REACTION CONTROL AND PROTEIN ENGINEERING OF *Bacillus lehensis* G1 MALTOGENIC AMYLASE FOR HIGHER MALTO-OLIGOSACCHARIDE SYNTHESIS

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Untuk Mak dan Abah tercinta

Jasa dan pengorbananmu tidak ternilai harganya

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"Say, If the sea were ink for [writing] the words of my Lord, the sea would be exhausted before the words of my Lord were exhausted, even if We brought the like of it as a supplement." (Qur'an 18:109)

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ABSTRACT

A multi-functional maltogenic amylase (MAG1) from alkaliphilic *Bacillus lehensis* G1 exhibited remarkable hydrolysis and transglycosylation activity to produce malto-oligosaccharides of various lengths. MAG1 demonstrated hydrolysis activity over wide range of substrates. Kinetic analysis revealed that the enzyme hydrolyzed small substrate more efficiently than the larger substrate. This was shown by lower Michaelis constant (K_m) value and higher turnover number (k_{cat}) and second order rate constant (k_{cat}/K_m) values for β-cyclodextrin compared to that of soluble starch. Malto-oligosaccharide synthesis by transglycosylation activity of MAG1 faces problem of product re-hydrolyzation due to the hydrolysis activity of the enzyme. An equilibrium-control reaction approach has been successfully employed to improve malto-oligosaccharides production by decreasing hydrolysis activity. A yield of 38% transglycosylation products was obtained with the presence of malto-oligosaccharides longer than maltoheptaose. The addition of organic solvents demonstrated an increase in the transglycosylation-to-hydrolysis ratio from 1.29 to 2.15. The transglycosylation activity of MAG1 was also successfully enhanced by using structure-guided protein engineering approach. A molecular modeling and substrate docking was performed to study the structure-function relationship for rational design. A unique subsite structure which has not been reported in other maltogenic amylases was revealed and the information was used to design mutants that have active sites with reduced steric interference and higher hydrophobicity properties to increase the transglycosylation activity. Mutations decreased the hydrolysis activity of the enzyme and caused various modulations in its transglycosylation property. W359F, Y377F and M375I mutations caused reductions in steric interference and alteration of subsite occupation. In addition, the mutations increased internal flexibility to accommodate longer donor/acceptor molecule for transglycosylation, resulted in increased transglycosylation to hydrolysis ratio of up to 4.0-fold. The increase of the active site hydrophobicity from W359F and M375I mutations reduced concentration of maltotriose used as donor/acceptor for transglycosylation to 100 mM and 50 mM, respectively compared to 200 mM of the wild-type. The improvement of the transglycosylation to hydrolysis ratio by 4.3-fold was also demonstrated by both mutants. Interestingly, reductions of both steric interference and hydrolysis by Y377F and W359F mutations caused a synergistic effect to produce malto-oligosaccharides with higher degree of polymerization than the wild-type. These findings showed that the transglycosylation activity of MAG1 was successfully improved by controlling water activity and modification of the active site structure. The high transglycosylation activity of MAG1 and mutants offers a great advantage for synthesizing malto-oligosaccharides and rare carbohydrates.

ABSTRAK

Amilase maltogenik pelbagai fungsi (MAG1) daripada bakteria alkalifilik, *Bacillus lehensis* G1 menunjukkan aktiviti hidrolisis dan pentransglikosilan tinggi untuk menghasilkan malto-oligosakarida yang mempunyai panjang rantai yang berbeza. MAG1 menunjukkan hidrolisis terhadap pelbagai jenis substrat. Analisis kinetik menunjukkan ia menjalankan hidrolisis lebih berkesan terhadap substrat kecil berbanding substrat besar. Ini dibuktikan melalui nilai pemalar Michaelis (K_m) yang lebih rendah dan nombor perolehan (k_{cat}) dan pemalar kadar tertib kedua (k_{cat}/K_m) yang lebih tinggi oleh β-siklodekstrin berbanding kanji larut. Sintesis malto-oligosakarida oleh amilase maltogenik sering berhadapan dengan hidrolisis semula produk disebabkan kebolehan enzim tersebut menjalankan aktiviti hidrolisis. Kaedah pengawalan keseimbangan tindakbalas telah digunakan untuk mengurangkan aktiviti hidrolisis justeru meningkatkan penghasilan malto-oligosakarida. Sebanyak 38% produk pentransglikosilan dengan kehadiran malto-oligosakarida lebih panjang daripada maltoheptosa berjaya dihasilkan. Penambahan pelarut organik meningkatkan nisbah pentransglikosilan kepada hidrolisis daripada 1.29 kepada 2.15. Aktiviti pentransglikosilan MAG1 juga ditingkatkan menggunakan kaedah kejuruteraan protein berpandukan struktur. Pemodelan molekul dan dok substrat dijalankan untuk mengkaji hubungkait struktur-fungsi untuk melaksanakan reka bentuk rasional. Struktur unik subtapak yang tidak pernah dilaporkan oleh amilase maltogenik lain telah dikenalpasti dan maklumat ini digunakan untuk mereka bentuk mutan yang mempunyai ciri tapak aktif yang kurang gangguan sterik dan tinggi hidrofobisiti untuk meningkatkan aktiviti pentransglikosilan enzim. Mutasi mengurangkan aktiviti hidrolisis enzim dan menyebabkan pelbagai perubahan pada ciri-ciri pentransglikosilan. Mutasi W359F, Y377F dan M375I mengurangkan gangguan sterik dan mengubah pendudukan subtapak. Mutasi turut meningkatkan fleksibiliti struktur dalaman untuk menampung molekul penderma/penerima yang lebih panjang dan meningkatkan nisbah pentransglikosilan kepada hidrolisis sebanyak 4.0 kali ganda. Peningkatan hidrofobisiti tapak aktif melalui mutasi W359F dan M375I menyebabkan pengurangan kepekatan maltotriosa yang digunakan sebagai molekul penderma/penerima untuk pentransglikosilan kepada masing-masing 100 mM dan 50 mM berbanding 200 mM oleh MAG1. Kenaikan nisbah pentransglikosilan kepada hidrolisis sebanyak 4.3 kali ganda juga ditunjukkan oleh kedua-dua mutan. Menariknya, pengurangan gangguan sterik dan aktiviti hidrolisis melalui mutasi Y377F dan W359F memberikan kesan sinergi untuk menghasilkan malto-oligosakarida yang lebih panjang daripada MAG1. Keputusan ini menunjukkan aktiviti pentransglikosilan MAG1 berjaya ditingkatkan dengan pengawalan aktiviti air dan pengubahsuaian struktur tapak aktif. Aktiviti pentransglikosilan yang tinggi ditunjukkan oleh MAG1 dan mutan menawarkan kelebihan yang besar untuk mensintesis malto-oligosakarida dan karbohidrat nadir.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

2-ME	-	2-mercaptoethanol
3D	-	three-dimensional
A280	-	absorbance at optical density of 280 nm
A600	-	absorbance at optical density of 600 nm
Ala or A	-	alanine
Al^{3+}	-	aluminium ion
Arg or R	-	arginine
Asn or N	-	asparagine
Asp or D	-	aspartic acid
B. clausii	-	*Bacillus clausii*
B. lehensis	-	*Bacillus lehensis*
B. licheniformis	-	*Bacillus licheniformis*
B. stearothermophilus	-	*Bacillus stearothermophilus*
B. subtilis	-	*Bacillus subtilis*
BLAST	-	Basic Local Alignment Search Tool
B. adolescentis	-	*Bifidobacterium adolescentis*
BSA	-	bovine serum albumin
C	-	carbon
Ca^{2+}	-	calcium ion
CD	-	cyclodextrin
Co^{2+}	-	cobalt ion
Cu^{2+}	-	cuprum ion
Cys or C	-	cysteine
CV	-	column volume
Da	-	Dalton
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleotide

DMSO	-	dimethyl sulphoxide
E. coli	-	*Escherichia coli*
EC	-	Enzyme Commission
EDTA	-	ethylenediamine tetraacetic acid
Fe^{2+}	-	ferrous ion
Fe^{3+}	-	ferric ion
FPLC	-	Fast protein liquid chromatography
g	-	gram
GC	-	guanine and cytosine
G. thermoleovorans	-	*Geobacillus thermoleovorans*
GH	-	Glycosyl Hydrolase
Gln or Q	-	glutamine
Glu or E	-	glutamic acid
Gly or G	-	glycine
h	-	hour
H	-	hydrogen
Hg^{2+}	-	mercury ion
His or H	-	histidine
HPLC	-	high-performance liquid chromatography
I^{2+}	-	iodide ion
I or Ile	-	isoleucine
IPTG	-	isopropyl β-D-1-thiogalactopyranoside
K	-	Kelvin
K^{+}	-	potassium ion
kcal	-	kilo calorie
kDa	-	kilodalton
kJ	-	kilo joule
l	-	liter
L. gasseri	-	*Lactobacillus gasseri*
L or Leu	-	Leucine
LB	-	Luria-Bertani
Li^{2+}	-	lithium ion
Lys or K	-	lysine
m	-	mili

M	-	molar
M1	-	glucose
M2	-	maltose
M3	-	maltotriose
M4	-	maltotetraose
M5	-	maltopentaose
M6	-	maltohexaose
M7	-	maltoheptaose
MAG1	-	maltogenic amylase from *Bacillus lehensis* G1
Met or M	-	methionine
mg	-	milligram
Mg^{2+}	-	magnesium ion
$MgSO_4$	-	magnesium sulphate
min	-	minute
ml	-	milliliter
mM	-	milimolar
Mn^{2+}	-	manganese ion
MW	-	molecular weight
Mx	-	malto-oligosaccharides longer than maltoheptaose
Na^{+}	-	sodium ion
NEB	-	New England Biolabs
NCBI	-	The National Center for Biotechnology Information
Ni^{2+}	-	nickel ion
nm	-	nanometer
O	-	oxygen
OH	-	hydroxyl
Pb^{2+}	-	lead ion
PCR	-	polymerase chain reaction
F or Phe	-	phenylalanine
pI	-	isoelectric point
PMSF	-	phenylmethylsulfonyl fluoride
Pro or P	-	proline
PSI-BLAST	-	Position-Specific Iterated BLAST
RMSD	-	root mean square deviation

rpm	-	revolutions per minute
s	-	second
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser or S	-	serine
sp.	-	species
T. vulgaris	-	*Thermoactinomyces vulgaris*
T. pendens	-	*Thermofilum pendens*
T. volcanium	-	*Thermoplasma volcanium*
T. maritime	-	*Thermotoga maritime*
T. thermophiles	-	*Thermus thermophiles*
Thr or T	-	threonine
TLC	-	thin-layer chromatography
Trp or W	-	tryptophan
Tyr or Y	-	tyrosine
U	-	unit
USD	-	United State Dollar
UV	-	ultraviolet
UV-VIS	-	ultraviolet-visible spectrophotometry
V	-	volt
V or Val	-	valine
v/v	-	volume per volume
w/v	-	weight per volume
w/w	-	weight per weight
X. dendrorhous	-	*Xanthophyllomyces dendrorhous*
Zn^{2+}	-	zinc ion
α	-	alpha
Å	-	Angstrom
β	-	beta
β-CD	-	β-cyclodextrin
°C	-	degree celcius
γ	-	gamma
µ	-	micro

μm	-	micro meter
-	-	minus
%	-	percent
π	-	pi
φ	-	phi
+	-	plus
Ψ	-	psi

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background: The catalyst of life

A living cell may look tiny and insignificant, but inside, it is a sea of tremendous interrelated biochemical activities that sustain life. The biochemical activities inside the cell involved various chemical transformations to maintain growth. The rate of a biochemical reaction is far too slow to support metabolism of a cell if it occurs spontaneously. These metabolic reactions are therefore accelerated a million times faster by a biochemical catalyst called enzyme. The word 'enzyme' (from the Greek, meaning 'in yeast') was proposed by Wilhelm Kühne in 1878 to differentiate between the chemical substances having catalytic properties with 'ferments', the microbes (Barnett, 2003). A new science of biochemistry emerged in the late of the eighteenth century, after a chemist, Eduard Büchner discovered that a 'juice' that was extracted from yeast, is capable of converting sugar to alcohol and carbon dioxide. The discovery led to the award of a Nobel Prize and the birth of modern biochemistry (Manchester, 2000). Today, the knowledge has expanded that the use of enzymes for catalyzing various in vitro biochemical reactions has become so common in everyday applications.

When the human genome sequence was available, researchers were surprised by the fewer number of the protein encoded for such a very complex organism. Then, they discovered that many proteins or enzymes were actually multi-functional (Jeffery, 2004). Enzymes are highly specific to their substrates and catalyzing the

reactions. However, some enzymes are also capable of catalyzing more than one reaction. It was first observed in 1890s when Arthur Croft Hill found that the hydrolysis of maltose to glucose by yeast maltase was incomplete due to another reaction that took place at the same time. He discovered that in a concentrated solution, polymerization of glucose by a reversible process occurred along with hydrolysis until the equilibrium state was reached (Manchester 2000). This proved that some enzymes are indeed capable of catalyzing more than one reaction.

Due to its growing interest, numerous multi-functional enzymes are being discovered. Glycosyl hydrolase enzymes are one of the enzymes that can catalyze two reactions, which are hydrolysis that cleaves substrate to smaller products, or transglycosylation that joins two molecules to produce a larger or longer product. Maltogenic amylase (glucan-1,4-alpha-maltohydrolase EC 3.2.1.133) is an amylolytic enzyme from glycosyl hydrolase family 13 (GH 13) that exhibits multi-functional property. Unlike typical α-amylases, maltogenic amylase demonstrates multi-substrates specificity and prefers cyclodextrins (CDs) as a substrate. In addition to hydrolysis, maltogenic amylase demonstrates synthetic activity known as transglycosylation to produce sugar molecules with various lengths (Kim *et al.*, 1999b). This enzyme with multiple catalytic activities becomes an interesting subject to explore. It is important to elucidate the mechanism beneath its multi-functionality of performing both hydrolysis and transglycosylation. The enzyme can act as a biochemical switch which response is regulated by the changes of the surrounding condition (Jeffery, 2004). The switching point can be explained through structural basis and the understanding of this biological role will lead to a subsequent improvement of the existing biochemical catalyst.

Maltogenic amylase can be specified as a promiscuous enzyme characterized by having a catalytic domain that employs various substrates and executes multiple biochemical reactions. Extensive researches are being carried out on this valuable enzyme with substrate and catalytic promiscuity as it has the potential to be exploited in synthetic application and development of novel synthesis pathway through protein engineering (Hult and Berglund, 2007). The transglycosylation activity of maltogenic amylase offers a great advantage in the industry for the synthesis of

oligosaccharides. Oligosaccharides offer various health benefits and diverse applications in improving physicochemical properties of foods. Conventionally, oligosaccharides were synthesized using chemical methods. However, due to its laborious steps, high cost and low yield, enzymatic synthesis has become a preferred alternative. Glycosyl transferase and glycosyl hydrolase enzymes have been employed, but the latter is favored because it uses simpler and inexpensive substrates. However, the bottleneck is synthesis reaction competes with hydrolysis resulting in a low yield of oligosaccharides produced.

The increased market demand for oligosaccharides has heightened the need of an efficient biocatalyst. The major improvement in DNA technology and bioinformatics over the years has promoted the advances of enzyme or protein engineering. Now, the discovery of novel enzymes and the availability of complete crystal structure data have enabled researchers to tailor the existing biocatalyst to fit the reaction specifications (Bornscheuer *et al.*, 2012). The study of a crystal structure of maltogenic amylase has elucidated the structure-function relationship to explain the multi-substrate and multi-functional properties of the enzyme. The role of extra N-terminal residues in the formation of domain-swapped homodimer was responsible for the multi-substrate specificity of the enzyme (Kim *et al.*, 1999a; Lee *et al.*, 2002a). In addition, scientists have discovered an extra space that resides at the bottom of the active site cleft for accommodating small acceptor sugar molecules that involved in transglycosylation (Kim *et al.*, 1999a). This understanding has provided a crucial base in re-designing the existing maltogenic amylase (MAG1) from *Bacillus lehensis* G1 for improving transglycosylation property to achieve the objective of the current study.

1.2 Problem statement

Oligosaccharides can be synthesized chemically, but enzymatic synthesis has generally been the first choice because it employs milder conditions, involves simpler steps and eliminates the need for hydroxyl group protection (Hansson *et al.*,

2001). Glycosyl transferase (EC 2.4) and glycosyl hydrolase (EC 3.2) are the two candidate enzyme classes that have been used in oligosaccharides synthesis (Bucke, 1996). Glycosyl hydrolase is preferred for oligosaccharides production because this enzymes can use simple and inexpensive acceptor sugar molecules (Hansson *et al.*, 2001). However, its major shortcoming is the inevitable hydrolysis activity of the enzyme that causes the synthesized oligosaccharides to be hydrolyzed again (Hinz *et al.*, 2006). Various strategies have been employed to overcome the problem, including the control of thermodynamic equilibrium of the enzyme reaction by the elimination of water, which is a competing nucleophile, for transglycosylation. The incorporation of an organic medium into the reaction mixture has been shown to increase the synthesis of galacto-oligosaccharide by β-glycosidase (Cruz-Guerrero *et al.*, 2006). However, no study on the influence of organic solvents on malto-oligosaccharide synthesis by maltogenic amylase has been reported to date. Protein engineering approach has also become a common practice to improve the transglycosylation activity and decrease the hydrolysis activity of the enzyme especially when the use of high substrate concentration is not favorable for industrial application and organic solvent is often avoided when the product is targeted for food additive. Therefore, through protein engineering, the enzyme with desired properties can be obtained by re-constructing or re-designing the active site.

1.3 Objectives of the study

The objectives of this research are to improve the transglycosylation property of MAG1 by using reaction equilibrium control and protein engineering approach for production of malto-oligosaccharide and to study amino acids that are important for hydrolysis and transglycosylation.

1.4 Scopes of the study

This study focusses on the improvement of transglycosylation activity of MAG1 for malto-oligosaccharide production by appropriate strategies. Hence, the following scopes were outlined to achieve the objective:

i. Cloning, expression, purification and characterization of MAG1 in *Escherichia coli* expression system.
ii. Kinetic study of MAG1 hydrolysis activity on various substrates and products determination.
iii. Reaction study of transglycosylation activity by MAG1 on various sugar donors and acceptors.
iv. Reaction equilibrium control to reduce hydrolysis activity and increase transglycosylation activity by suppressing water activity.
v. Construction of the 3D structure of MAG1, docking of substrates into MAG1 active site and subsite structure determination.
vi. Rational design targeted for suppressing hydrolysis activity and improving transglycosylation activity of MAG1 for higher malto-oligosaccharide synthesis.
vii. Mutant construction using site-directed mutagenesis, cloning, expression, purification, characterization and reaction studies (hydrolysis and transglycosylation) of the mutants.

1.5 Rationale and novelty of the study

Exploring new enzyme source is important because a different source of enzymes exhibits their own unique characteristic even though they have some similarities in terms of function, substrate they act upon or their structure. Moreover, continuous offering of data regarding this enzyme could be beneficial to the scientific community and provide more knowledge in understanding this type of the biocatalyst. The potential of maltogenic amylase for the synthesis of carbohydrate has been recognized by researchers. Many scientific studies dealing with this

enzyme for transglycosylation are only focusing on the production of transfer products from specific carbohydrate donor and acceptor. The production of malto-oligosaccharide by maltogenic amylase has a few been reported. However, the use of reaction equilibrium control approach to reduce the hydrolysis activity of maltogenic amylase for increased transglycosylation has yet to be reported in literature and little is known about the effects of organic solvents on the production of malto-oligosaccharide by maltogenic amylase. This report is the first to describe the optimization of reaction conditions and the incorporation of a water-miscible organic solvent to suppress hydrolysis activity during malto-oligosaccharide production by maltogenic amylase. The present study showed that the addition of an organic solvent could be used to produce malto-oligosaccharides with degrees of polymerization higher than maltoheptaose. The findings demonstrated that MAG1 is a promising candidate for carbohydrate synthesis applications.

Analysis of homology modeling and docking of malto-oligosaccharides in this study reveals a novel finding regarding the subsite structure of MAG1 which is different from the reported subsite structures of other maltogenic amylases. This finding leads to a proposed mode of action for β-cyclodextrin (β-CD) hydrolysis that describes how maltose is mainly produced from the hydrolysis. Protein engineering combined with site-directed mutagenesis has also been successfully employed in this study to shift the enzyme activity toward favoring transglycosylation and subsequently to increase the production of malto-oligosaccharide. Although the protein engineering approach has commonly been employed to improve transglycosylation activity of maltogenic amylase, the production of longer malto-oligosaccharides has not yet been reported. The mutants MAG1 have produced malto-oligosaccharides longer than that of the wild-type. Longer oligosaccharides are desirable as prebiotics because they are less fermentable and, therefore, can reach the most distal area of the colon (Voragen, 1998). Moreover, the understanding of the structural modifications generated from the mutation of specific amino acids in this study will also contribute knowledge for better understanding of the structure-function of maltogenic amylase and related enzymes.

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APPENDIX A: List of publications

1. Nor Hasmaliana Abdul Manas, Mohd Anuar Jonet, Abdul Munir Abdul Murad, Farah Diba Abu Bakar, Nor Muhammad Mahadi, Rosli Md. Illias (2016). Computational docking simulations and subsite structure analysis of a maltogenic amylase from *Bacillus lehensis* G1 provide insights into hydrolysis product specificity. Journal of Molecular Graphics and Modeling. Manuscript submitted.
2. Nor Hasmaliana Abdul Manas, Mohd Anuar Jonet, Abdul Munir Abdul Murad Nor Muhammad Mahadi and Rosli Md. Illias (2015). Modulation of transglycosylation and improved maltooligosaccharide synthesis by protein engineering of maltogenic amylase from *Bacillus lehensis* G1. Process Biochemistry. 50: 1572–1580
3. Nor Hasmaliana Abdul Manas, Samson Pachelles, Nor Muhammad Mahadi and Rosli Md. Illias (2014). The characterisation of an alkali-stable maltogenic amylase from *Bacillus lehensis* G1 and improved malto-oligosaccharide production by hydrolysis suppression. PLoS ONE. 9(9): e106481.
4. Rosli Md. Illias, Nor Hasmaliana Abdul Manas, Mohd Anuar Jonet and Abdul Munir Abdul Murad (2016). Improvement of transglycosylation activity of Bacillus lehensis G1 maltogenic amylase for higher malto-oligosaccharide synthesis. The 7th AFOB Regional Symposium 2016. January 28-30, 2016. Hue City, Vietnam.
5. Nor Hasmaliana Abdul Manas and Rosli Md Illias (2015). Structural Interpretation for Hydrolysis and Transglycosylation Characteristics of Maltogenic Amylase from Bacillus lehensis G1. The 20th Biological Sciences Graduate Congress (20th BSGC). December 9-11, 2015. Bangkok, Thailand.
6. Rosli Md Illias, Goh Kian Mau, Nor Hasmaliana Abdul Manas. (2014). Structural insight into the catalytic site of multifunctional starch degrading enzymes. *The 2nd International Symposium and Workshop on Functional Genomics and Structural Biology.* 21 – 24 January 2014. Selangor, Malaysia.

7. Nor Hasmaliana Abdul Manas, Samson Pachelles, Abdul Munir Abdul Murad, Nor Muhammad Mahadi and Rosli Md Illias. (2013). High Maltotriose-Producing Maltogenic Amylase from *Bacillus lehensis* G1 and Preliminary Investigation on its Multifunctionality. *International Congress of the Malaysian Society for Microbiology (ICMSM2013).* 12 – 15 December 2013. Langkawi, Malaysia.

8. Nor Hasmaliana Abdul Manas, Samson Pachelles, Nor Muhammad Mahadi, Rosli Md. Illias. (2013). A Novel Hydrolysis Pattern of a High Maltotriose Producing Maltogenic Amylase from *Bacillus lehensis* G1. *Kolokium Biologi Struktur Ke-V.* 3 June 2013. Melaka, Malaysia.

9. Nor Hasmaliana Abdul Manas, Abdul Munir Abdul Murad, Nor Muhammad Mahadi, Rosli Md. Illias. (2010). Enhancement of Solubility in *Escherichia coli* and Characterization of Cold-Adapted α-Amylase from *Leucosporidium antarcticum* PI12. *Zamalah Graduate Colloquium*. 15 July 2010. Skudai, Malaysia.

10. Nor Hasmaliana Abdul Manas, Rosli Md Illias, Noor Azah Jema'on, Nor Muhammad Mahadi and Amir Rabu. (2010). Transformation of Na^+/H^+ Antiporter Gene from Alkaliphilic *Bacillus lehensis* G1 Increases Salt Tolerance to *Escherichia coli*. *3rd International Conference Southeast Asian Natural Resources and Environmental Management (SANREM 2010).* 3 – 5 August 2010. Sabah, Malaysia.

11. Nor Hasmaliana Abdul Manas, Rosli Md Illias, Noor Azah Jema'on, Nor Muhammad Mahadi and Amir Rabu (2010). Expression and Functional Studies of Na^+/H^+ Antiporter from Alkaliphilic *Bacillus lehensis* G1. *11th Symposium of Malaysian Society of Applied Biology*. 13 – 15 June 2010. Kelantan, Malaysia.