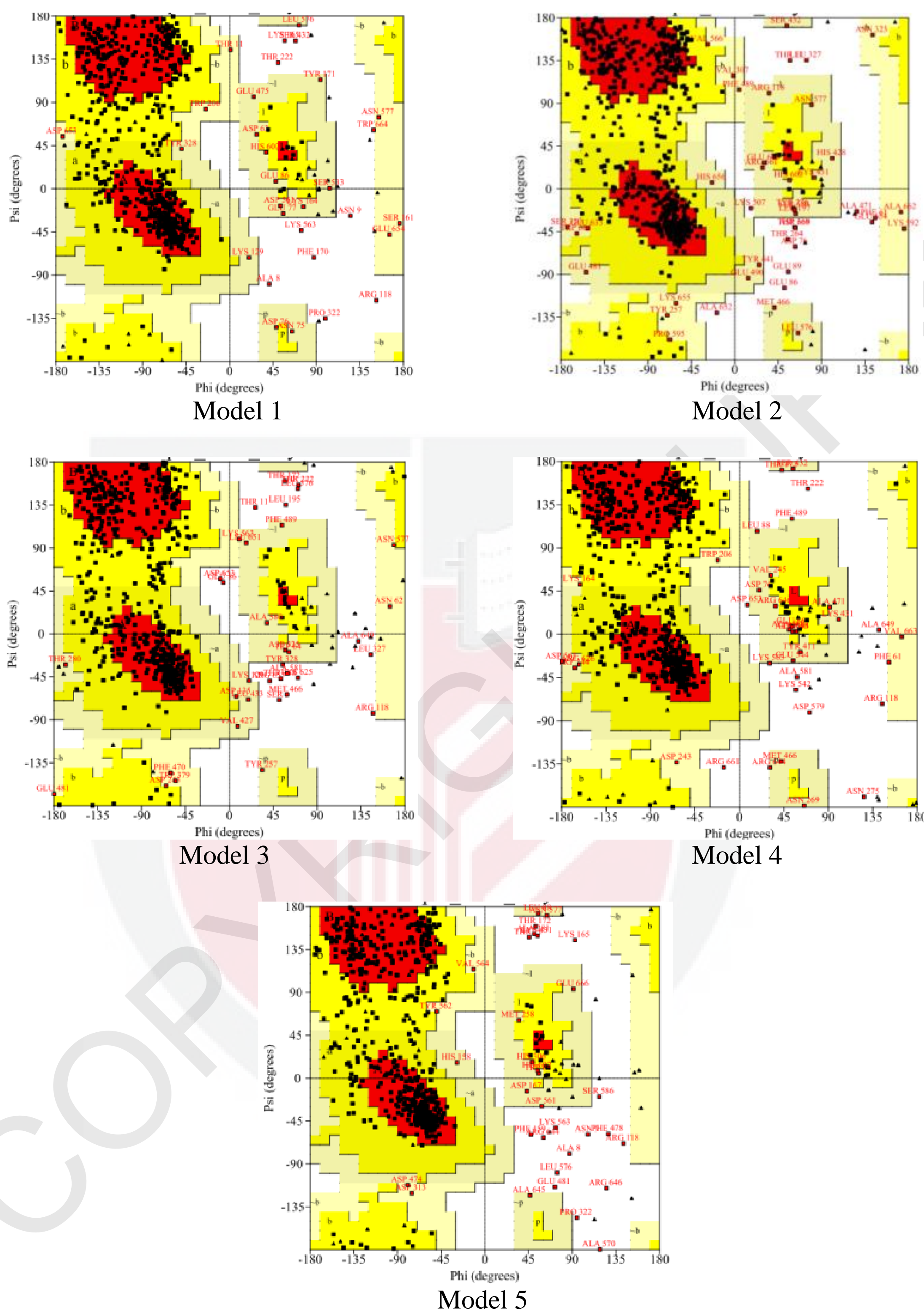

Figure 10. Ramachandran plots. These plots were produced by PROCHECK for the five models of GBE from *Geobacillus* sp. Geo5 predicted by I-TASSER. The red, dark yellow, light yellow and white regions represent the favoured, additionally allowed, generously allowed and disallowed region. ▲: glycine residues. ■: other amino acid residues.

Table 6. Summary of the PROCHECK analysis.

Models	Most favoured region (%)	Additionally allowed region (%)	Generously allowed region (%)	Disallowed region (%)
1	68.6	26.4	3.4	1.6
2	72.1	20.9	4.8	2.2
3	68.1	25.9	2.9	3.1
4	69.7	24.1	4.3	1.9
5	70.2	24.3	2.6	2.9

Note: the values represent the percentage of amino acid residues in respected regions.

Looking back at the predicted structures, I-TASSER suggested that the residues involved in the binding site of the enzyme are Try^{208}, His^{248}, Asp^{313}, Ala^{314}, Glu^{356} and Asp^{424}. Three of these residues, Asp^{313}, Glu^{356} and Asp^{424}, were the conserved residues that ought to be in charge of the catalysis reaction of the enzyme. The corresponding residues in *M. tuberculosis* branching enzyme are Asp^{411}, Glu^{464} and Asp^{532} (Pal *et al.*, 2010). These residues were located in model 1 of the predicted structure given by I-TASSER using a visualization tool, PyMOL (Figure 11).

These negatively charged amino acids in the catalytic cavity are important for sugar-protein interactions (Pal *et al.*, 2010; Abad *et al.*, 2002). As proposed by Pal *et al.* (2010), Glu^{356} (Glu^{464} in *M. tuberculosis* numbering) acts as the acid catalyst that protonates the oxygen of the 1,4-glycosidic bond while Asp^{313} (Asp^{411} in *M. tuberculosis* numbering) acts as the attacking nucleophile, which reacts with the OH group of C6 of the neighbouring glucose residue and thus forming the 1,6- glycosidic bond (refer to Figure 2, p. 11). Three other residues that are proposed by

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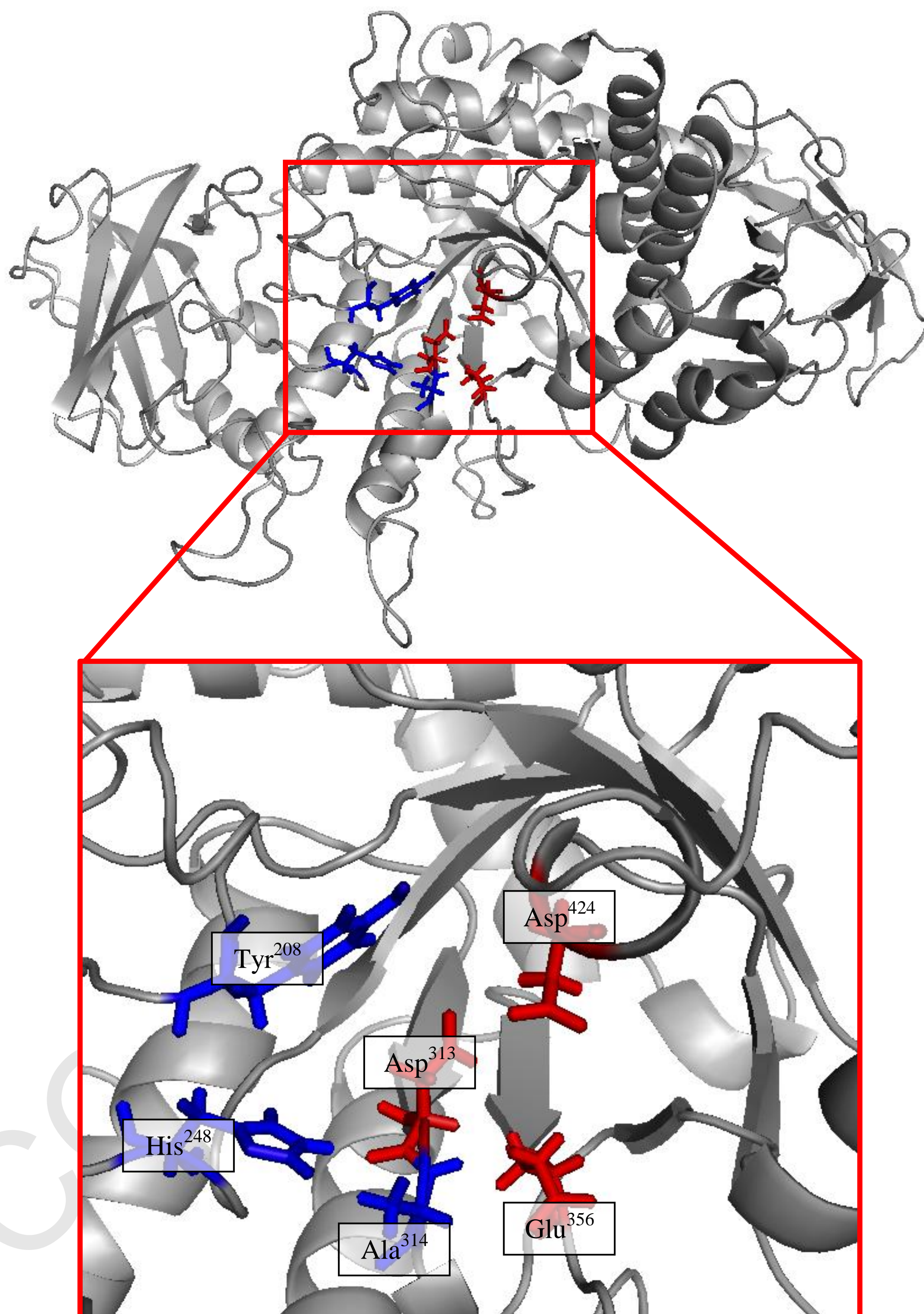

Figure 11. The catalytic site of glycogen branching enzyme from *Geobacillus* sp. Geo5. The protein structure was predicted by I-TASSER (model 1). The red coloured amino acids are the catalytic residues (Asp^{424}, Asp^{313} and Glu^{356}). The blue coloured amino acids are the other binding residues predicted by I-TASSER (Tyr^{208}, His^{248} and Ala^{314}).

I-TASSERT, Tyr^{208}, His^{248} and Ala^{314} are possibly involve in the substrate binding and specificity since they are located near the entrance of the catalytic α/β-barrel domain (Figure 11), and His^{248} is one of the conserved residues in region IV (Table 3, p. 57) (van der Maarel *et al.*, 2003; Abad *et al.*, 2002).

4.3 Culturing *Geobacillus* sp. Geo5

Geobacillus sp. Geo5 was subcultured in nutrient agar, thermus agar, nutrient broth, and thermus broth from the stock provided by MGI. On nutrient agar plate, the morphology of the *Geobacillus* sp. Geo5 colonies were round, mucoid and tranluscent white with a diameter of about 5 mm. These descriptions correlate with colony morphology of *Geobacillus* sp. (Nazina *et al.*, 2001; Zeigler, 2001).

4.4 Genomic DNA extraction of *Geobacillus* sp. Geo5

Genomic DNA of *Geobacillus* sp. Geo5 was successfully extracted using Qiagen DNeasy® Blood and Tissue Kit. The gel electrophoresis result showed that the DNA purified was pure as a single intact band was observed. The concentration of the purified genomic DNA was 50 µg/mL and the optical density ratio of $A_{260/280}$ was 1.88. The ratio of 1.8 to 2.0 means that the extracted genomic DNA was pure from contaminations, such as proteins and residual chemicals from the extraction protocol (Wilfinger *et al.*, 1997).

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4.5 Polymerase chain reaction of *glgB*

glgB was amplified from genomic DNA of *Geobacillus* sp. Geo5 by PCR. PCR was carried out using *Pfu* DNA polymerase, an enzyme found in the hyperthermophilic archaeon *Pyrococcus furiosus*. *Pfu* DNA polymerase has 3' to 5' exonuclease proofreading capability, which would produce blunt end products. This proofreading activity also makes PCR products by *Pfu* DNA polymerase having lower error rate that is 1 in 1.3 million base pairs compared to frequently used *Taq* DNA polymerase (isolated from *Thermus aquaticus*), 1 in 9000 base pairs (Cline *et al.*, 1996). Therefore, *Pfu* DNA polymerase was chosen as the PCR products would be suitable for directional TOPO cloning later on.

PCR primers were designed based on the open reading frame sequence of *glgB* obtained from genome mining. Based on the genome mining result, the expected size of the amplification product was 2013 bp. However, the result from PCR showed larger size than what was expected, that is around 3500 bp (Figure 12). From the gel electrophoresis result, it can be seen that the PCR products gave a single band, meaning that both forward and reverse PCR primers designed were specifically bound to the template and able to amplify the gene. Therefore, the PCR products were purified and sent for DNA sequencing.

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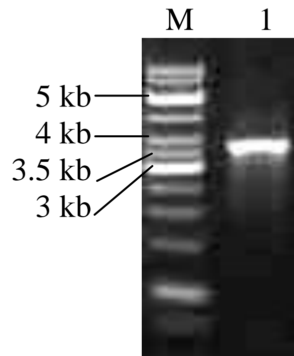

Figure 12. Gel electrophoresis of PCR products. M: GeneRuler 1 kb DNA Ladder. Lane 1: PCR product with the size of about 3.5 kb.

4.5.1 DNA sequencing of PCR product

Both forward and reverse PCR primers were used to sequence the purified PCR products. Due to the large size of the PCR product, additional two primers were designed to sequence the PCR product by primer walking. The forward primer (F4) was designed at nucleotides 647 to 664 and the reverse primer (R3) was designed at nucleotide 1346 to 1369.

Forward primer, F4: 5'– TGA CAA GCC GCT ATG GCA –3'

Reverse primer, R3: 5'– CCA TCA TGT AGC CAT ACA ATA GGC –3'

The results were analysed by aligning the DNA sequencing results with the expected gene sequence in Figure 8, p. 56. The sequencing result shows 1226 bp from the 5' end of the gene and 775 bp from 3' end of gene have 99% identities when compared to the putative gene sequence. However, the sequence in the middle of the gene, which made up of about 2000 bp, was foreign to the glycogen branching enzyme gene. The DNA sequencing results were BLAST in NCBI database and show that the foreign sequence was a transposase IS116/IS110/IS902 family protein (GenBank:

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ADU95388.1) (Appendix F). The BLAST result shows that this transposase was also present in *Geobacillus* sp. Y412MC52 and *Bacillus* sp. JF8. It is the insertion of transposase gene in glycogen branching enzyme gene that has caused the PCR product to have larger size than what was expected.

Transposase is an enzyme located at the end of a transposon that catalyses the movement of the transposon to another part of the genome (Lodish *et al.*, 2004). Transposon, also known as jumping gene, is a mobile DNA element that can move its location within the genome (Kulakov *et al.*, 1999). Transposon in bacterial is usually for antibiotic resistance or for disabling a certain gene. As in this case, transposon has to be removed as it would interfere with the translation process during protein synthesis and consequently the protein expressed would be structurally different and not functional.

4.6 Polymerase chain assembly

In order to remove the transposase that was inserted in the middle of the gene, three sets of PCR were carried out. Step 1 was to amplify the 5' end of the gene before the occurrence of the transposase, step 2 was to amplify the 3' end of the gene after the occurrence of the transposase and step 3 was to assemble those two steps of PCR. That final step is a technique called polymerase chain assembly (PCA) or assembly PCR. This method is generally used to construct genes synthetically and the PCR primers have to be designed very carefully to get the perfect gene sequence, as PCR is very specific and sensitive (TerMaat *et al.*, 2009; Wu *et al.*, 2006).

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The PCR products of step 1, step 2 and PCA gave the expected size of 1239 bp, 788 bp and 2013 bp, respectively (Figure 13). These PCR products were sent for DNA sequencing for verification. Primers F1, F4, R1 and R3 were used to sequence the PCR products. The DNA sequencing results were aligned with the predicted gene sequence (refer to Figure 8, p. 56) and it is confirmed that the gene, *glgB*, has been amplified without the transposase integration as the sequences matched the predicted gene sequence. The gene sequence was deposited to GenBank (accession number: KC951870).

4.7 Cloning of PCR products

The PCR product, which was the amplified *glgB*, was cloned into pET102/D-TOPO® vector in *E. coli* TOP10. To use this system, the PCR product must have 5' overhang that was made by adding four bases (CACC) to the forward primer, F3. The PCR product was directionally cloned into the vector as the 5' overhang of the PCR product annealed to the 3' overhang of the vector by topoisomerase. When the cloning reaction was spread on LB/Ampicillin plates, less than 10 colonies were observed. According to the manufacturer's instructions, it is expected that the colonies obtained would be 2 to 5-fold fewer compared to traditional bidirectional TOPO TA cloning and high-copy number TOPO® plasmids. This is because pET102/D-TOPO® vector has a pBR322 origin for low-copy replication. The manufacturer also stated that even though only a few colonies were obtained, more than 90% of these colonies contain plasmid with the gene insert in the correct orientation. All these colonies were isolated and subcultured in LB/Ampicillin broths. The recombinant plasmids were extracted and used for further analysis.

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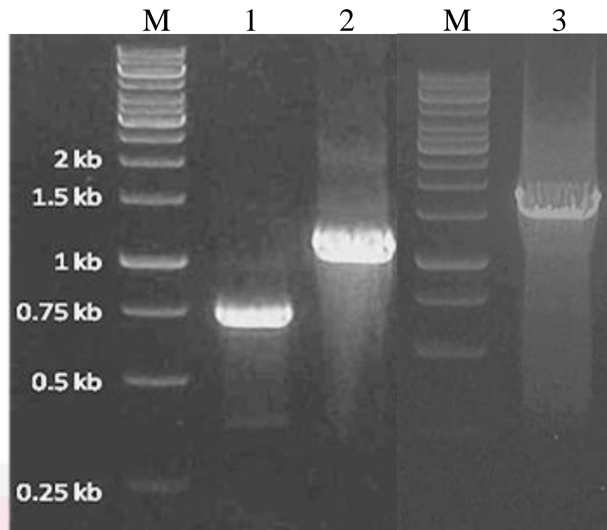

Figure 13. Gel electrophoresis of PCR and PCA products. M: GeneRuler 1 kb DNA Ladder. Lane 1: Second step PCR product. Lane 2: First step PCR product. Lane 3: PCA product.

4.8 Analysing transformed recombinant plasmids

Recombinant plasmid DNA was purified from 5 mL LB/Ampicillin broth and analysed to ensure that the gene was successfully cloned into the vector in the correct orientation. PCR was done using pET/GBE as the DNA template. Two sets of PCR primers were used, set 1 (PCR primers, F1 and R1) and set 2 (TrxFus forward and T7 reverse). The expected sizes of the amplification product were 2013 bp and 2249 bp, respectively. The PCR product using the primers provided by the kit was larger as these primers would anneal to the sites on the vector and therefore would amplify some part of the vector together with the gene (Appendix C). The results from agarose gel electrophoresis meet the expected sizes (Figure 14).

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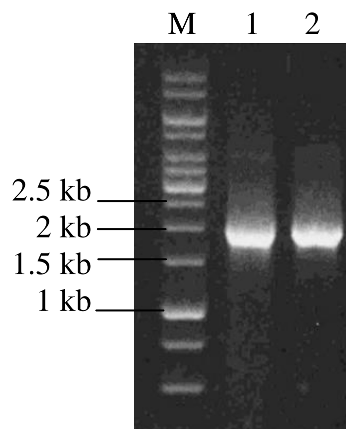

Figure 14. Gel electrophoresis of PCR product from pET/GBE. M: GeneRuler 1 kb DNA Ladder. Lane 1: PCR product using PCR primers from set 1. Lane 2: PCR product using primers from set 2.

Another way to analyse the recombinant plasmid was done by restriction enzyme digestion. Restriction enzymes would digest specific sites on the DNA and the digestion pattern could be observed from gel electrophoresis. Plasmid DNA might exist in circular, nicked, supercoiled and linear form. Each form of DNA would migrate at a different rate in gel electrophoresis although they have the same size. The digested plasmid DNA would have a linear DNA structure and therefore the estimation of the DNA size on the gel electrophoresis would be better compared to circular plasmid DNA. This is because the DNA ladder used as the size guideline is also in linear form. To see if the cloned plasmid harbours the gene of interest, *glgB*, restriction enzymes *Pst*I and *Bgl*II was used.

The agarose gel electrophoresis results show the expected patterns of the pET/GBE digestion where single a digestion with *Pst*I was 8328 bp and double digestion with *Pst*I and *Bgl*II gave the products of 3839 bp and 4489 bp (lane 2 and 3 in Figure 15). Instead, single digestion of an empty plasmid gave the size of

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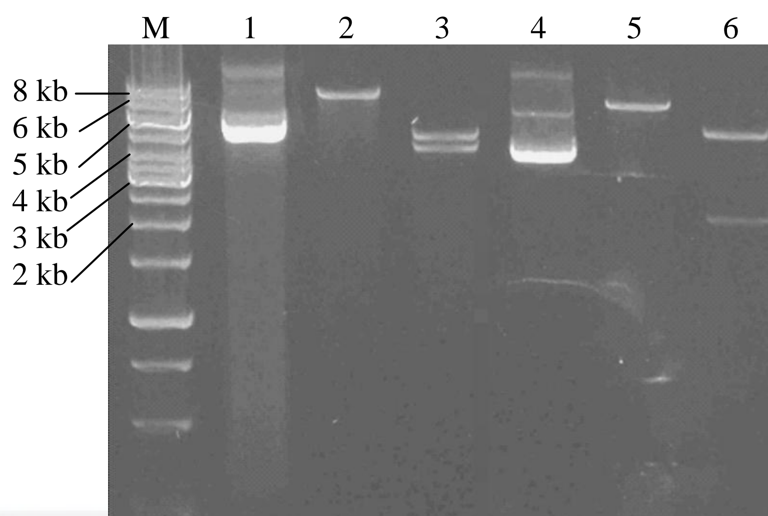

Figure 15. Gel electrophoresis of restriction enzyme digestion of pET/GBE. M: GeneRuler 1 kb DNA Ladder. Lane 1: Purified pET/GBE, undigested. Lane 2: Recombinant plasmid digested with *Pst*I. Lane 3: Recombinant plasmid digested with *Pst*I and *Bgl*II. Lane 4: undigested pET102. Lane 5: pET102 digested with *Pst*I. Lane 6: pET102 digested with *Pst*I and *Bgl*II.

6315 bp and double digestion gave fragments of 1826 bp and 4489 bp (lane 5 and 6 in Figure 15).

To confirm further, the recombinant plasmids were sent for DNA sequencing as mentioned in section 3.11.3. The results were analysed by aligning the DNA sequencing result with the predicted gene sequence using ClustalW (Figure 8, p. 56). The results show that the gene was successfully integrated into the vectors and in the correct orientation with the reading frame.

4.9 Expression of GBE in *Escherichia coli*

GBE was expressed in *E. coli* system. There were two *E. coli* expression systems studied in this research, that is, intracellular expression and secretory expression. In

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intracellular expression, the expressed G5GBE would be accumulated in the cell cytoplasm, while in secretory expression, G5GBE would be secreted to the extracellular environment, that is the culture medium, with the help from BRP. Since the intracellular expression produced higher enzyme expression in shorter time, therefore the enzyme produced by this system was chosen to be purified and used for enzyme characterisation.

4.9.1 Intracellular expression of G5GBE

Recombinant plasmid was transformed into *E. coli* BL21 Star™ (DE3) to express the GBE. Protein expression using expression vector pET102/D-TOPO® is regulated by a strong bacteriophage T7 promoter that have a *lac* operator sequence. For the gene of interest to be expressed, T7 RNA polymerase that was supplied by *E. coli* BL21 Star™ (DE3) will bind to the promoter and initiate the transcription of the gene of interest. Three parameters that prominently affect the level of protein expression have been optimised to achieve a high level of protein expression; the inducer concentration, induction temperature and induction time (Berrow *et al.*, 2006; Baneyx, 1999; Markrides, 1996).

4.9.1.1 Effect of IPTG concentration

Isopropylthio-β-galactoside (IPTG) was used as the inducer that allows the expression of T7 RNA polymerase. T7 RNA polymerase is very selective for T7 promoter that is naturally absent in *E. coli* (Studier and Moffatt, 1986). Therefore it can be used to regulate the expression of the gene of interest. The *E. coli* BL21

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Star^{TM} (DE3) harbouring pET/GBE was induced with different concentrations of IPTG up to 1 mM of final concentration to optimise GBE production (Figure 16). Expression was done for 12 hours of induction at 37°C.

The expression was found to be at the highest level when induced with 0.75 mM IPTG with the enzyme activity of 50.18 U/mL and went down to 48 U/mL when the IPTG was increased to 1 mM. The activity, which indicates the amount of active enzyme produced, was slightly lower when 1 mM of IPTG was used. This might due to the inclusion bodies formation. Inclusion bodies are the proteins that are incorrectly folded due to high rate of protein synthesis (Gonzalez-Montalban *et al.*, 2007; Berrow *et al.*, 2006). The possibility of protein to misfold is high with the use of strong promoters and also with high inducer concentration (Baneyx and Mujacic, 2004). Basal expression was detected since 12 U/mL of branching enzyme activity was observed during the absence of inducer. This basal expression is common in T7 promoter and will not cause any problem unless the recombinant protein expressed is toxic and that may cause plasmid instability or even cell death (Studier and Moffatt, 1986).

The expressed protein was visualised in SDS-PAGE (Figure 17). There are obvious bands near the 116 kDa marker that could be G5GBE as the expected size of the fusion protein was 91 kDa. The band appears the brightest when expression was induced with 0.75 mM, but the expression level of active enzyme cannot be determined accurately. Therefore the expression level had to be confirmed by doing enzyme assay.

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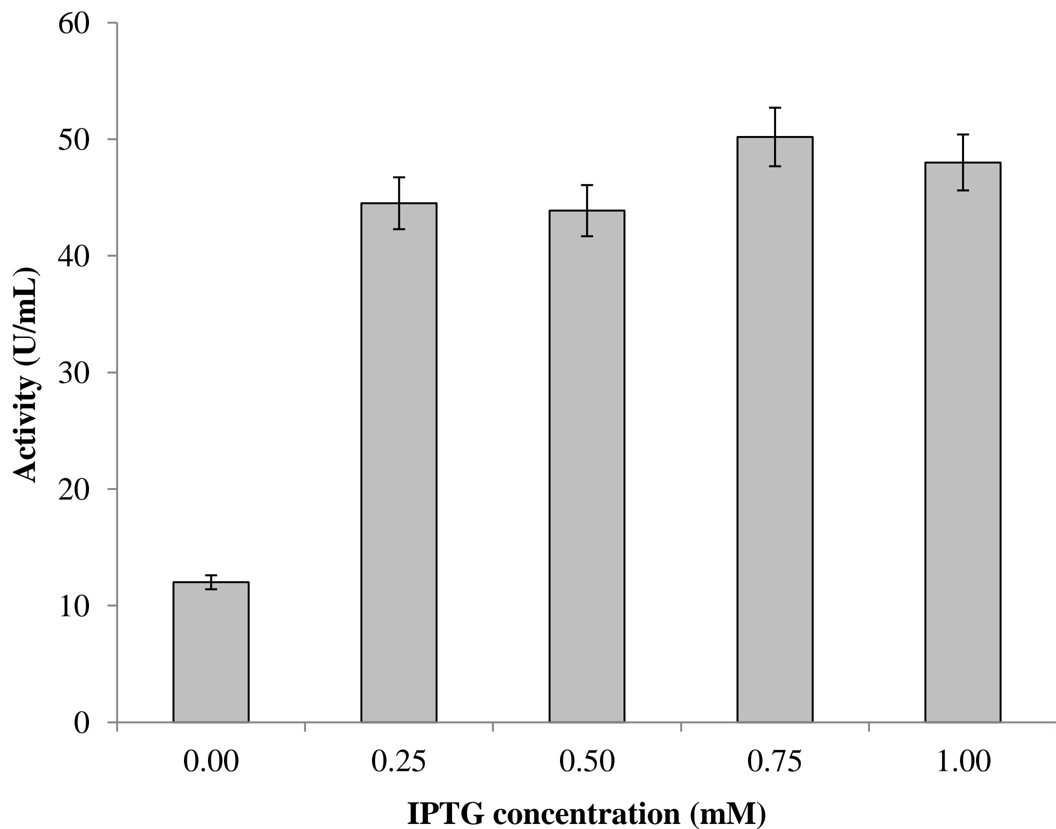

Figure 16. Expression of G5GBE by *Escherichia coli* BL21 Star^{TM} (DE3) when induced with different concentrations of IPTG. Note: Data represents mean ± SE (n=3).

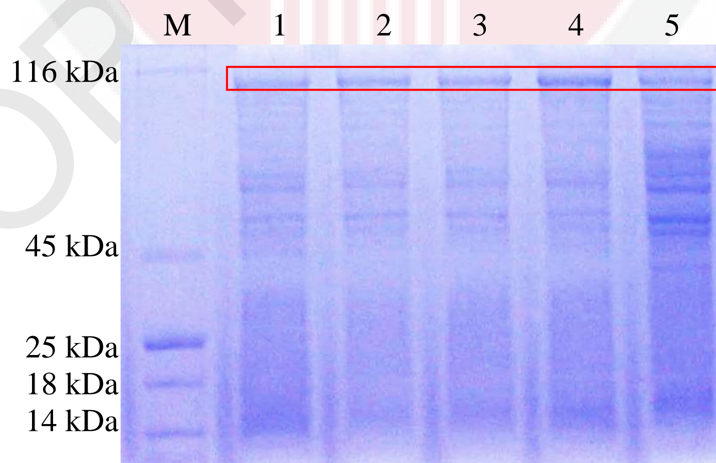

Figure 17. SDS-PAGE of expressed G5GBE when induced with different concentrations of IPTG. The red box represents the expected protein, which is 91 kDa. M: Unstained protein molecular weight marker (Fermentas). Lane 1 to 5: The expression induced with concentration of IPTG of 0 mM, 0.25 mM, 0.5 mM, 0.75 mM and 1.0 mM, respectively.

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4.9.1.2 Effect of induction temperature

To investigate the effect of induction temperature on the level of active enzyme production, expression was induced with 0.75 mM of IPTG for 12 hours. The effect of induction temperature on G5GBE expression is shown in Figure 18. Cultivation at 30°C resulted in the best of G5GBE production where 51.7 U/mL of enzyme activity was detected. Cultivation at 37°C however hampered the expression level of active enzyme to 48.6 U/mL.

The induction temperature is known to affect the synthesis of recombinant protein. The growth of *E. coli* is optimum at 37°C. The cells grow rapidly at this temperature and therefore the rate of recombinant protein expression is high. However, the rapid protein expression at this temperature may cause the expressed protein to misfold and aggregates (Baneyx and Mujacic, 2004). The misfolded proteins, also called as inclusion bodies, are not active. As a result, expression level of active enzyme, which is determined by enzyme activity, at temperature above 30°C is usually lower when compared to expression level at lower temperature. The reason behind this is at lower temperature, the synthesis rate is slower and that would promote proper protein folding. Nonetheless, expression at temperature lower than 20°C seems to have no significant effect (Berrow *et al.*, 2006). This phenomenon was also observed in the expression of preS2-S'-β-galactosidase in *E. coli* where the level of active enzyme obtained was higher when the induction temperature was shifted from 37°C to 25°C and 20°C (Vasina and Baneyx, 1996). Thus, for the purpose of this research, the cultivation temperature of 30°C was chosen for further optimisation study. The expressed proteins were visualised in SDS-PAGE (Figure 19). The bands that

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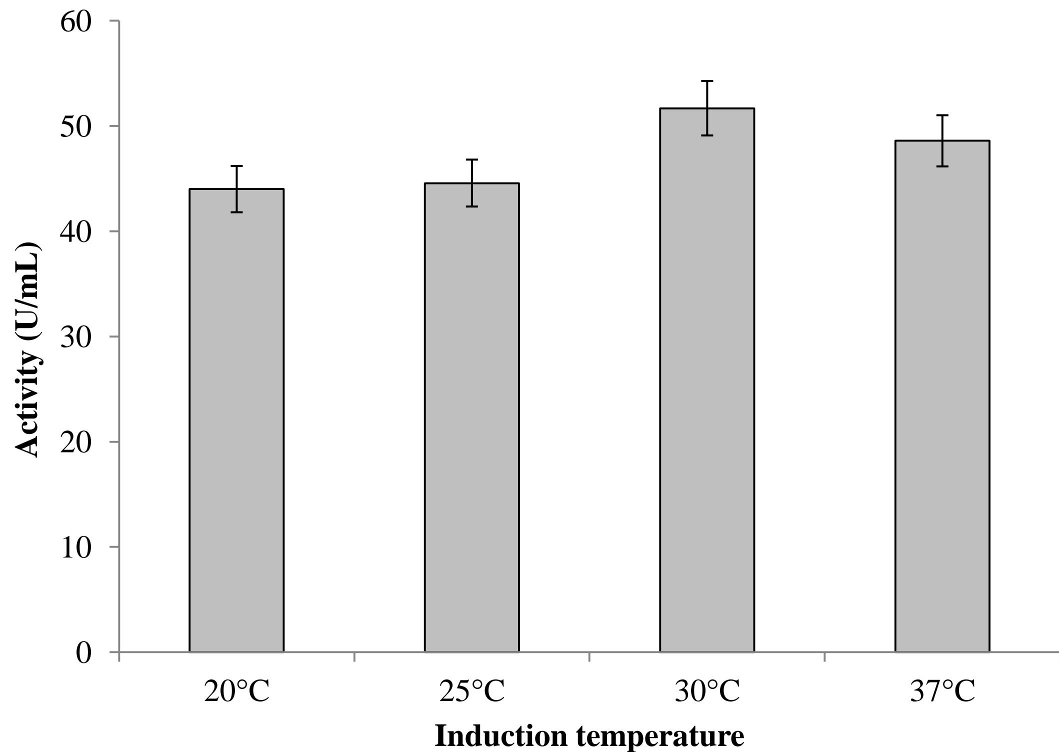

Figure 18. Expression of G5GBE by *Escherichia coli* BL21 Star™ (DE3) when induced at different temperatures. Note: Data represents mean ± SE (n=3).

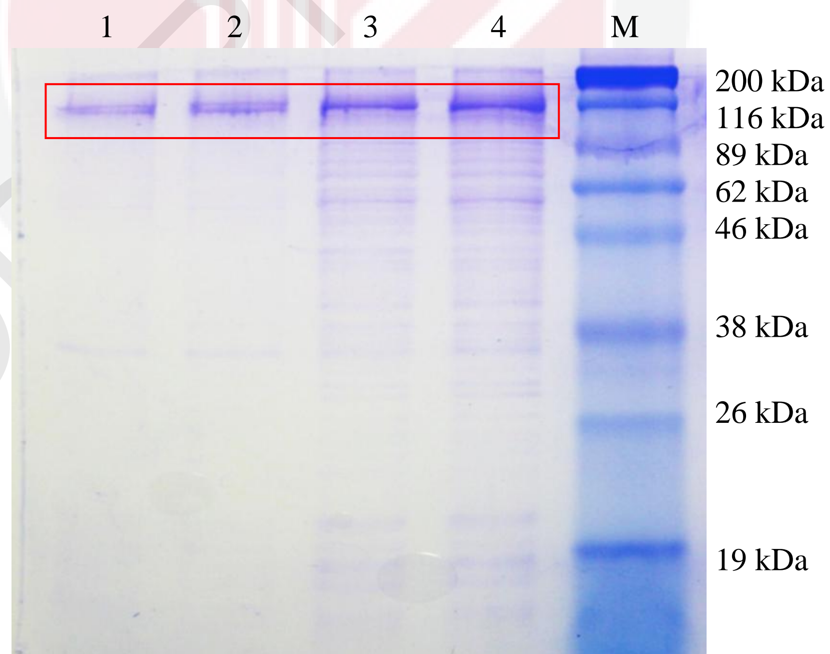

Figure 19. SDS-PAGE of expressed G5GBE when induced at different temperatures. The red box represents the expected size of the protein, which is 91 kDa. M: Broad Range Prestained Protein Marker (Nacalai). Lane 1 to 4: The expression induced at temperature of 20°C, 25°C, 30°C and 37°C, respectively.

represent expression at 30°C and 37°C appear to be thicker than expression at 20°C and 25°C. However, the SDS-PAGE result is not as conclusive as the enzyme assay.

4.9.1.3 Effect of induction time

The effect of induction time was determined by inducing the culture with 0.75 mM of IPTG at 30°C. The expression of active enzyme and cell growth was determined every 4 hours for 28 hours (Figure 20). Basal protein expression was observed as enzyme activity was detected at 0 hour of induction. The expression increased almost 2-fold from 17.7 U/mL to 32.5 U/mL after 4 hours of induction. The enzyme activity continued to increase drastically to 39.6 U/mL when expression was induced for 8 hours. The expression reached a maximum level at 12 hours of induction with the activity of 41.6 U/mL.

Even that so, 8 hours was chosen as the optimum expression level with enzyme activity of 39.6 U/mL as the growth curve shows that the culture was still in log phase at that hour and already gone to stationary phase at 12 hours. Moreover, there was not much difference in enzyme activity at 8 hours and 12 hours. As the growth entered the stationary phase after 12 hours of induction, the expression level gradually dropped. This is probably caused by the depletion of oxygen and nutrient in the medium and to adapt to that condition, the cells focus on their metabolisms to maintain their viability. Therefore, protein expression is decreased (Sezonov *et al.*, 2007; Studier, 2005).

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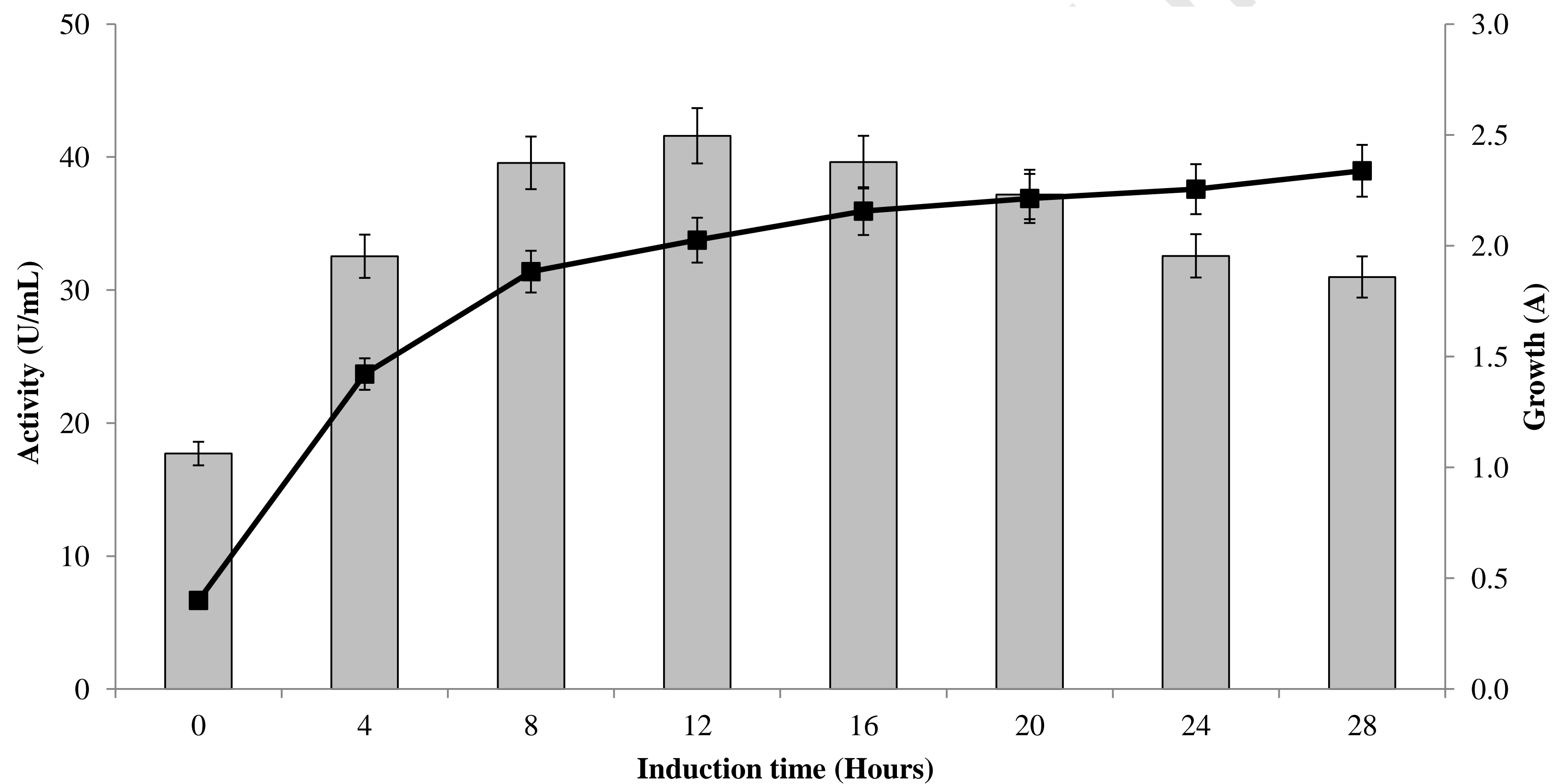

Figure 20. Expression of G5GBE by *Escherichia coli* BL21 Star^{TM} (DE3) when induced up to 28 hours and the growth curve of the *Escherichia coli*. ( ) is the enzyme activity and ( ) indicates the bacterial growth. Note: Data represents mean ± SE (n=3).

4.9.2 Secretory expression of G5GBE

When both pET/GBE and pSW1 were expressed in the same host (*E. coli* BL21 (DE3) Star^{TM}), BRP, which was expressed by pSW1 vector, would form permeable zones allowing the secretion of GBE into the extracellular environment (van der Wal *et al.*, 1995).

4.9.2.1 Analysis of positive transformants

To confirm that the host cells are harbouring pET/GBE and pSW1 vector, the positive transformants were analysed by digesting the purified plasmids with restriction enzyme *Hin*dIII so that the plasmids became linear. Linear plasmids would give better accuracy of size estimation on gel electrophoresis compared to circular plasmids. From the digestion reactions, two bands are seen on the gel electrophoresis result (Figure 21). The sizes of the bands meet the expectation, 8.3 kb for pET/GBE and 4 kb for pSW1 vector.

4.9.2.2 Quasi-lysis test

Growth of transformed cells in media containing the appropriate antibiotics for selection did not confirm that pSW1 vector would be expressing a functional BRP. The vectors that were transformed into the host cells have to be checked for inducible BRP function using a quasi-lysis test (van der Wal *et al.*, 1995). The basis of this test is the decline in culture turbidity and cell viability when BRP is expressed in the absence of divalent metal ions (van der Wal *et al.*, 1998).

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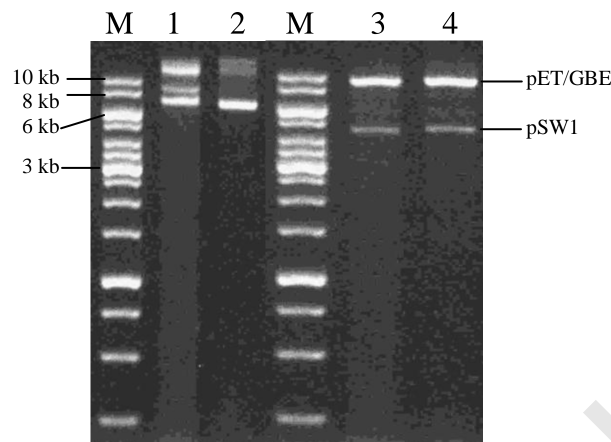

Figure 21. Gel electrophoresis of restriction enzyme digestion of pSW1 and pET/GBE. M: GeneRuler 1 kb DNA Ladder. Lane 1 and 2: Undigested pSW1 vector and pET/GBE. Lane 3 and 4: pSW1 and pET/GBE digested with *Hin*dIII.

In Figure 22, it is shown that BRP was expressed and functional as the fully induced culture declined in turbidity right after an hour of induction. The growth of the culture induced with 20 ng/mL of mitomycin C also declined but after 4 hours of induction. The control culture, which harboured pSW1 vector but was not induced with mitomycin C, continued to grow during the 6 hours studied period. These results show that induction with mitomycin C affects the expression of BRP and consequently hampers the cell growth by inducing cell lysis.

4.9.2.3 Effect of mitomycin C concentration on secretory expression

The *E. coli* BL21 Star^{TM} (DE3) harbouring both pET/GBE and pSW1 vector was induced with both IPTG and mitomycin C at the same time when optical density at A_{600} reached 0.5. In order to see the effect of mitomycin C on secretory

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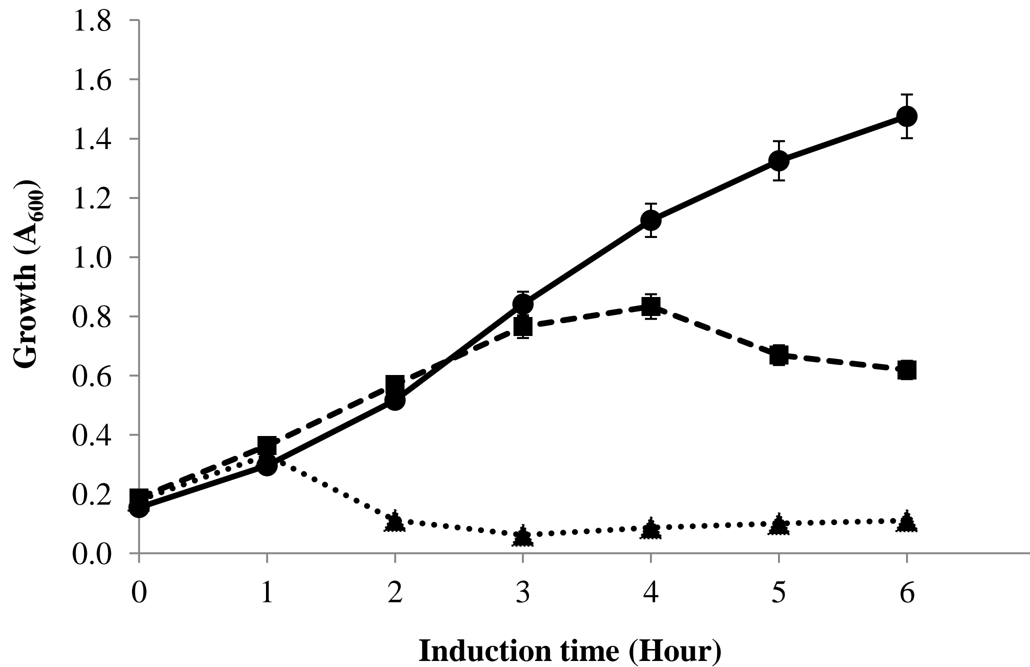

Figure 22. Quasi-lysis test done on *Escherichia coli* BL21 (DE3) Star™ harbouring pSW1 and pET/GBE. The symbols used: (—●—), the culture was not induced with mitomycin C; (- -■- -), the culture was induced with 20 ng/mL of mitomycin C; (•••▲••), the culture was induced with 500 ng/mL of mitomycin C. Note: Data represents mean ± SE (n=3).

expression, the concentration of IPTG was kept constant at 0.75 mM. Induction was done at 30°C for 12 hours. A control for expression was done with the expression host that has BRP plasmid in absent and was only carrying pET/GBE. Referring to Figure 23, the best concentration of mitomycin C to express GBE extracellularly was 5 ng/mL where the activity was 34.4 U/mL. However, the enzyme activity was almost the same when compared to induction with 10 ng/mL and 15 ng/mL of mitomycin C where the activity was 33 U/mL and 32.7 U/mL, respectively. Enzyme activity decreased to 25 U/mL when expression was induced with of 20 ng/mL and 25 ng/mL of mitomycin C. The decreased in activity is probably due to high concentration of mitomycin C kills the cells and therefore less

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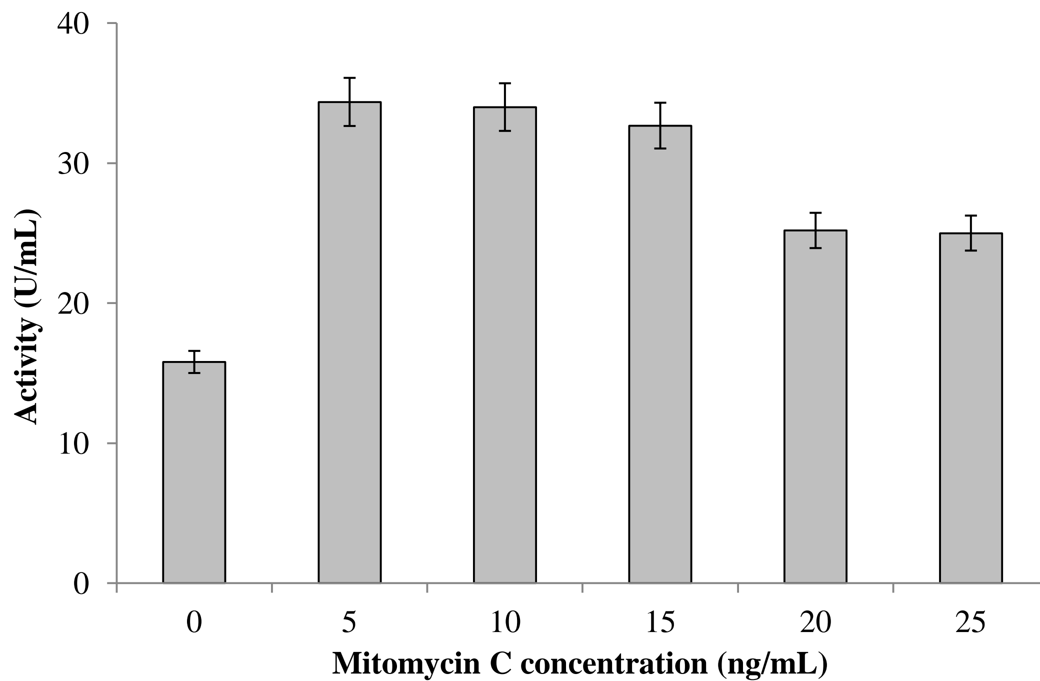

Figure 23. Effect of mitomycin C concentration on secretory expression of G5GBE. Note: Data represents mean ± SE (n=3).

amount of protein was produced (van der Wal *et al.*, 1995). The expression of BRP was not tightly regulated and therefore the basal activity can be observed where 15.8 U/mL of enzyme activity was detected when expression was not induced. Extracellular activity was not observed when expression was done for the expression host that harbours only pET/GBE, without the BRP plasmid. Therefore, it can be concluded that the secretory expression is solely contributes by the expression of BRP by pSW1 vector.

The total enzyme expression of intracellular and extracellular, and the growth cell culture were then analysed every 4 hours for 28 hours of induction time (Figure 24). The expression was induced with the optimum concentration of mitomycin C for the secretion of GBE that is 5 ng/mL. The control in this study was expression where

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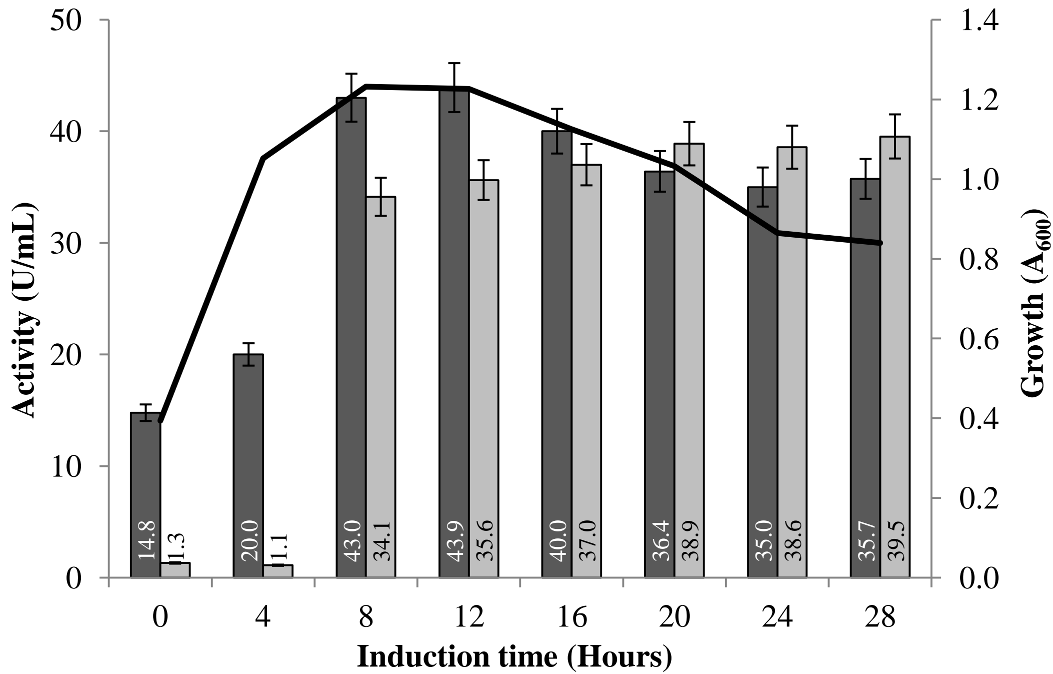
A



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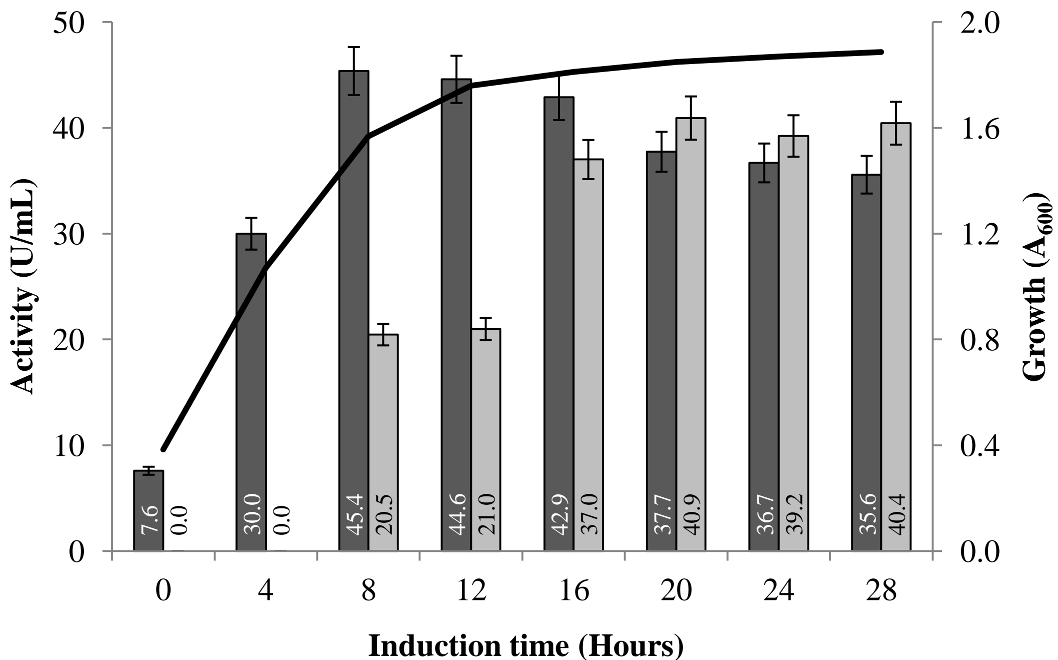

Figure 24. Secretory expression of G5GBE. A: Secretory expression was induced with 5 ng/mL of mitomycin C. B: Secretory expression was not induced with mitomycin C as a control. The symbols used: (■), enzyme activity from intracellular protein; (■), enzyme activity from extracellular protein; (▬), bacterial growth. The values inside the bars are the values of Y-axis, activity (U/mL). Note: Data represents mean ± SE (n=3).

only pET/GBE was induced while the BRP plasmid was not (Figure 24B). The protein started to be secreted into the extracellular environment after 8 hours of induction when expression was induced with mitomycin C. The extracellular enzyme activity was 34.1 U/mL, which is 66% more than activity obtained when the expression was not induced with mitomycin C, 20.5 U/mL. The secretion of enzyme increased gradually parallel with the induction time. The highest expression was achieved at 20 hours of induction with the enzyme activity of 39.5 U/ mL.

As shown in Figure 24A, the induced expression was going down after 8 hours. This indicates that cell lysis has occurred (van der Wal *et al.*, 1995). The effect of this cell lysis was reflected in the enzyme expression as the total of intracellular and extracellular enzyme activity decreased. As more cells died, fewer amount of enzyme was produced. As seen in Figure 24A, although secretory expression was induced, the enzyme was still entrapped in the cell since intracellular expression was present. However, the intracellular enzyme became lessen as time prolonged while extracellular enzyme increase. The cell lysis, which represented in the drop of the growth curve, promotes the release of recombinant protein into the culture media (van der Wal *et al.*, 1995).

As for the control (Figure 24B), the total activity (79.9 U/mL) was higher compared to the induced expression (77.0 U/mL) when induction was done for 16 hours and more. The reason behind this is that the total viable cells are more in the non-induced expression compared to the induced expression where the cell growth was declining. Hence, the amount of protein produced by the control expression, which was detected as the enzyme activity, was higher. Although the control expression was not

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induced with mitomycin C, the expressed protein was still secreted out of the cell. As mentioned before, this is the basal expression of BRP since the expression of BRP is not tightly regulated (van der Wal *et al.*, 1995). In conclusion, secretory expression using pSW1 vector was not very successful in secreting all the expressed recombinant protein to the extracellular environment.

4.10 Purification of G5GBE

G5GBE produced by pET102/D-TOPO® expression vector has His-Patch thioredoxin fused to the protein. His-Patch thioredoxin is a mutated thioredoxin that have metal binding domain, which has been shown to have high affinity for divalent cations. Therefore the fusion protein can be purified using metal chelating resins like nickel sepharose. Fusion GBE was purified using HisTrap HP column by affinity chromatography performed using Äkta Explorer system (GE Healthcare). As presented in the chromatogram, the enzyme was eluted at about 25%B, which means 25% of elution buffer (Figure 25). The pooled fractions (fraction B5 and B6) was run in SDS-PAGE and assayed for enzyme activity and protein content. As shown in Table 7, the recovery obtained was 84% with a purification fold of 10. Figure 26 shows the SDS-PAGE result where a single band is observed for the purified enzyme (lane 3). This means that the enzyme was successfully purified. It is important to purify the enzyme before proceeds to enzyme characterisation to ensure the accuracy and validity of the results because the contaminants might affect the enzyme activity and stability.

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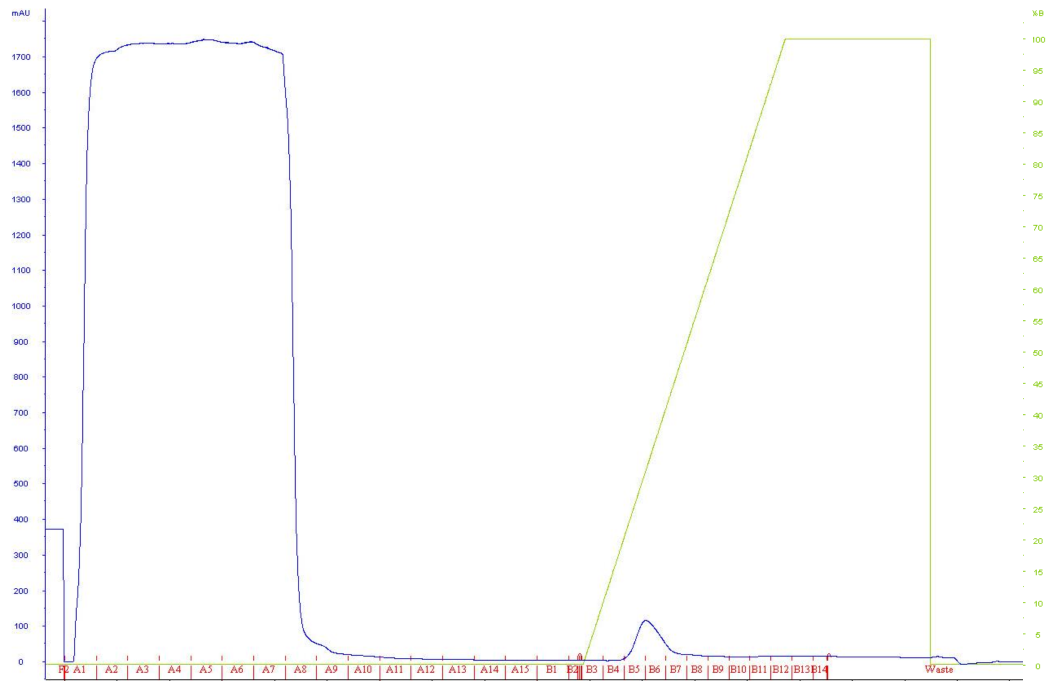

Figure 25. Chromatogram of affinity chromatography performed using Äkta Explorer. The blue line indicates the absorbance reading at A_{280} in milli absorbance unit (mAU) and the green line indicates the %B (% of elution buffer).

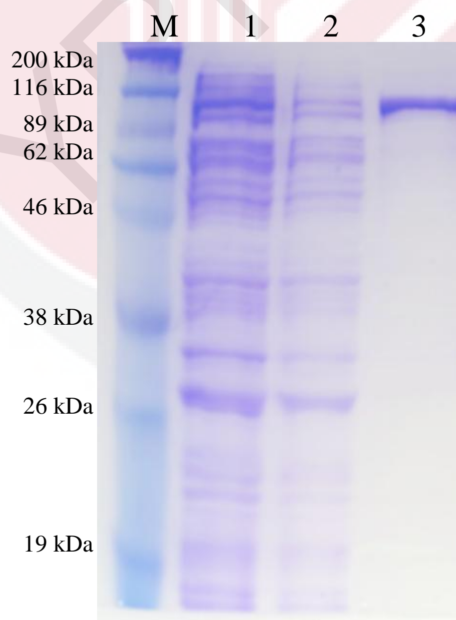

Figure 26. SDS-PAGE of purified enzyme. M: Broad Range Prestained Protein Marker (Nacalai). Lane 1: Crude enzyme. Lane 2: Protein in flowthrough fractions. Lane 3: Purified enzyme.

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Table 7. Purification of GBE from *Geobacillus* sp. Geo5 using affinity chromatography.

Sample	Total protein (mg)	Total activity (u)	Specific activity (u/mg)	Purification fold	Recovery (%)
Cell extract	4.86	1314.50	270	1	100
Purified GBE	0.43	1105.28	2598	10	84

4.10.1 MALDI TOF/TOF analysis

To verify that the protein purified was GBE, SDS-PAGE gel band was excised and sent for MALDI TOF/TOF analysis to First BASE Laboratories Sdn Bhd. The protein sample was digested by trypsin and the peptides were analysed by MALDI TOF/TOF mass spectrometer using a 4800 Proteomics Analyzer (AB Sciex). Each peptide was fragmented within the mass spectrometer to produce ions that would give information on the amino acid sequence (Gogichaena, *et al*., 2007). The spectra of each peptide ion data was analysed and matched to possible amino acid sequences in the database using Mascot sequence matching software (Matrix Science) and Ludwig NR Database. The result confirmed that the sample was from *glgB* or the 1,4-alpha-glucan branching enzyme. The peptide sequences showed two hits in the database. The hits matched the branching enzyme from *Geobacillus* sp. (strain Y412MC52) and *Geobacillus thermoleovorans* CCB_US3_UF5 (Figure 27). The peptide sequences also matched with the amino acid sequence of GBE from *Geobacillus* sp. Geo5 with the sequence coverage of 36%.

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Geobacillus sp. Geo5. Protein sequence coverage: 36%

```
  1  MRSSLIAANP  TDLEVYLFHE  GRLYQSYELF  GAHVIRDGGA  VGTRFCVWAP
 51  HAREVRLVGS  FNDWNGANSP  LTKVNDEGVW  TIVVPENLEG  HLYKYEIITP
101  DGRVLLKADP  YAFYSELRPH  TASIVYDLKG  YEWNDSSWQR  KKRRKRIYDQ
151  PMVIYELHFG  SWKKKPDGRF  YTYREMADEL  IPYVLERGFT  HIELLPLVEH
201  PLDRSWGYQG  TGYYSVTSRY  GTPHDFMYFV  DRCHQAGLGV  IIDWVPGHFC
251  KDAHGLYMFD  GAPTYEYANE  KDRENYVWGT  ANFDLGKPEV  RSFLISNALF
301  WLEYYHVDGF  RVDAVANMLY  WPNNDRLYEN  PYAVEFLRQL  NEAVFAYDPS
351  VLMIAEDSTD  WPRVTAPTYD  GGLGFNYKWN  MGWMNDMLKY  METPPHERKY
401  AHNQVSFSLL  YAYSENFILP  FSHDEVVHGK  KSLLNKMPGS  YEEKFAQLRL
451  LYGYMMAHPG  KKLLFMGSEF  AQFDEWKFEG  ELDWVLFDFE  LHRKMDEYVK
501  QLIACYKRYK  PFYELDHDPR  GFEWIDVHNA  EQSIFSFIRR  GKKEGDVLVI
551  VCNFTNQAYD  DYKVGVPLLA  PYREVLNSDA  AEFGGSGHVN  GKRLPAFHEP
601  FHGKPYHVRM  TIPPFGISIL  RPVQKRGERK  QNEEEVHRHV  IGRRARKPAS
651  LADEKHRETG  RAVWGEVPDH
```

Geobacillus sp. (strain Y412MC52). Protein sequence coverage: 32%

```
  1  MYGHHFASER  MRSSLIAANP  TDLEVYLFHE  GRLYQSYELF  GAHVIRDGGA
 51  VGTRFCVWAP  HAREVRLVGS  FNDWNGANSP  LTKVNDEGVW  TIVVPENLEG
101  HLYKYEIITP  DGRVLLKADP  YAFYSELRPH  TASIVYDLKG  YEWNDSSWQR
151  KKRRKRIYDQ  PMVIYELHFG  SWKKKPDGRF  YTYREMADEL  IPYVLERGFT
201  HIELLPLVEH  PLDRSWGYQG  TGYYSVTSRY  GTPHDFMYFV  DRCHQAGLGV
251  IIDWVPGHFC  KDAHGLYMFD  GAPTYEYANE  KDRENYVWGT  ANFDLGKPEV
301  RSFLISNALF  WLEYYHVDGF  RVDAVANMLY  WPNNDRLYEN  PYAVEFLRQL
351  NEAVFAYDPN  VLMIAEDSTD  WPRVTAPTYD  GGLGFNYKWN  MGWMNDMLKY
401  METPPHERKY  AHNQVSFSLL  YAYSENFILP  FSHDEVVHGK  KSLLNKMPGS
451  YEEKFAQLRL  LYGYMMAHPG  KKLLFMGSEF  AQFDEWKFEG  ELDWVLFDFE
501  LHRKMDEYVK  QLIACYKRYK  PFYELDHDPR  GFEWIDVHNA  EQSIFSFVRR
551  GKKDGDLLVI  VCNFTNQAYD  DYKVGVPLLA  PYREVLNSDA  AEFGGSGHVN
601  GKRLPAFSEP  FHGKPYHVRM  TIPPFGISIL  RPVQKRGERK  QNEEEVHRHV
651  IGRRARKPAS  LADEKHRETS  RAVWGEVPDH
```

Geobacillus thermoleovorans CCB_US3_UF5. Protein sequence coverage: 33%

```
  1  MIAANPTDLE  VYLFHEGRLY  QSYELFGAHV  IRGGGAVGTR  FCVWAPHARE
 51  VRLVGSFNDW  NGANSSLTKV  NDEGVWTIVV  PENLEGHLYK  YEIITPDGRV
101  LLKADPYAFY  SELRPHTASI  VYDLKGYEWN  DSSWQRKKRR  KRIYDQPMVI
151  YELHFGSWKK  KPDGRFYTYR  EMADELIPYV  LERGFTHIEL  LPLVEHPLDR
201  SWGYQGTGYY  SVTSRYGTPH  DFMYFVDRCH  QAGLGVIIDW  VPGHFCKDAH
251  GLYMFDGAPT  YEYANEKDRE  NYVWGTANFD  LGKPEVRSFL  ISNALFWLEY
301  YHVDGFRVDA  VANMLYWPNN  DRLYENPYAV  EFLRQLNEAV  FAYDPNVWMI
351  AEDSTDWPRV  TAPTYDGGLG  FNYKWNMGWM  NDMLKYMETP  PHERKYAHNQ
401  VSFSLLYAYS  ENFILPFSHD  EVVHGKKSLL  NKMPGSYEEK  FAQLRLLYGY
451  MMAHPGKKLL  FMGSEFAQFD  EWKFAEELDW  VLFDFELHRK  MDEYVKQLIA
501  CYKRYKPFYE  LDHDPRGFEW  IDVHNAEQSI  FSFIRRGKKE  GDVLVIVCNF
551  TNQAYDDYKV  GVPLLAPYRE  VLNSDAAEFG  GSGHVNGKRL  PAFSEPFHGK
601  PYHVRMTIPP  FGISILRPVQ  KRGERKQNEE  EVHRHVIGRR  ARKPASLADE
651  KHRETSRAVW  GEVPDH
```

Figure 27. Hits from the MALDI TOF/TOF analysis. Matched peptides shown in red.

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4.11 Biochemical characterisation of purified GBE

Three basic biochemical properties (temperature, pH and metal ions) of fusion GBE were characterised. GBE, which was isolated from a thermophilic bacterium, *Geobacillus* sp. Geo5, was stable and active at high temperature. Apart from temperature, pH and metal ions too affected the enzyme activity.

4.11.1 Effect of temperature on GBE activity and stability

As seen on Figure 28, GBE was generally active at 45°C to 60°C as more than 80% of GBE activity was obtained and enzyme activity was highest at 55°C. This optimum temperature of GBE from *Geobacillus* sp. Geo5 was higher than GBEs isolated from other thermophilic bacteria, *G. stearothermophilus* and *Anaerobranca gottschalkii* which has the optimum temperature of 50°C (Thiemann *et al.*, 2006; Takata *et al.*, 1994). However, GBEs isolated from extreme thermophilic bacteria, *Rhodothermus obamensis*, *Rhodothermus marinus* and *Aquifex aeolicus* showed higher optimum temperature, that is between 65°C to 80°C (Yoon *et al.*, 2007; van der Maarel *et al.*, 2003; Shinohara *et al.*, 2001). These bacteria produce enzymes that are active at higher temperature because the optimal growth temperatures for these bacteria are higher than *Geobacillus* sp. Geo5, that is between 65°C to 80°C.

The activation energy of the enzyme was achieved as the assay temperature rise and therefore, the rates of reactions catalysed by the enzyme increase (Voet and Voet, 2011). But as temperature increased further, GBE activity decreased drastically. The residual enzyme activity was only about 20% when enzyme was assayed at 70°C and

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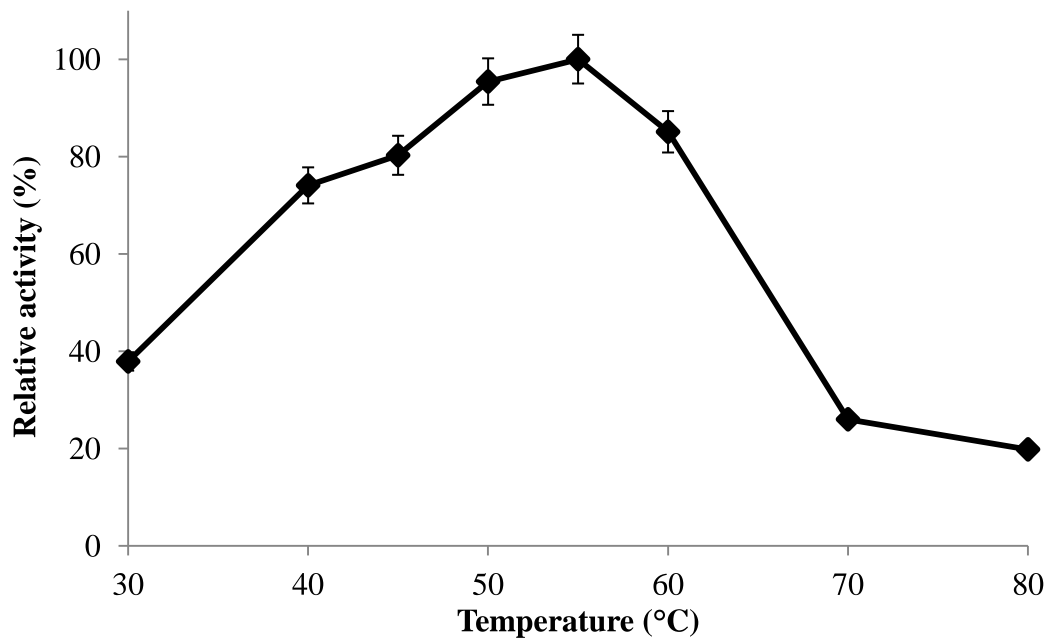

Figure 28. Effect of temperature on enzyme activity. GBE activity was assayed at temperature between 30°C to 80°C. Note: Data represents mean ± SE (n=3).

80°C. This must be due to protein denaturation. As an enzyme is a protein, in an elevated temperature, the structure of the enzyme including its active site would be disrupted and therefore the denatured enzyme is not functional and consequently causes the lost in enzyme activity (Bischof and He, 2005).

To test the thermal stability, the enzyme was heated at 40°C to 80°C. Enzyme activity was assayed every 4 hours. Referring to Figure 29, enzyme was stable up to 60°C for 24 hours. The enzyme maintained almost all of its activity for 16 hours when treated at 40°C and 50°C. For 24 hours, the enzyme manage to retained 78% and 70% when incubated at 40°C and 50°C, respectively. Nonetheless, enzyme activity drastically drop within 12 hours when treated at 70°C and was totallyinhibited at 80°C. The half-life of the enzyme at 60°C was 24 hours while at 70°C, 5 hours.

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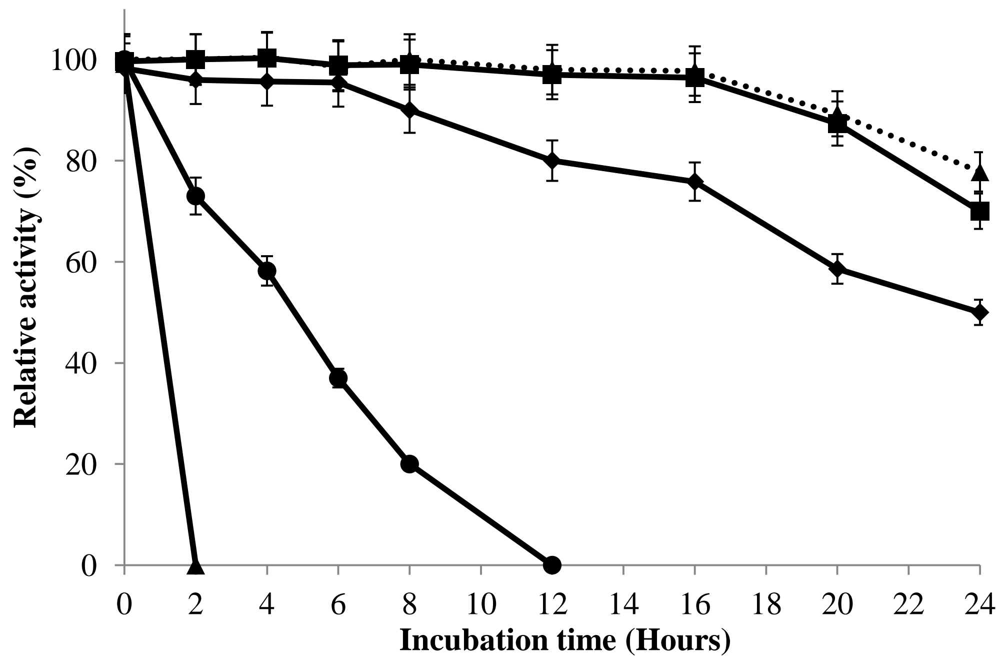

Figure 29. Effect of temperature on enzyme stability. GBE was incubated at 40°C (••▲••), 50°C (━■━), 60°C (━◆━), 70°C (━●━) and 80°C (━▲━) prior to enzyme assay. Note: Data represents mean ± SE (n=3).

Since this enzyme does not have any disulphide bonds, therefore the stability of this enzyme is possibly due to the high composition of aromatic amino acid residues. This GBE is more stable compared to GBE from *G. stearothermophilus* that has lost 20% of enzyme activity at 60°C in just 30 minutes and *A. gottschalkii* that has a half-life of only 55 minutes at 55°C (Thiemann *et al.*, 2006; Takata *et al.*, 1994). According to the composition of aromatic amino acid residues, *G. stearothermophilus* has more, which is 16.3% while *Geobacillus* sp. Geo5 has only 15.3%. However, the composition of asparagine and glutamine, which often result in inactivation of an enzyme exposed to high temperature, in *G. stearothermophilus* is more, which is 7.7% while *Geobacillus* sp. Geo5 has only 6.3% (van der Maarel *et al.*, 2003). This could be the reason why GBE from *Geobacillus* sp. Geo5 survives longer in high temperature compared to *G. stearothermophilus*.

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However, if compared to GBE isolated from hyperthermophilic bacteria, *R. obamensis* and *R. marinus*, at 80°C, their half-life was 16 hours and 73.7 minutes, respectively, while *A. aeolicus* retained 70% of its activity at 90°C. (Yoon *et al.*, 2007; van der Maarel *et al.*, 2003; Shinohara *et al.*, 2001). This could also be related to the composition of aromatic amino acid residues, asparagine and glutamine; as GBEs isolated from those bacteria have more aromatic amino acid residues and less asparagine and glutamine residues compared to *Geobacillus* sp. Geo5. Therefore, this result confirmed the genome mining result discussed in section 4.1.

4.11.2 Effect of pH on GBE activity and stability

Enzymes are pH sensitive. pH affect the enzyme on four factors that would influence the enzyme activity; substrate binding, catalytic reaction, ionisation of substrate and the protein structure (Voet and Voet, 2011). pH can change the ionization state of the side chains of acidic and basic amino acids, Asp, Glu, Lys, Arg, and His, that made up the enzyme. The change in ionization state will alter the ionic bonds that establish the 3D structure of the enzyme and consequently affect the enzyme activity. The change in pH not only affects the enzyme but it may have effect on the properties of the substrate too that could inhibit the enzyme-substrate binding.

To determine the effect of pH on GBE activity, iodine stain assay was done at various pHs. GBE displayed relatively high activity with more than 60% of enzyme activity in a broad pH range, which is from pH 5 to pH 9 (Figure 30). GBE was

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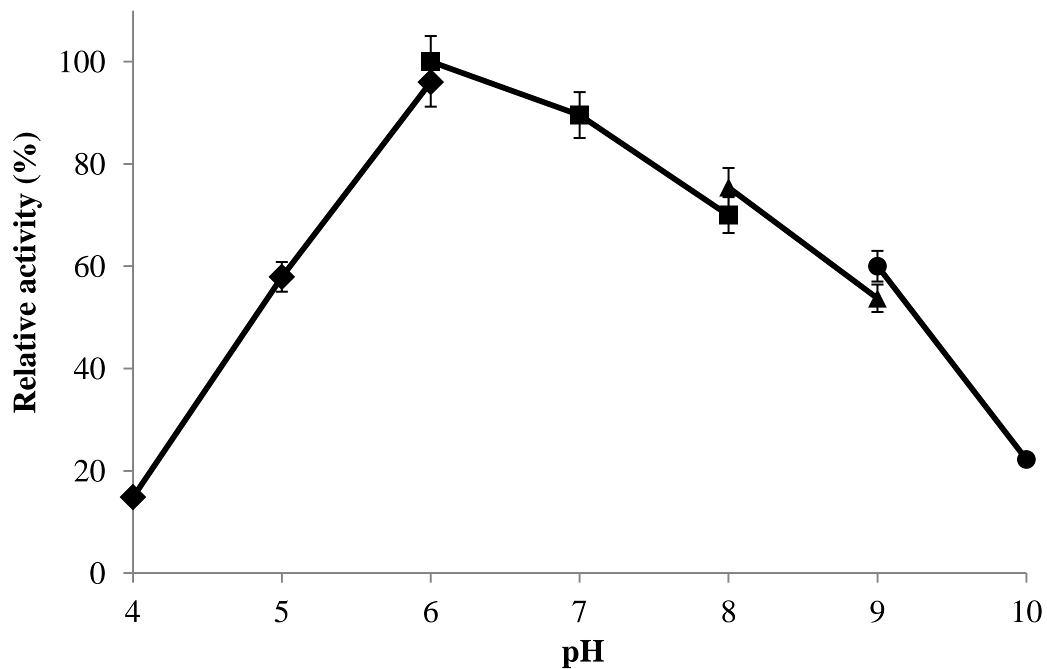

Figure 30. Effect of pH on enzyme activity. GBE activity was assayed at pH 4 to pH 10. Symbols used are (—◆—), acetate buffer; (—■—), sodium phosphate buffer; (—▲—), tris-HCl buffer; (—●—), glycine buffer. Note: Data represents mean ± SE (n=3).

found to be most active at pH 6 and this is similar to GBE isolated from *R. obamensis* and *R. marinus* (Yoon *et al.*, 2007; Shinohara *et al.*, 2001). However, about 80% of enzyme activity was drastically lost when enzyme was assayed at pH 4 and pH 10. Since the amino acids involve in catalysis are ionisable residues, Asp and Glu, therefore pH difference affects the ionization state of those residues and consequently alter the conformation of the active site and leads to the lost of enzyme activity (Nielsen *et al.*, 2001).

The stability test shown that the enzyme was stable between pH 5 to pH 9 where more than 50% of enzyme activity remained after the 1 hour of pH treatment (Figure 31). This is probably related to its isoelectric point (pI). When a protein is in

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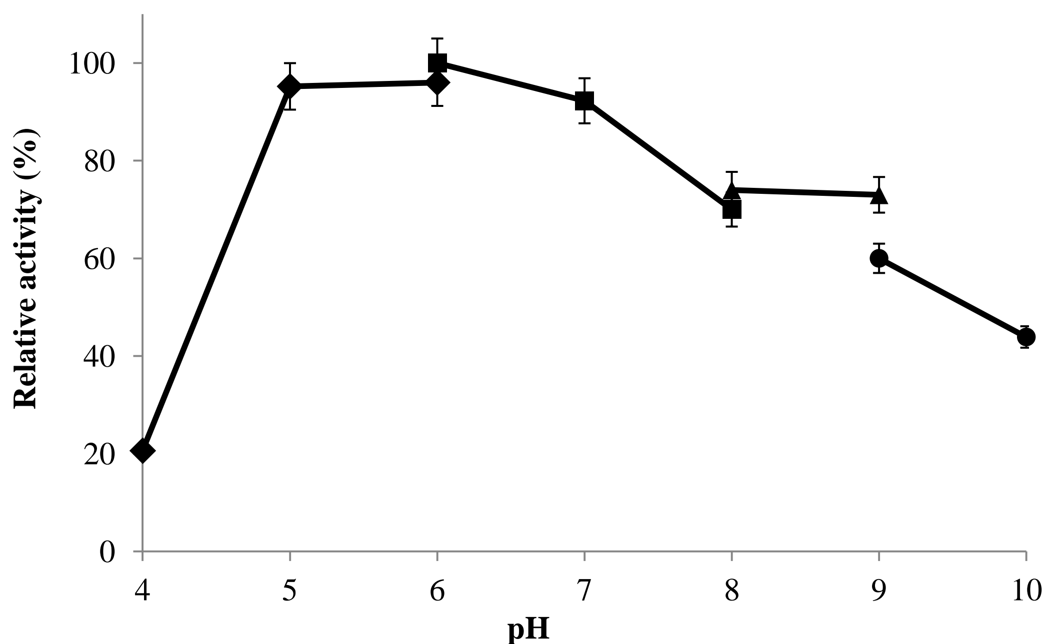

Figure 31. Effect of pH on enzyme stability. GBE was incubated for 1 hour at pH 4 to pH 10 prior to assay at pH 7.0. Symbols used are (—◆—), acetate buffer; (—■—), sodium phosphate buffer; (—▲—), tris-HCl buffer; (—●—), glycine buffer. Note: Data represents mean ± SE (n=3).

an environment with pH too far from its pI, the protein tends to aggregates due to the lost of electrostatic repulsions (Nielsen *et al.*, 2001; Voet and Voet, 2011). This aggregation, which is irreversible, impairs the active site and thus causes the protein to lose its activity. This recombinant GBE, G5GBE, has a theoretical pI of 6.1 and therefore it can be the reason why enzyme cannot tolerates pH lower than pH 5 and greater that pH 9. GBEs isolated from other sources in general are also active in this pH range, pH 5 to 9 (Lee *et al.*, 2008; Garg *et al.*, 2007; Thiemann *et al.*, 2006; Takata *et al.*, 2005; van der Maarel *et al.*, 2003; Takata *et al.*, 1994). It is important for GBE to be active in wide range of pH if this enzyme were to be applied in industries.

4.11.3 Effect of metal ions on GBE activity

Metals can participate in catalysis whether it is bound to the enzyme or acquired from the reaction solution along with the substrate (Nelson and Cox, 2005). GBE is not a metalloenzyme and so does not have metal binding site in its structure. However, the presence of metal ions could act as an inorganic cofactor to enhance enzyme activity. There are three properties of metal ions that involve in enzyme mechanism of reaction (Berg, 1987). Firstly, the electrophilic nature of metal ions could act as Lewis acids for substrate binding and substrate activation. Secondly, metal ions can engage in different types of oxidation-reduction processes since they are stable in various oxidation states. Finally, metal ions can act as cross-linking agents by binding several protein side chains and consequently enhance the stability of the enzyme. On the other hand, metal ion may also interrupt the ionic interaction of a readily stable structure of an enzyme and thus changing the conformation of the enzyme. In this case, metal ions would show inhibitory effect on the enzyme activity.

Metal ions had different effects on GBE activity but none of the metal ions experimented enhanced the enzyme activity (Figure 32). Two alkaline earth metals of group 2 elements (Mg^{2+} and Ca^{2+}) were tested. Mg^{2+} had no effect on enzyme activity. Also, there was no change in enzyme activity when the enzyme was incubated with 1 mM Ca^{2+}. However, the activity was slightly lowered to 73% when the concentration of Ca^{2+} increased to 5 mM. Similar results are also observed in GBE from *M. tuberculosis* as well as *R. marinus*, although Mg^{2+} seems to enhance the activity of GBE by 15% for *R. marinus* (Garg *et al.*, 2007; Yoon *et al.*, 2007).

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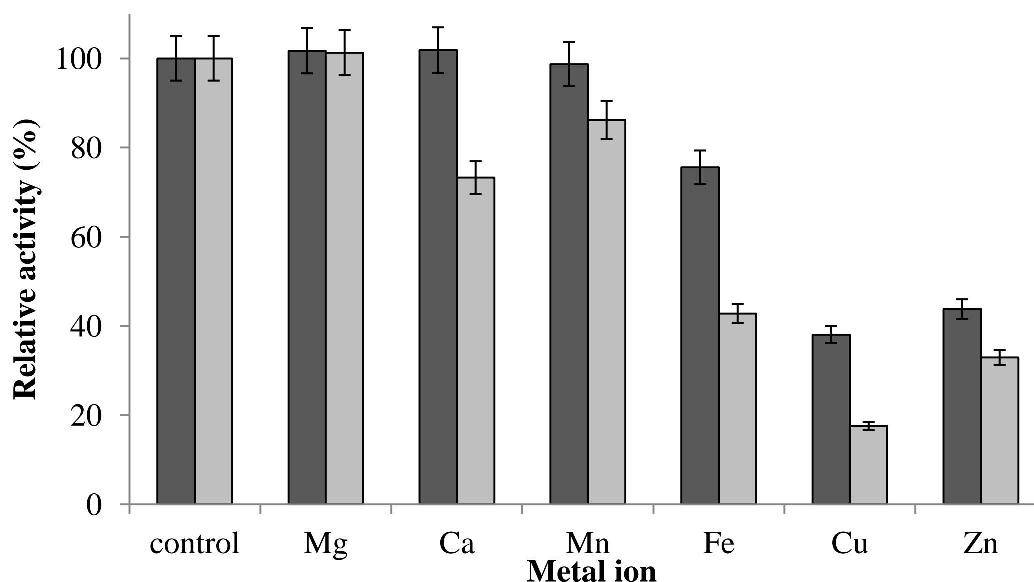

Figure 32. Effect of metal ion on enzyme activity. Enzyme activity was assayed with two concentrations of metal ions, 1mM (■) and 5 mM (■). Note: Data represents mean ± SE (n=3).

Four transition metals (Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}) were also tested out to observe the effect on GBE activity. Mn^{2+} did not affect enzyme activity when the enzyme was incubated in 1 mM of the metal ions and the activity was slightly decreased by 14% when the enzyme was incubated in 5 mM of the metal ions. Mn^{2+} too showed slight inhibition on GBE activity isolated from *A. gottschalkii* and *R. marinus* (Yoon *et al.*, 2007; Thiemann *et al.*, 2006). Zn^{2+} and Cu^{2+} repressed the enzyme as only 40% and less of the enzyme activity was remaining. These metal ions also appear to restrain GBE activity of other bacteria, *A. gottschalkii*, *R. marinus* and *M. tuberculosis* (Garg *et al.*, 2007; Yoon *et al.*, 2007; Thiemann *et al.*, 2006). Meanwhile, 1 mM of Fe^{2+} did not really affect GBE activity as 76% of enzyme activity retained but 5 mM of Fe^{2+} inhibit the enzyme by 60%. 5 mM of Fe^{2+} also inhibit GBE from *R. marinus* by 60% (Yoon *et al.*, 2007). In conclusion, GBE isolated from *Geobacillus* sp. Geo5 does not require metal ions to perform its catalytic reaction.

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CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

One of the criteria sought after in industrial enzymes are enzymes that are able to withstand and active at high temperature. That is why thermophiles are targeted in the search for biocatalysts that are functional in hot environment. The aim of this study is achieved as a thermostable glycogen branching enzyme was successfullly isolated from *Geobacillus* sp. Geo5 by genome mining. Although the genome sequence used was not fully assembled and annotated, it was good enough for obtaining the gene sequence and to be used for structural prediction. The result that was analysed by computational method postulated that the enzyme is thermostable and this was confirmed by the results produced by molecular biology experiments.

With the help from genome mining result, the gene for GBE was easily isolated using PCR. Without the genome mining result, a gene of an unknown sequence has to be isolated by primer walking, which would take longer time and more effort. From there, GBE was successfully expressed in *E. coli* system. Two systems were tested, intracellular expression system and secretory expression system using BRP. Although secretory expression system is expected to be advantageous especially for protein purification, it was found less performing than the intracellular expression system. For that reason, the enzyme produced by intracellular expression system was used for subsequent experiments.

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The characterisation of the purified enzyme shown that the enzyme was most active at 55°C and stable up to 60°C for 24 hours. The optimum pH for enzyme activity was at pH 6 and stable in pH 5 to pH 9. Metal ions, Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+} and Ca^{2+}, seem to have inhibitory effect on enzyme activity while Mg^{2+} does not. The characteristics of this enzyme would make it very applicable for industrial use particularly those engaging in food and beverages other than in paper and textile industries. This enzyme also has potential to be use in medical research regarding the treatment for branching enzyme deficiency disease, a rare hereditary metabolic disorder in human and animals.

5.2 Recommendations

This research can be used as a basis to further study the thermostable glycogen branching enzyme. This enzyme has a potential to be crystallised since there are no crystal structure of thermostable branching enzyme to date. The crystal structure of this enzyme, which would be more accurate compared to the predicted models, would bring more knowledge on factors that determine the enzyme stability, information on enzyme mechanism and factors that can improve enzyme activity and stability. On the contrary, the study can also focus on inactivation of this enzyme in order to disable pathogenic microorganisms for the purpose of medical research.

Another prospective research that can be done on this particular thermostable GBE isolated from *Geobacillus* sp. Geo5 is to experiment the enzyme for different chemical reactions, using substrates other than starch components. This might lead to the discovery of novel compounds that are potentially valuable.

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APPENDICES

Appendix A

Materials	Manufacturers
Chemicals	
Acetic acid	Merck, Germany
Agarose	Seakem, USA
Amylose, from potato	Sigma, USA
Ampicillin	Calbiochem, Germany
Ammonium persulfate	Sigma, USA
Bis-acrylamide, 40% solution	Merck, Germany
Calcium chloride dihydrate	Merck, Germany
Calsium sulfate	R & M Chemicals, UK
Copper (II) sulfate	R & M Chemicals, UK
Di-Sodium hydrogen phosphate	Merck, Germany
Dimethyl sulfoxide	Sigma, USA
dNTP mix	Fermentas, Canada
Glycerol	R & M Chemicals, UK
Glycine	Merck, Germany
Hydrochloric acid	Merck, Germany
Imidazole	Merck, Germany
Iodine	Sigma, USA
IPTG	Merck, Germany

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Iron (II) sulfhate	R & M Chemicals, UK
Magnesium sulfate	R & M Chemicals, UK
Manganese (II) sulfate	R & M Chemicals, UK
Methanol	Fisher scientific, UK
Mitomycin C	Sigma, USA
Pfu DNA polymerase	Fermentas, Canada
Potassium iodide	Sigma, USA
Proteinase K	Promega, USA
Quick Start™ Bradford 1× reagent	Bio-Rad, USA
FastDigest® Restriction enzymes	Fermentas, Canada
Sodium acetate	Merck, Germany
Sodium chloride	Merck, Germany
Sodium dihydrogen phosphate	Merck, Germany
Sodium dodecyl sulphate	Merck, Germany
Sodium hydroxide	Merck, Germany
Taq DNA Polymerase	Fermentas, Canada
TEMED	Sigma, USA
Tetracycline	Sigma, USA
Tris	Invitrogen, USA
Zinc sulphate	R & M Chemicals, UK
β-mercaptoethanol	Merck, Germany

DNA and protein ladder

Broad Range Prestained Protein Marker	Nacalai Tesque, Japan
GeneRuler 1 kb DNA Ladder	Fermentas, USA

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Lambda DNA/*Hin*dIII marker	Fermentas, USA
Unstained protein molecular weight marker	Fermentas, USA

Media

Bacteriological agar	Merck, Germany
Luria-Bertani agar	Merck, Germany
Luria-Bertani broth	Merck, Germany
Nutrient agar	Oxoid, England
Nutrient broth	Merck, Germany
Peptone	Oxoid, England
Yeast extracts	Oxoid, England

Kits

Champion™ pET Directional TOPO® Expression Kit	Invitrogen, USA
DNeasy® Blood and Tissue Kit	Qiagen, Germany
QIAquick® Gel Extraction Kit	Qiagen, Germany
QIAprep® Spin Miniprep Kit	Qiagen, Germany

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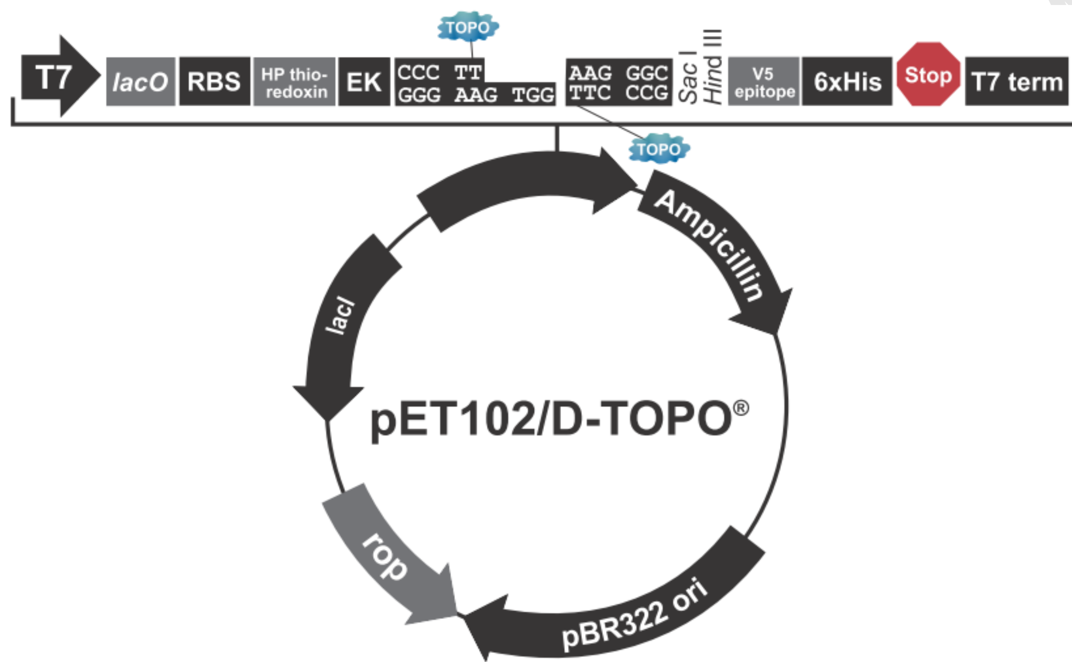
Appendix B

pET102/D-TOPO ® (6315 bp) vector map and features.



	pET102/D-TOPO®
T7 promoter	209-225
T7 promoter/priming site	209-228
lac operator (lacO)	228-252
Ribosome binding site (RBS)	282-288
His-patch (HP) thioredoxin ORF	298-627
TrxFus forward priming site	607-624
EK recognition site	643-657
TOPO® Cloning site (directional)	670-683
V5 epitope	700-741
Polyhistidine (6xHis) region	751-768
T7 reverse priming site	822-841
T7 transcription termination region	783-911
bla promoter	1407-1505
Ampicillin (*bla*) resistance gene	1506-2366
pBR322 origin	2511-3184
ROP ORF (complementary strand)	3552-3743
lacI ORF (complementary strand)	5055-6146

Appendix C

Cloning site and priming sites (TrxFus forward and T7 reverse) of pETl02/D-TOPO®

```
                                                                                      T7 promoter/priming site
                                                                                 T7 promoter
151  GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC CGCGAAATTA ATACGACTCA CTATAGGGGA

                lac operator                                              RBS
231  ATTGTGAGCG GATAACAATT CCCCTCTAGA AATAATTTTG TTTAACTTTA AGAAGGAGAT ATACATA ATG GGA TCT GAT
                                                                                   Met Gly Ser Asp
                                              His-patch (HP) thioredoxin
310  AAA ATT ATT CAT CTG ACT GAT GAT TCT TTT GAT ACT GAT GTA CTT AAG GCA GAT GGT GCA ATC CTG
     Lys Ile Ile His Leu Thr Asp Asp Ser Phe Asp Thr Asp Val Leu Lys Ala Asp Gly Ala Ile Leu

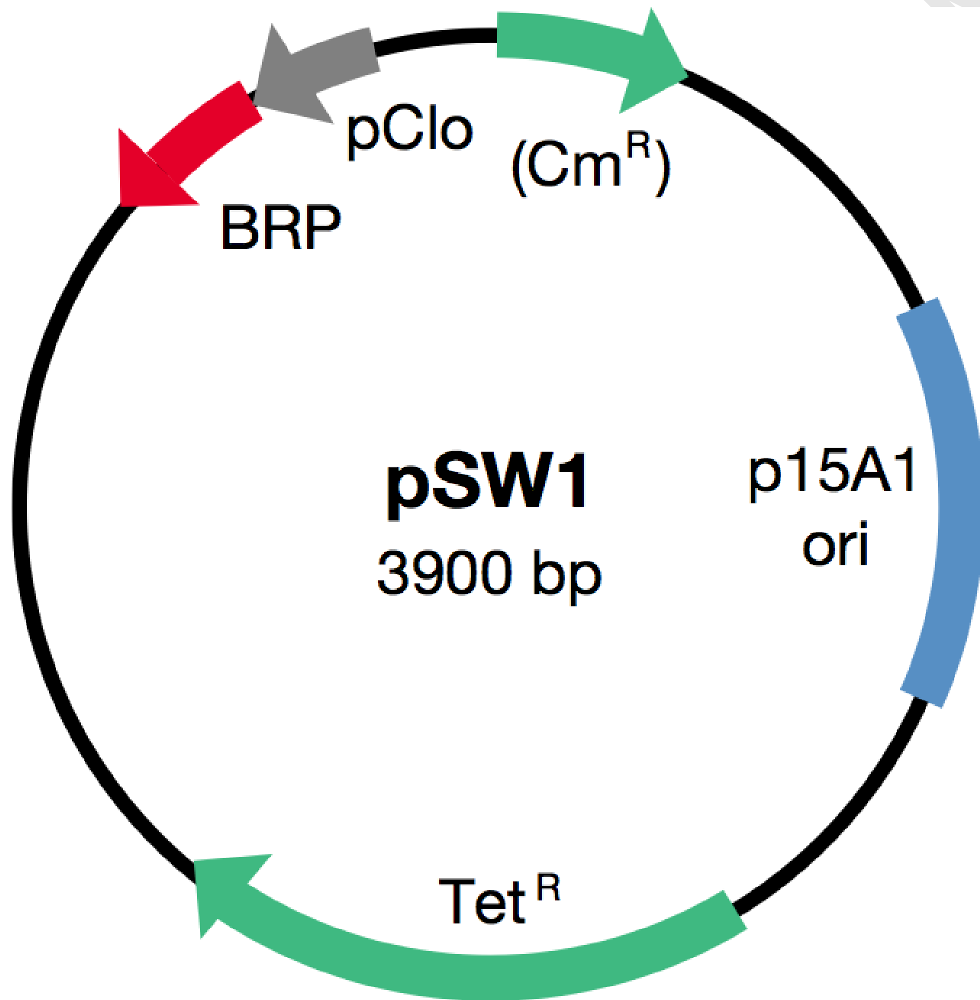
376  GTT GAT TTC TGG GCA CAC TGG TGC GGT CCG TGC AAA ATG ATC GCT CCG ATT CTG GAT GAA ATC GCT
     Val Asp Phe Trp Ala His Trp Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala

442  GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAC AAC CCG GGC ACT GCG CCG
     Asp Glu Tyr Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp His Asn Pro Gly Thr Ala Pro

508  AAA TAT GGC ATC CGT GGT ATC CCG ACT CTG CTG CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA
     Lys Tyr Gly Ile Arg Gly Ile Pro Thr Leu Leu Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys
                                                       TrxFus forward priming site
574  GTG GGT GCA CTG TCT AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCC GGC TCT GGA TCC
     Val Gly Ala Leu Ser Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala Gly Ser Gly Ser
         Enterokinase (EK) recognition site  EK cleavage site                          Sac I  Hind III
640  GGT GAT GAC GAT GAC AAG CTG GGA ATT GAT CCC TTC ACC ... ... AAG GGC GAG CTC AAG CTT GAA
                                                 GGG AAG TGG ... ... TTC CCG CTC
     Gly Asp Asp Asp Asp Lys Leu Gly Ile Asp Pro Phe Thr             Lys Gly Glu Leu Lys Leu Glu
                                                          G TGG
                                 V5 epitope                              Age I  Polyhistidine (6xHis) region
700  GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC
     Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His
                                                                        T7 reverse priming site
766  CAT TGA GTTTGATCC GGCTGCTAAC AAAGCCCGAA AGGAAGCTGA GTTGGCTGCT GCCACCGCTG AGCAATAACT AGCA
     His ***
```

Appendix D

Bacteriocin release protein (BRP) expression plasmid pSW1 map and features.



Appendix E

Result of multiple alignment using ClustalW.

```
Geobacillus_sp._MGI_                G---CTACTGGCGAATAGGAATCAATGTTGTGTATGGCCATCATTTTGCT
G._thermodenitrificans_NG80-2       -----------------------------ATGTGTGGCCATCATCTTGCT
Geobacillus_sp._Y412MC10            ------------------------------------TTGAACCAAGCAGT
Bacillus_sp._NBRC_15313             --------------------------------------------------
Geobacillus_sp._Y412MC61            -----------------------------GTGTATGGCCATCATTTTGCT
G.stearothermophilus                GGAATTTATGGAATCGCTGTGGAATATAAGTAACAACGGTAAGAAACTTT


Geobacillus_sp._MGI_                TCGGAAAGGATGCGATCCAGCTTGATTGCGGCGAATCCGACAGATTTAGA
G._thermodenitrificans_NG80-2       TTGGAAAGGATGCGATACGGTTTGATTGCGGCGAATCCGACGGATTTGGA
Geobacillus_sp._Y412MC10            TAGGGAAGGAAACATGACTCCGG-----------------------AACA
Bacillus_sp._NBRC_15313             TCGGAAAGGATGCGATCCAGCTTGATTGCGGCGAATCCGACAGATTTAGA
Geobacillus_sp._Y412MC61            TCGGAAAGGATGCGATCCAGCTTGATTGCGGCGAATCCGACAGATTTAGA
G.stearothermophilus                AAGGAAAGGATGCGATACAGATTGATCGCCGTCGGTCCCACTGATTTAGA
                                    : **.*****:.*.: .*                            :. *

Geobacillus_sp._MGI_                AGTGTATTTGTTTCATGAAGGCCGTTTGTATCAAAGTTATGAGCTGTTCG
G._thermodenitrificans_NG80-2       AGTGTATTTGTTTCATGAAGGCAGCTTGTATAAAAGTTACGAGCTGTTTG
Geobacillus_sp._Y412MC10            AGTGTATTTATTTCATGAAGGAACCTGGTTTCACAGTTACCAATGTATGG
Bacillus_sp._NBRC_15313             AGTGTATTTGTTTCATGAAGGCCGTTTGTATCAAAGTTATGAGTTGTTCG
Geobacillus_sp._Y412MC61            AGTGTATTTGTTTCATGAAGGCCGTTTGTATCAAAGTTATGAGCTGTTCG
G.stearothermophilus                AATCTATTTATTTCATGAAGGCAGCTTATATAAAAGTTATGAATTGTTTG
                                    *.* *****.***********..  * .*:*.*.*****  *.   :* *

Geobacillus_sp._MGI_                GCGCTCATGTCATCCGCGACGGCGGAGCGGTCGGCACTCGCTTTTGCGTG
G._thermodenitrificans_NG80-2       GCGCCCATGTGATTAATGAGGGCGGGAAGGTCGGCACCCGTTTTTGTGTT
Geobacillus_sp._Y412MC10            GTGCACACCTTGCCACAGAAGAAGGAGAAGAGGGCGTGCGTTTTACGGTG
Bacillus_sp._NBRC_15313             GCGCTCATGTCATCCGCGACGGCGGAGCGGTCGGCACTCGCTTTTGCGTG
Geobacillus_sp._Y412MC61            GCGCTCATGTCATCCGCGACGGCGGAGCGGTCGGCACTCGCTTTTGCGTG
G.stearothermophilus                GTGCACATGTGATAAAGAAAAATGGCATGGTCGGAACCCGGTTTTGTGTA
                                    * ** **  * .  .  .* .. ** . .*: **..  ** ***:  **

Geobacillus_sp._MGI_                TGGGCGCCCCATGCGCGGGAAGTCCGTCTTGTCGGCAGTTTCAACGATTG
G._thermodenitrificans_NG80-2       TGGGCGCCGCACGCGCGCGAGGTGCGTCTTGTCGGCAGTTTCAACGATTG
Geobacillus_sp._Y412MC10            TGGGCTCCGAATGCCAGGCAGGTAGGACTTGCCGGAGACTGGAACGGCTG
Bacillus_sp._NBRC_15313             TGGGCGCCCCATGCGCGGGAAGTCCGTCTTGTCGGCAGTTTCAACGATTG
Geobacillus_sp._Y412MC61            TGGGCGCCCCATGCGCGGGAAGTCCGTCTTGTCGGCAGTTTCAACGATTG
G.stearothermophilus                TGGGCACCCCATGCGCGGGAAGTGCGATTAGTCGGCAGTTTTAATGAATG
                                    ***** ** .* ** .*  *.**  *: *:* ***... *  ** *. **

Geobacillus_sp._MGI_                GAATGGGGCGAATTCCCCCCTGACGAAGGTGAACGACGAAGGGGTATGGA
G._thermodenitrificans_NG80-2       GGACGGGACGGATTTTCGCCTTGAGAAAGTGAATGATGAAGGGGTATGGA
Geobacillus_sp._Y412MC10            GGATGGTTCCCAAGACCCGTTATATAGGATGCCCGATTCGGGAATTTGGA
Bacillus_sp._NBRC_15313             GAATGGGGCGAATTCCCCCCTGACGAAGGTGAACGACGAAGGGGTATGGA
Geobacillus_sp._Y412MC61            GAATGGGGCGAATTCCCCCCTGACGAAGGTGAACGACGAAGGGGTATGGA
G.stearothermophilus                GAACGGAACTAATTTTAACCTTATGAAAGTAAGTAATCAAGGCGTATGGA
                                    *.* **  *  *:   .   *    *...*..  .*  ..** .*:****

Geobacillus_sp._MGI_                CGATCGTTGTTCCAGAAAACTTGGAAGGGCATCTCTATAAATATGAGATC
G._thermodenitrificans_NG80-2       CGATTGTTGTCCCCGAAAACTTGGAAGGGCATTTATATAAGTATGAGATT
Geobacillus_sp._Y412MC10            GTCGGTTTTTCCCTGGGATGGAGACTGGAACTTTTTACAAATATCAGATC
Bacillus_sp._NBRC_15313             CGATCGTTGTTCCAGAAAACTTGGAAGGGCATCTCTATAAATATGAGATC
Geobacillus_sp._Y412MC61            CGATCGTTGTTCCAGAAAACTTGGAAGGGCATCTCTATAAATATGAGATC
G.stearothermophilus                TGATTTTTATTCCTGAAAACTTAGAAGGGCATTTATATAAATACGAAATT
                                      .   ** * ** *..*:  :...:**...* * ** **.**  *.**

Geobacillus_sp._MGI_                ATCACACCGGATGGCCGTGTTCTGTTGAAAGCCGACCCGTACGCCTTTTA
G._thermodenitrificans_NG80-2       GTTACGCCGGACGGACAGGTGCTGTTCAAAGCCGACCCGTACGCTTTTTA
Geobacillus_sp._Y412MC10            ACGGGGCCCTTCGGAGAAACATTCCTAAAGGCCGATCCTTATGCATTTCA
Bacillus_sp._NBRC_15313             ATCACACCGGATGGCCGTGTTCTGTTGAAAGCCGACCCGTACGCCTTTTA
Geobacillus_sp._Y412MC61            ATCACACCGGATGGCCGTGTTCTGTTGAAAGCCGACCCGTACGCCTTTTA
G.stearothermophilus                ACGACGAACGATGGGAATGTTCTGTTAAAATCGGATCCATACGCGTTTTA
                                    .  . ...  : **  . .   *  * **. * ** ** ** ** *** *
```

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Geobacillus_sp._MGI_                CTCCGAATTGCGCCCTCATACCGCCTCGATTGTCTACGATTTGAAAGGAT
G._thermodenitrificans_NG80-2       CTCCGAATTGCGTCCTCATACCGCCTCGATTGCCTACGATCTGAAAGGAT
Geobacillus_sp._Y412MC10            TGCCGAAGTACGACCTGCGACGGCATCGGTGGTGACCGACCTCTCAGGAT
Bacillus_sp._NBRC_15313             CTCCGAATTGCGCCCTCATACCGCCTCGATTGTCTACGATTTGAAAGGAT
Geobacillus_sp._Y412MC61            CTCCGAATTGCGCCCTCATACCGCCTCGATTGTCTACGATTTGAAAGGAT
G.stearothermophilus                CTCCGAGTTGCGTCCCCATACTGCTTCCATTGTCTACAACATAAAAGGAT
                                      ****. *.** **  . ** ** ** .* *  :.*.*  * :.*****

Geobacillus_sp._MGI_                ACGAGTGGAATGATTCATCTTGGCAGCGGAAGAAACGGCGAAAGCGGATT
G._thermodenitrificans_NG80-2       ACCAGTGGAACGATCAATCTTGGAAGCGGAAGAAGCGACGAAAACGGATT
Geobacillus_sp._Y412MC10            ATCGCTGGAATGATGCCGCATGGAGAAGGAAGAACAAAT------CCCCA
Bacillus_sp._NBRC_15313             ACCAGTGGAATGATTCATCTTGGCAGCGGAAGAAACGGCGAAAGCGGATT
Geobacillus_sp._Y412MC61            ACGAGTGGAATGATTCATCTTGGCAGCGGAAGAAACGGCGAAAGCGGATT
G.stearothermophilus                ATCAATGGAATGACCAGACATGGCGACGGAAGAAACAGCGAAAGCGAATT
                                    *  . ***** **  .  *:***....******* ...         . :

Geobacillus_sp._MGI_                TATGACCAACCGATGGTCATTTATGAACTTCATTTCGGTTCGTGGAAAAA
G._thermodenitrificans_NG80-2       TATGATCAGCCCATGGTGATTTATGAACTCCATTTCGGTTCGTG---GAA
Geobacillus_sp._Y412MC10            TATGCCAAGCCGGTGAACATTTATGAAATGCACTTCGGAACCTGGCGGCA
Bacillus_sp._NBRC_15313             TATGACCAACCGATGGTCATTTATGAACTTCATTTCGGTTCGTGGAAAAA
Geobacillus_sp._Y412MC61            TATGACCAACCGATGGTCATTTATGAACTTCATTTCGGTTCGTGGAAAAA
G.stearothermophilus                TATGACCAGCCTTTGTTCATTTATGAACTTCACTTTGGTTCGTGGAAAAA
                                    ****. .*.**  ** : *********.* ** ** **::* **   ..*

Geobacillus_sp._MGI_                GAAACCGGACGGCCGCTTTTATACGTACCGTGAGATGGCCGACGAACTCA
G._thermodenitrificans_NG80-2       GAAAAAAGATGGGCGTTTTTATACGTACCGTGAGATGGCCGATGAACTGA
Geobacillus_sp._Y412MC10            AAAAGAGGACGGTTCGTTCTTTACATATCGGGAATTGGCCGATCAGCTGA
Bacillus_sp._NBRC_15313             GAAACCGGACGGCCGCTTTTATACGTACCGTGAGATGGCCGACGAACTCA
Geobacillus_sp._Y412MC61            GAAACCGGACGGCCGCTTTTATACGTACCGTGAGATGGCCGACGAACTCA
G.stearothermophilus                GAAAGAGGACGGCAGTTTTTATACATATCAAGAGATGGCAGAGGAGCTAA
                                    .***  ..** **    ** *:***.** *. **.:****.**  *.** *

Geobacillus_sp._MGI_                TTCCGTACGTGCTGGAGCGCGGATTTACGCACATTGAGCTGCTTCCGCTT
G._thermodenitrificans_NG80-2       TCCCGTATGTGCTCGATCATGGGTTTACGCACATTGAGTTGCTTCCTCTT
Geobacillus_sp._Y412MC10            TTCCTTATTTGCAGGAAATGAGTTATACCCACGTAGAATTATGCCCTTG
Bacillus_sp._NBRC_15313             TTCCGTACGTGCTGGAGCGCGGATTTACGCACATTGAGCTGCTTCCGCTT
Geobacillus_sp._Y412MC61            TTCCGTACGTGCTGGAGCGCGGATTTACGCACATTGAGCTGCTTCCGCTT
G.stearothermophilus                TCCCTTATGTTCTCGAACATGGGTTTACTCATATTGAGCTGCTCCCACTC
                                    * ** **  * *: **  .  .* *:*** ** .*:**. * .* **  *

Geobacillus_sp._MGI_                GTCGAGCATCCGCTCGATCGTTCGTGGGGATATCAAGGGACCGGCTATTA
G._thermodenitrificans_NG80-2       GTCGAGCATCCGCTCGACCGCTCGTGGGGCTATCAAGGAACAGGGTATTA
Geobacillus_sp._Y412MC10            GCGGAGCATCCCTACGATCTTTCGTGGGGCTATCAGGGCACAGGATTTTA
Bacillus_sp._NBRC_15313             GTCGAGCATCCGCTCGATCGTTCGTGGGGATATCAAGGGACCGGCTATTA
Geobacillus_sp._Y412MC61            GTCGAGCATCCGCTCGATCGTTCGTGGGGATATCAAGGGACCGGCTATTA
G.stearothermophilus                GTCGAGCATCCGTTCGATCGTTCTTGGGGATATCAGGGAATAGGTTATTA
                                    *  ********  :*** *  ** *****.*****.** * .** *:***

Geobacillus_sp._MGI_                TTCGGTGACAAGCCGCTATGGCACGCCGCACGATTTCATGTATTTCGTCG
G._thermodenitrificans_NG80-2       TGCGGTAACGAGTCGCTATGGTACGCCACACGACTTCATGTACTTCGTCG
Geobacillus_sp._Y412MC10            TGCGCTGACGAGCAGATACGGAACTCCCCATGATTTCATGTTTCTCGTCG
Bacillus_sp._NBRC_15313             TTCGGTGACAAGCCGCTATGGCACGCCGCACGATTTCATGTATTTCGTCG
Geobacillus_sp._Y412MC61            TTCGGTGACAAGCCGCTATGGCACGCCGCACGATTTCATGTATTTCGTCG
G.stearothermophilus                TTCAGCAACAAGCCGCTACGGAACACCGCATGATTTGATGTATTTTATTG
                                    * *.  .**.** .*.** ** ** ** ** ** ****:  * .* *

Geobacillus_sp._MGI_                ACCGCTGCCATCAAGCGGGGCTTGGCGTCATCATCGACTGGGTGCCGGGG
G._thermodenitrificans_NG80-2       ACCGTTGCCATCAGGCGGGAATCGGGGTCATTATGGACTGGGTGCCGGGG
Geobacillus_sp._Y412MC10            ACCAGCTGCACCAAGCCGGCATCGGCGTGCTGCTCGACTGGGTGCCCGCA
Bacillus_sp._NBRC_15313             ACCGCTGCCATCAAGCGGGGCTTGGCGTCATCATCGACTGGGTGCCGGGG
Geobacillus_sp._Y412MC61            ACCGCTGCCATCAAGCGGGGCTTGGCGTCATCATCGACTGGGTGCCGGGG
G.stearothermophilus                ACCGCTGTCACCAAGCTGGAATAGGCGTCATTCTCGATTGGGTTCCTGGC
                                    ***.     ** **.** ** .* ** ** .* .* ** ***** ** *

Geobacillus_sp._MGI_                CATTTTTGCAAGGACGCCCACGGGCTGTACATGTTTGACGGCGCACCGAC
G._thermodenitrificans_NG80-2       CATTTTTGCAAGGACGCCCATGGGTTATATATGTTTGATGGCGCCCCGAC
Geobacillus_sp._Y412MC10            CATTTTGCCAAGGATGCCCACGGGCTTCGGCAGTTTGACGGGGCGCCTCT
Bacillus_sp._NBRC_15313             CATTTTTGCAAGGACGCCCACGGGCTGTACATGTTTGACGGCGCACCGAC
Geobacillus_sp._Y412MC61            CATTTTTGCAAGGACGCCCACGGGCTGTACATGTTTGACGGCGCACCGAC
G.stearothermophilus                CACTTTTGTAAAGATTCCCATGGGTTATATATGTTTGATGGCGCACCGGC
                                    ** ***    **.**  **** *** *  . .:****** ** ** **
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Geobacillus_sp._MGI_               GTATGAATACGCGAATGAAAAAGACCGAGAAAATTACGTCTGGGGGACGG
G._thermodenitrificans_NG80-2      GTATGAATACGCGAATGAAAAAGACCGAGAAAATTACGTTTGGGGGACGG
Geobacillus_sp._Y412MC10           TTATGAGTATGCAGACCCCCAAAAAGCCGAGAAACCTGGCTGGGGAACAT
Bacillus_sp._NBRC_15313            GTATGAATACGCGAATGAAAAAGACCGAGAAAATTACGTCTGGGGGACGG
Geobacillus_sp._Y412MC61           GTATGAATACGCGAATGAAAAAGACCGAGAAAATTACGTCTGGGGGACGG
G.stearothermophilus               ATATGAATATGCCAACATGCAAGACCGGGAAAATTACGTATGGGGAACGG
                                    *****.** ** .*    .**.*.   **.**: . *  *****.**.

Geobacillus_sp._MGI_               CGAATTTTGACTTGGGCAAGCCGGAAGTGCGCAGTTTTCTGATCTCCAAT
G._thermodenitrificans_NG80-2      CCAATTTTGATTTAGGCAAGCCGGAAGTGCGCAGTTTTCTCATCTCGAAC
Geobacillus_sp._Y412MC10           TGTCTTTCGATTATGCCAAGCCGGAGGTAATTTCATTCCTCATCTCCAAT
Bacillus_sp._NBRC_15313            CGAATTTTGACTTGGGCAAGCCGGAAGTGCGCAGTTTTCTGATCTCCAAT
Geobacillus_sp._Y412MC61           CGAATTTTGACTTGGGCAAGCCGGAAGTGCGCAGTTTTCTGATCTCCAAT
G.stearothermophilus               CAAACTTTGACCTTGGCAAGCCGGAAGTCCGCAGCTTTTTGATTTCCAAT
                                     :. ** **  : * *********.** .  :  **  * ** ** **

Geobacillus_sp._MGI_               GCGTTGTTTTGGCTGGAGTATTACCATGTGGACGGGTTTCGCGTCGATGC
G._thermodenitrificans_NG80-2      GCATTGTTTTGGCTCGAGTATTACCATATCGACGGGTTCCGGGTCGATGC
Geobacillus_sp._Y412MC10           GCCTTATTTTGGATGGATATGTATCATATTGATGGTCTTCGCGTTGATGC
Bacillus_sp._NBRC_15313            GCGTTGTTTTGGCTGGAGTATTACCATGTGGACGGGTTTCGCGTCGATGC
Geobacillus_sp._Y412MC61           GCGTTGTTTTGGCTGGAGTATTACCATGTGGACGGGTTTCGCGTCGATGC
G.stearothermophilus               GCGTTATTTTGGATGGAATATTTCCATGTGGACGGGTTTCGTGTAGATGC
                                   ** **.******.* ** :: *: ***.* ** **  * ** ** *****

Geobacillus_sp._MGI_               GGTCGCTAATATG------------------------------CTTTATT
G._thermodenitrificans_NG80-2      GGTTGCCAATATG------------------------------CTTTATT
Geobacillus_sp._Y412MC10           CGTGACGAGCATGATCCGTCTTGATTTTGAGAAGCGTGAGGGTCAGTACA
Bacillus_sp._NBRC_15313            GGTCGCCAATATG------------------------------CTTTATT
Geobacillus_sp._Y412MC61           GGTCGCCAATATG------------------------------CTTTATT
G.stearothermophilus               TGTTGCCAATATG------------------------------TTATATT
                                    ** .* *. ***                               : ** :

Geobacillus_sp._MGI_               GGCCGAACAACGACCGGCTCTATGAAAATCCGTATGCGGTCGAGTTTTTG
G._thermodenitrificans_NG80-2      GGCCGAACAATGACAGGCTGTACGAGAACCCGTATGCGGTCGAGTTTTTG
Geobacillus_sp._Y412MC10           GCCTTAACGAGCACGGAGGCTTGGAAAACCTTGAGGCGATATCCTTTCTG
Bacillus_sp._NBRC_15313            GGCCGAACAATGACCGGCTCTATGAAAATCCGTATGCGGTCGAGTTTTTG
Geobacillus_sp._Y412MC61           GGCCGAACAACGACCGGCTCTATGAAAATCCGTATGCGGTCGAGTTTTTG
G.stearothermophilus               GGCCAAACAGCGACGTACTATACAAAAATACGTATGCCGTGGAGTTCTTG
                                   * *  ***..  **  .   *: .*.** .   * ** .*  . **  **

Geobacillus_sp._MGI_               CGCCAGTTGAATGAGGCGGTGTTTGCCTATGACCCGAGCGTCTTGATGAT
G._thermodenitrificans_NG80-2      CGCAAGTTAAACGAAGCGGTGTTTGCCTATGATCCGAATGTGCTGATGAT
Geobacillus_sp._Y412MC10           CAGGAATTGAATAAAGCCGTGTTTCGATATTACCCGAATGCCTTGATGAT
Bacillus_sp._NBRC_15313            CGCCAGTTGAATGAGGCGGTGTTTGCCTATGACCCGAACGTCTTGATGAT
Geobacillus_sp._Y412MC61           CGCCAGTTGAATGAGGCGGTGTTTGCCTATGACCCGAACGTCTTGATGAT
G.stearothermophilus               CAAAAATTAAATGAAACGGTATTCGCCTATGATCCGAACATATTAATGAT
                                   *.  *.**.** .*..* **.**   .*** * ****. .   *.*****

Geobacillus_sp._MGI_               CGCTGAAGATTCGACCGACTGGCCTCGGGTGACCGCGCCGACGTACGATG
G._thermodenitrificans_NG80-2      TGCGGAAGATTCGACTGACTGGCCGAAGGTGACCGCGCCGACGTATGAAG
Geobacillus_sp._Y412MC10           GGCCGAGGAGTCGAGCGCATGGGCGGGGGTTACGGCACCTGTGCATGAAG
Bacillus_sp._NBRC_15313            CGCTGAAGATTCGACCGACTGGCCTCGGGTGACCGCGCCGACGTACGATG
Geobacillus_sp._Y412MC61           CGCTGAAGATTCGACCGACTGGCCTCGGGTGACCGCGCCGACGTACGATG
G.stearothermophilus               TGCCGAAGATTCGACAGACTGGCCGCGCGTCACTGCTCCAACATACGACG
                                    ** **.** ****  *..*** *  . ** ** ** ** . . * ** *

Geobacillus_sp._MGI_               GCGGCCTTGGGTTTAACTACAAGTGGAACATGGGCTGGATGAACGACATG
G._thermodenitrificans_NG80-2      GCGGACTCGGCTTTAATTATAAATGGAACATGGGCTGGATGAACGACATG
Geobacillus_sp._Y412MC10           GGGGTCTTGGCTTCAATTACAAATGGAATATGGGCTGGATGAACGACACG
Bacillus_sp._NBRC_15313            GCGGCCTTGGGTTTAACTACAAGTGGAACATGGGCTGGATGAACGACATG
Geobacillus_sp._Y412MC61           GCGGCCTTGGGTTTAACTACAAGTGGAACATGGGCTGGATGAACGACATG
G.stearothermophilus               GAGGATTAGGATTTAACTATAAATGGAACATGGGATGGATGAACGATATT
                                   * **  * ** ** ** ** **.***** *****.*********** *

Geobacillus_sp._MGI_               CTGAAGTATATGGAAACGCCGCCGCATGAGCGGAAATACGCCCATAACCA
G._thermodenitrificans_NG80-2      CTGAAGTACATGGAAACACCGCCGTATGAGCGGAGGCATGTGCATAACCA
Geobacillus_sp._Y412MC10           CTGTCTTATATTGAACAAGAGTTCGATCAGCGGCCTACCCATCATAATTT
Bacillus_sp._NBRC_15313            CTGAAGTATATGGAAACGCCGCCGCATGAGCGGAAATACGCCCATAACCA
Geobacillus_sp._Y412MC61           CTGAAGTATATGGAAACGCCGCCGCATGAGCGGAAATACGCCCATAACCA
G.stearothermophilus               TTAACTTATATGGAAACGCCGCCTGAACATCGAAAATACGTGCACAATAA
                                    *.:. ** ** ***... .*    *: * **..   .    ** **  :
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Geobacillus_sp._MGI_                 AGTCAGTTTTTCCCTCCTTTATGCGTATTCGGAAAATTTCATTTTGCCAT
G._thermodenitrificans_NG80-2        AGTAACGTTCTCCCTCCTTTATGCGTATTCGGAAAATTTCATTTTGCCGT
Geobacillus_sp._Y412MC10             ACTGACATTTCCCATTTGTTATGCCTATTCGGAGAATTTTACATTGCCGC
Bacillus_sp._NBRC_15313              AGTCAGTTTTTCCCTCCTTTATGCGTATTCGGAAAATTTCATTTTGCCAT
Geobacillus_sp._Y412MC61             AGTCAGTTTTTCCCTCCTTTATGCGTATTCGGAAAATTTCATTTTGCCAT
G.stearothermophilus                 AGTAACATTTTCCCTCTTGTATGCGTATTCGGAAAATTTCATTTTACCTT
                                     * * *  **  **.*    ***** ********.***** * :**.**

Geobacillus_sp._MGI_                 TTTCCCATGATGAAGTTGTGCATGGCAAAAAATCGCTGCTCAATAAAATG
G._thermodenitrificans_NG80-2        TTTCCCACGATGAAGTCGTGCATGGCAAAAAATCGCTGCTCAATAAAATG
Geobacillus_sp._Y412MC10             TTTCACACGATGAAGTCGTCCATGGAAAGAAATCGCTGCTTAATAAAATG
Bacillus_sp._NBRC_15313              TTTCCCATGATGAAGTTGTGCATGGCAAAAAATCGCTGCTCAATAAAATG
Geobacillus_sp._Y412MC61             TTTCCCATGATGAAGTTGTGCATGGCAAAAAATCGCTGCTCAATAAAATG
G.stearothermophilus                 TTTCCCATGACGAGGTCGTACATGGAAAAAAATCGCTGTTAAGTAAAATG
                                     ****.** ** **.** ** *****.**.********* * *.*******

Geobacillus_sp._MGI_                 CCTGGGTCGTACGAAGAGAAGTTCGCCCAGCTGCGCCTATTGTATGGCTA
G._thermodenitrificans_NG80-2        CCAGGGTCGTATGAAGAGAAGTTCGCCCAGCTGCGCCTCTTGTACGGCTA
Geobacillus_sp._Y412MC10             CCGGGAAGCTACGAACAAAAGTTTGCGGGACTGCGGATTTTGCTTGGATA
Bacillus_sp._NBRC_15313              CCTGGGTCGTACGAAGAGAAGTTCGCCCAGCTGCGCCTATTGTATGGCTA
Geobacillus_sp._Y412MC61             CCTGGGTCGTACGAAGAGAAGTTCGCCCAGCTGCGCCTATTGTATGGCTA
G.stearothermophilus                 CCGGGGACATATGAGGAAAAGTTTGCGCAATTAAGGTTGCTGTATGGATA
                                     ** **.:  ** **. *.***** **  .. *..*  *  ** : **.**

Geobacillus_sp._MGI_                 CATGATGGCCCACCCTGGGAAAAAGCTGCTGTTTATGGGCAGTGAGTTTG
G._thermodenitrificans_NG80-2        CATGATGGCTCATCCGGGGAAAAAGCTGTTGTTTATGGGCAATGAATTTG
Geobacillus_sp._Y412MC10             CCAGATCACCCAGCCCGGGAAGAATCTGCTGTTCATGGGGGAGAGTTCG
Bacillus_sp._NBRC_15313              CATGATGGCCCACCCTGGGAAAAAGCTGCTGTTTATGGGCAGTGAGTTTG
Geobacillus_sp._Y412MC61             CATGATGGCCCACCCTGGGAAAAAGCTGCTGTTTATGGGCAGTGAGTTTG
G.stearothermophilus                 TTTGTTGACGCATCCTGGTAAGAAATTATTGTTTATGGGCGGCGAATTTG
                                       :*:* .* ** ** ** **.**  *. **** ***** .. **.** *

Geobacillus_sp._MGI_                 CCCAGTTTGATGAATGGAAGTTTGAGGGAGAGCTCGACTGGGTGCTGTTC
G._thermodenitrificans_NG80-2        CTCAGTTTGATGAATGGAAGTTTGAGGATGAACTCGATTGGGTGCTGTTT
Geobacillus_sp._Y412MC10             GCCAGTTTATCGAATGGAAGGATCAGGAGCAGCTCGACTGGCTGCTGCTG
Bacillus_sp._NBRC_15313              CCCAGTTTGATGAATGGAAGTTTGAGGGAGAGCTCGACTGGGTGCTGTTC
Geobacillus_sp._Y412MC61             CCCAGTTTGATGAATGGAAGTTTGAGGGAGAGCTCGACTGGGTGCTGTTC
G.stearothermophilus                 GCCAGTTTGATGAATGGAAAGATTTAGAGCAGCTGGATTGGATGCTTTTT
                                       ******.: ********. :* :.*.  *.** ** *** ****  *

Geobacillus_sp._MGI_                 GATTTTGAATTGCACCGGAAAATGGACGAATATGTGAAGCAGCTGATCGC
G._thermodenitrificans_NG80-2        GATTTTGAGCTGCACCGGAAGATGAACGATTACATGAAAGAGTTAATCGC
Geobacillus_sp._Y412MC10             GACTATGAAAGCCACCGCAAGCAGTTGGCATATACGGCAGCTCTAAACCG
Bacillus_sp._NBRC_15313              GATTTTGAATTGCACTGGAAAATGGACGAATATGTGAAGCAGCTGATCGC
Geobacillus_sp._Y412MC61             GATTTTGAATTGCACCGGAAAATGGACGAATATGTGAAGCAGCTGATCGC
G.stearothermophilus                 GATTTTGATATGCATCGGAATATGAATATGTATGTGAAAGAATTGTTGAA
                                     ** *:***    **  * ** .:* : .  ** . *... .  *.::

Geobacillus_sp._MGI_                 CTGCTATAAACGGTATAAGCCGTTTTACGAGCTTGATCATGATCCGAGGG
G._thermodenitrificans_NG80-2        CTGCTATAAACGGTATAAGCCGTTTTACGAATTGGATCATGACCCGCAAG
Geobacillus_sp._Y412MC10             CATGTACGTGGATGAAAAGGCGCTATGGGAGCAGGATCACCGATGGGAAG
Bacillus_sp._NBRC_15313              CTGCTATAAACGGTATAAGCCGTTTTACGAGCTTGATCATGATCCGAGGG
Geobacillus_sp._Y412MC61             CTGCTATAAACGGTATAAGCCGTTTTACGAGCTTGATCATGATCCGAGGG
G.stearothermophilus                 ATGTTATAAGCGCTATAAACCGCTTTATGAGTTAGACCACTCTCCAGATG
                                     .:  ** .:. .  *:**. ** *:*. **. : ** **      . . *

Geobacillus_sp._MGI_                 GGTTTGAATGGATTGACGTTCATAATGCCGAGCAAAGTATTTTCTCATTC
G._thermodenitrificans_NG80-2        GATTTGAATGGATTGACGTTCACAACGCTGAACAAAGCATTTTCTCATTC
Geobacillus_sp._Y412MC10             GCTTCGAATGGCTCAGCGCCGATGATCATGAGCAGAGTGTCGTATCGTAT
Bacillus_sp._NBRC_15313              GGTTTGAATGGATTGACGTTCATAATGCCGAGCAAAGTATTTTCTCATTC
Geobacillus_sp._Y412MC61             GGTTTGAATGGATTGACGTTCATAATGCCGAGCAAAGCATTTTCTCGTTC
G.stearothermophilus                 GATTCGAGTGGATTGATGTTCATAACGCCGAACAAAGTATTTTCTCATTC
                                     * ** **.***.* .. *   * .*  . **.**.** .*  *.**.*:

Geobacillus_sp._MGI_                 ATCCGCCGGGGGAAAAAAGAAGGTGATGTGCTGGTCATTGTTTGTAATTT
G._thermodenitrificans_NG80-2        ATCCGCCGCGGGAAAAAAGAAGATGATGTGCTTGTTATTGTTTGCAATTT
Geobacillus_sp._Y412MC10             ATGAGAATGGGCACCAAGCCCGGCGATACGCTGATCATCGTCATTAATTT
Bacillus_sp._NBRC_15313              ATCCGCCGGGGGAAAAAAGAAGGTGATGTGCTGGTCATTGTTTGTAATTT
Geobacillus_sp._Y412MC61             GTCCGCCGCGGGAAAAAAGACGGCGATCTATTGGTAATTGTTTGCAATTT
G.stearothermophilus                 ATTCGCAGAGGAAAAAAAGAGGATGATTTGCTTATTGTTGTGTGTAATTT
                                     .* .*..  ** *..**. . *. ***  . * .* .* ** :  *****
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Geobacillus_sp._MGI_               CACAAATCAGGCGTATGACGATTACAAAGTCGGTGTGCCGCTTTTGGCGC
G._thermodenitrificans_NG80-2      CACAAATCAGGCGTATGACGACTACAAAGTTGGAGTGCCGTTGCTCGTAC
Geobacillus_sp._Y412MC10           TCAGCCAAAGGCATATGACCAATATCGGGTCGGTTTGCCGAGAGCGGGGC
Bacillus_sp._NBRC_15313            CACAAATCAGGCGTATGACGATTACAAAGTCGGCGTGCCGCTTTTGGCGC
Geobacillus_sp._Y412MC61           CACAAATCAAGCGTATGACGATTACAAAGTCGGCGTGCCGCTTTTGGCGC
G.stearothermophilus               CACAAATAAAGTATACCACGGTTATAAAGTTGGTGTTCCGTTATTTACAA
                                    .....:.*.* .**  ** . ** ...** **  * ***      . ..

Geobacillus_sp._MGI_               CGTACCGCGAAGTGCTGAACAGCGATGCAGCGGAGTTTGGCGGTTCGGGA
G._thermodenitrificans_NG80-2      CGTATCGGGAAGTGCTGAATAGCGATGCGGTCACGTTTGGTGGATCGGGG
Geobacillus_sp._Y412MC10           AATATGTGGAAATTCTGAATTCCGATCATGCCGATTACGGCGGCTCCGGA
Bacillus_sp._NBRC_15313            CGTACCGCGAAGTGCTGAACAGCGATGCAGCGGAGTTTGGCGGATCAGGG
Geobacillus_sp._Y412MC61           CGTACCGCGAAGTGCTTAACAGCGATGCAGCGGAGTTTGGCGGTTCGGGA
G.stearothermophilus               GATATCGGGAAGTAATCAATAGCGATGCAATCCAATTCGGCGGCTTTGGG
                                    .**    ***.* .* ** : **** . .   . *: ** ** *  **.

Geobacillus_sp._MGI_               CATGTCAACGGGAAGCGGCTTCCCGCTTTCCATGAGCCGTTTCATGGAAA
G._thermodenitrificans_NG80-2      CATGTCAATGGGAAACGGCTTTCCGCCTTCAATGAGCCGTTTCATGGTAA
Geobacillus_sp._Y412MC10           CTGCTGAACGACGGTGAGATGAAAGCGGAGAAGAAGACCTGGCATGGCCA
Bacillus_sp._NBRC_15313            CATGTCAATTCGAAGCGGCTTTCCGCTTTCCATGAGCCGTTTCATGGAAA
Geobacillus_sp._Y412MC61           CATGTCAACGGGAAGCGGCTTCCCGCTTTCAGTGAGCCGTTTCATGGAAA
G.stearothermophilus               AATATCAATCCAAAACCGATTGCGGCGATGGAAGGGCCGTTTCACGGAAA
                                   .:  * **    ..   *.*  . **  :  . ..*.* *  ** ** .*

Geobacillus_sp._MGI_               ACCGTACCATGTGCGCATGACGATTCCGCCGTTTGGCATTTCCATTTTGC
G._thermodenitrificans_NG80-2      ACCATACCACGTGCGCATGACGATTCCGCCATTTGGCATTTCCATTTTAC
Geobacillus_sp._Y412MC10           AACCCACAGCCTGGAGATCAAGCTGCCTCCATTAGGCATGGTTGTTTTAA
Bacillus_sp._NBRC_15313            ACCGTACCATGTGCGCATGACGATTCCGCCGTTTGGCATTTCCATTTTGC
Geobacillus_sp._Y412MC61           ACCGTACCATGTGCGCATGACGATTCCGCCGTTTGGCATTTCCATTTTGC
G.stearothermophilus               GCCATATCATATTCAGATGACGATCCCGCCGTTTGGCATTTCTATTTTAA
                                   ..*  * ..  *  . ** *.*.* ** **.**:*****    .****..

Geobacillus_sp._MGI_               GGCCAGTGCAAAAACGAG--------------------------------
G._thermodenitrificans_NG80-2      GGCCAGTGCAAAAACGAG--------------------------------
Geobacillus_sp._Y412MC10           AGAAAAAGCCGCCTCGAT--------------------------------
Bacillus_sp._NBRC_15313            GGCCAGTGCAAAAACGAG--------------------------------
Geobacillus_sp._Y412MC61           GGCCAGTGCAAAAACGAG--------------------------------
G.stearothermophilus               GACCAGTAAAAAAAGGTAGCGTCAAAAGTTTTATGAAAACTCCACATCCG
                                   ....*.:......: *:

Geobacillus_sp._MGI_               --------------------------------------------------
G._thermodenitrificans_NG80-2      --------------------------------------------------
Geobacillus_sp._Y412MC10           --------------------------------------------------
Bacillus_sp._NBRC_15313            --------------------------------------------------
Geobacillus_sp._Y412MC61           --------------------------------------------------
G.stearothermophilus               CCATCCCATGGAGCATCGTAAGGCATCCTTGGAGCCGGATTCGCCCTTGA

Geobacillus_sp._MGI_               --------------GGGAGAGAAAG-------------------------
G._thermodenitrificans_NG80-2      --------------GGGAGAGAAAG-------------------------
Geobacillus_sp._Y412MC10           --------------CGAGGAAATCG-------------------------
Bacillus_sp._NBRC_15313            --------------GGGAGAGAAAG-------------------------
Geobacillus_sp._Y412MC61           --------------GGGAGAGAAAG-------------------------
G.stearothermophilus               CCAACACCCGCCAAAGGTGTGAAAGGGACGTCAAGGGCGACGGGGACAAA
                                                  *. *:.*:.*

Geobacillus_sp._MGI_               --------------------------------------------------
G._thermodenitrificans_NG80-2      --------------------------------------------------
Geobacillus_sp._Y412MC10           --------------------------------------------------
Bacillus_sp._NBRC_15313            --------------------------------------------------
Geobacillus_sp._Y412MC61           --------------------------------------------------
G.stearothermophilus               AAAGAGGGCATAGGAAAGCCGCCCTTGCCCTTACCGAATTTTACCTTTGA

Geobacillus_sp._MGI_               -------CAGAATGAAGAAGAAGTGCATCGCCATGTTATTGG--------
G._thermodenitrificans_NG80-2      -------CGAAATGAAAAAGAAATGCATCGCCATGTTATTGG--------
Geobacillus_sp._Y412MC10           -------CAAGATCGATCG--GACGCGTCGCTGAAGGCGGCA--------
Bacillus_sp._NBRC_15313            -------CAGAATGAAGAAGAAGTGCATCGCCATGTTATTGG--------
Geobacillus_sp._Y412MC61           -------CAGAATGAAGAAGAAGTGCATCGCCATGTTATTGG--------
G.stearothermophilus               CGAGGTTCGGTTGGTCAAGGGTTCGCTTCGCCGAATCCGGCTGTTCTTCT
                                          *.. :   . ..     ** **** .:.  .
```

```
Geobacillus_sp._MGI_               ------------CCGGCGGGCAAGGAAGCCGGCTTCGCTCGCTGACGAAA
G._thermodenitrificans_NG80-2      ------------CCGGCGGGCAAGGAAGTCGGCTTCGCTCGCTGACGACA
Geobacillus_sp._Y412MC10           ------------GCGGCAAGAGAGAAAACAGAACAAAAAACGAAACGGAG
Bacillus_sp._NBRC_15313            ------------CCGGCGGGCAAGGAAGCCGGCTTCGCTCGCTGACGAAA
Geobacillus_sp._Y412MC61           ------------CCGGCGGGCAAGGAAGCCGGCTTCGCTCGCTGACGAAA
G.stearothermophilus               GATCCATGGGCTCCGGCGGACAAAAAAGTTAGGCTGCCTCTTG-TTGGAG
                                                ****.....*..**.  ..  :  .:.    : *...

Geobacillus_sp._MGI_               AACATCGCGAAACCGGCCGTGCCGTTTGGGGGGAAGTACCGGATCATTGA
G._thermodenitrificans_NG80-2      AACATCGCTAA---------------------------------------
Geobacillus_sp._Y412MC10           CAAAAGGGGGAAGCAGGATTGA----------------------------
Bacillus_sp._NBRC_15313            AACATCGCGAAACCAGCCGTGCCGTTTGGGGGGAAGTACCGGATCATTGA
Geobacillus_sp._Y412MC61           AACATCGCGAAACCAGCCGTGCCGTTTGGGGGGAAGTACCGGATCATTGA
G.stearothermophilus               GAAATCTTGAGCCATGGCGATCAGCTTCGTCCACCGGGCCGGCATATGGG
                                    *.*:    ..

Geobacillus_sp._MGI_               TTTTACGTTGAGCAATTGCACGAACTCGGGTATTGACACAGTCGGGGTGT
G._thermodenitrificans_NG80-2      --------------------------------------------------
Geobacillus_sp._Y412MC10           --------------------------------------------------
Bacillus_sp._NBRC_15313            TTTTAC--------------------------------------------
Geobacillus_sp._Y412MC61           --------------------------------------------------
G.stearothermophilus               GCAGATCGG-----------CGAGCTC-----------------------
```

(*)indicates positions which have a single, fully conserved residue.

(:)indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

(.) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.

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Appendix F

```
>gb|CP002442.1| Download subject sequence CP002442 spanning the HSP Geobacillus sp.
Y412MC52, complete genome

Length=3628883
Sort alignments for this subject sequence by:
E value
Score
Percent
identity
Query start position
Subject start position

Features in this part of subject sequence:
hypothetical protein transposase IS116/IS110/IS902 family protein

 Score = 1053 bits (570),  Expect = 0.0
 Identities = 676/722 (94%), Gaps = 28/722 (3%)
 Strand=Plus/Plus

Query  545      TCCCTCCCTCTAGAAATATTGATCTATCAAGGCTTTTCGGCCCCTCAGGGGTGTCCAAAA  604
                ||||||||||||||||| ||||| ||||||||||||||||||||||||||||||||||||
Sbjct  3070184  TCCCTCCCTCTAGAAATGTTGATTTATCAAGGCTTTTCGGCCCCTCAGGGGTGTCCAAAA  3070243

Query  605      AATATTTTTCGACAAAATCCCTTACATCCTTTTTCAGTTTTGTGGATATCTTACAGACCT  664
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3070244  AATATTTTTCGACAAAATCCCTTACATCCTTTTTCAGTTTTGTGGATATCTTACAGACCT  3070303

Query  665      AGGGTGCGCTTCGCGGCCACGGCTGGACATATTTTCCTGCCGTGGG-AGGAAGCGTATTC  723
                ||||||||||||||||||||||||||  |||||||||| ||||||| || ||||||||||
Sbjct  3070304  AGGGTGCGCTTCGCGGCCACGGCTGGGGATATTTTCCTTCCGTGGGGAG-AAGCGTATTC  3070362

Query  724      GATGCACCCTTTGGATCTCCGCGTCGCTGATGAGGACGAACCCTCCCATGTCTCCGGGCG  783
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3070363  GATGCACCCTTTGGATCTCCGCGTCGCTGATGAGGACGAACCCTCCCATGTCTCCGGGCG  3070422

Query  784      TCTTCGCGCCAACATTCCCTCGTTCGGTCTCGTCATCAGGCGGAGGCCGGCCAAGCTCCT  843
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3070423  TCTTCGCGCCAACATTCCCTCGTTCGGTCTCGTCATCAGGCGGAGGCCGGCCAAGCTCCT  3070482

Query  844      TTGGGGACTCAAGGTCGAATGGATGAAATCACGCCAGCTTCTCCGGTGTCATCCTGTTGT  903
                |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  3070483  TTGGGGACTCAAGGTCGAATGGATAAAATCACGCCAGCTTCTCCGGTGTCATCCTGTTGT  3070542

Query  904      CCAACGATTCTTCCGTTCGCAGGGGGAGGCCTTAGGCCGCCCGTTGCACCTGGGACAAGA  963
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3070543  CCAACGATTCTTCCGTTCGCAGGGGGAGGCCTTAGGCCGCCCGTTGCACCTGGGACAAGA  3070602

Query  964      CGTCCTGTTTCATTCGCTCCGCGTCAAACGCTTGTTTCTTCGTACAAATCGCAA-CAGCA  1022
                |||||||||||||||||||||||||||| ||||||||||||||||||||||||| |||||
Sbjct  3070603  CGTCCTGTTTCATTCGCTCCGCGTCAAAGGCTTGTTTCTTCGTACAAATCGCAAACAGCA  3070662

Query  1023     CATTCAACAGCTTCCGGCACAG-GCGACGATGGACTGCTTTCCGGTCAGCGGATTGACGG  1081
                ||||||||||||||||||| |  |||||||||||||||||||||||||||||||||||||
Sbjct  3070663  CATTCAACAGCTTCCGGCATAATGCGACGATGGACTGCTTTCCGGTCAGCGGATTGACGG  3070722

Query  1082     GGCGGGTCGTATA-TACCTCATGCAGCTCGCGAAACGCTTCGTTGTGC-G-ATCAGCGGA  1138
                |||||||||| || ||  |||||||||||||||||||| ||||||||| | |||||||||
Sbjct  3070723  GGCGGGTCGTGTAATAG-TCATGCAGCTCGCGAAACGCCTCGTTGTGCCGGATCAGCGGA  3070781

Query  1139     -TCATCGCTCGAA-CAGCACCGATCGCAGCGGTTTC-GTCC--GATTTG-AGATGTGCTT  1192
                 ||||||| |||| |||||||||||||||| ||||| ||||  | |||| ||||||||||
Sbjct  3070782  ATCATCGCCCGAAACAGCACCGATCGCAGCCGTTTCCGTCCCCG-TTTGGAGATGTGCTT  3070840

Query  1193     T-GCC-TG-GC-CTGAC-G-AG-A-TTC-CTT-GAGCGTCAGGCTGGCCA-CTTTCAC-A  1240
                | ||| |  || ||| | | || | ||| ||| |||||||||||  |||| ||| ||| |
Sbjct  3070841  TTGCCCTTTGCGCTGGCCGGAGGAGTTCTCTTTGAGCGTCAGGCCCGCCAACTT-CACCA  3070899

Query  1241     AT  1242
                ||
Sbjct  3070900  AT  3070901
```


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BIODATA OF STUDENT

The student was born in Alor Setar, Kedah on May 10th 1984. She studied in Sekolah Rendah Kebangsaan Treacher Methodist, Taiping and Sekolah Rendah Seafield, Subang Jaya for primary education. She went to Sekolah Kebangsaan Seafield in 1999 for lower secodary and continued in MRSM Balik Pulau in 2002 for her upper secondary education. She received her degree of Bachelor of Science majoring in Biotechnology, Biochemistry and Molecular Biology from University of Melbourne, Australia in 2007. She then continued her study for Master of Science in the field of catalysis and molecular biology under Faculty of Science. After the completion of her Master degree, she plans to pursue her study for PhD for her passion in research.

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