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RECOMBINANT THERMOSTABLE MALTOGENIC AMYLASE FROM GEOBACILLUS SP. SK70 AND ITS VARIANTS

MOOHAMAD ROPANING SULONG

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By

MOOHAMAD ROPANING SULONG

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Chairman: **Prof. Dato' Abu Bakar Bin Salleh, PhD**

Institute: **Bioscience**

A thermostable maltogenic amylase-producing bacterium was successfully isolated from a hot spring located at Sungai Klah, Perak, at 70°C. The 16S rRNA gene sequence showed 99% similarity to *Geobacillus* sp. and was deposited in the GenBank with the accession number JN812978. A 1767 bp gene was successfully amplified and was cloned into pGEM-T cloning vector. The gene encodes for the extracellular enzyme revealed 97% identical to maltogenic amylase of *Geobacillus* sp. Gh6, α-cyclodextrinase of *Thermus* sp. YBJ-1 and *Geobacillus stearothermophilus*, and α-amylase of *Geobacillus* sp. Y412MC61. The gene was effectively expressed in *E. coli* BL21 (DE3) using pET102 Directional TOPO expression vector and showed highest specific activity of the intracellular enzyme (61 U/mg) after 12 h of post induction time using 0.02 mM IPTG at OD_{600} 0.5. Hence, the isolate was suggested as thermostable maltogenic amylase-producing *Geobacillus* sp. SK70 and was considered as the first ever to be expressed intracellularly using the pET102 Directional TOPO expression vector.

The intracellular enzyme was purified to homogeneity with 8.2 fold and 41% recovery through a single step purification using HisTrap HP affinity column chromatography. The optimum temperature and pH for the purified enzyme was at 55°C and pH 7.0 respectively, and showed broad range of pH stability ranging from pH 5.0 to pH 10.0. The activity of the purified enzyme was stable in the presence of 1 mM Ca^{2+}, was enhanced by 1 mM Zn^{2+} and 0.1 % (v/v) Tween-20, and was 20% inhibited by 1% (v/v) of 2-Mercaptolethanol, EDTA and SDS. Thus, the enzyme is considered as Ca^{2+}-independent that differs to most of reported maltogenic amylases.

Two single point mutations at positions Q294H and A550K were conducted to enhance the thermostability of the wild-type enzyme. The mutant Q294H revealed optimum temperature at 60°C as compared to the mutant A550K and the wild-type enzyme that showed optimum temperature at 55°C. The half-life of the mutant Q294H was found

to be 85 min comparing to 35 min of the wild-type enzyme and retained almost 80% of the enzyme activity in the presence of 1 mM of Mg^{2+}, $Ca^{2+}$and Fe^{2+} , and was not significantly affected by detergents, reducing agents and additives. Interestingly, the enzyme activity was enhanced by 1 mM Mn^{2+} and 0.1 % (v/v) Tween-20, thus demonstrated characteristic unlike other reported maltogenic amylases.

To study the effect of mutation on thermostability of the mutant enzymes, molecular dynamic (MD) simulation of both wild-type and mutant enzymes for 20 ns at 60°C was performed. The root mean square deviation (RMSD) for both wild-type and mutant enzymes showed no significant structural alteration has occurred. The relatively minor difference in radius of gyration (R_g) would provide a better structure compactness to the mutants. While lower solvent accessible surface area (SASA) value for the mutant Q294H comparing to the mutant A550K and the wild-type enzyme indicated the mutant Q294H would have better protein folding and subsequently would give better enzyme thermostability.

The circular dichroism (CD) spectra analysis for both mutant enzymes showed characteristic differences in their secondary structure as compared to the wild-type enzyme. Higher percentage of β-sheets (23.6%) than α-helices (16.7%) in the mutant Q294H comparing to the mutant A550K and the wild-type enzyme has given more stability to the protein folding. While higher melting temperature for mutant Q294H (79.31°C) as compared to the mutant A550K (41.66°C) and the wild-type enzyme (53.3°C) has offered good characteristics for industrial applications.

The results of the study of both wild-type enzyme and mutant Q294H demonstrated that these enzymes can be applied in various industrial sectors including foods industry and enzyme enriched detergent formulations. Further research for large scale production of mutant Q294H is recommended. Besides, the uniqueness of amylases produced by the other four isolates also should be further investigated.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

AMILASE MALTOGENIK REKOMBINAN YANG STABIL SUHU DARI *GEOBACILLUS* SP. SK70 DAN VARIANNYA

Oleh

MOOHAMAD ROPANING SULONG

Januari 2015

Pengerusi: **Prof. Dato' Abu Bakar Bin Salleh, PhD**

Institut: **Biosains**

Bakteria yang menghasilkan enzim maltogenik yang stabil pada suhu tinggi telah berjaya dipencilkan dari sumber mata air panas yang terletak di Sungai Klah, Perak pada suhu 70°C. Jujukan gen 16S rRNA telah menunjukkan 99% persamaan dengan *Geobacillus* sp. dan ianya telah di depositkan ke dalam GenBank dengan nomber aksesi JN812978. Gen sebanyak 1767 bp telah berjaya diamplifikasi dan diklon ke dalam pGEM-T vektor pengkolonan. Gen yang menghasilkan ekstrasel enzim itu telah menunjukkan 97% persamaan dengan amilase maltogenik dari *Geobacillus* sp. Gh6, α-siklodekstrinase dari *Thermus* sp. YBJ-1 dan *Geobacillus stearothermophilus*, dan α-amilase dari *Geobacillus* sp. Y412MC61. Gen tersebut telah diekspres dalam *E. coli* BL21 (DE3) dengan menggunakan pET102 Directional TOPO sebagai vektor pengekspresan dan telah menunjukkan enzim ekstrasel aktiviti spesefik tertinggi (61 U/mg) setelah 12 jam daripada masa post induksi dengan menggunakan 0.02 mM IPTG pada OD_{600} 0.5. Justeru itu, pencilan tersebut telah dicadangkan sebagai *Geobacillus* sp. SK70 yang menghasilkan maltogenik amilase intrasel tahan suhu panas dan ianya dianggap sebagai vektor pengekspresan pET102 Directional TOPO yang pertama kali digunakan untuk enzim tersebut.

Enzim intrasel tersebut telah ditulinkan sebanyak 8.2 kali ganda dengan 41% perolehan melalui penulinan langkah tunggal menggunakan kromatografi turus *HisTrap HP affinity*. Suhu dan pH optima bagi enzim yang telah ditulinkan itu masing masing adalah pada 55°C dan pH 7.0, dan telah menunjukan julat lebar kesatbilan pH antara pH 5.0 hingga pH 10.0. Aktiviti enzim tulin adalah stabil dengan adanya 1 mM Ca^{2+}, telah ditingkatkan dengan penambahan 1mM Zn^{2+} dan 0.1%(v/v)Tween20 dan sebahagiannya direncat dengan adanya 2-Mercaptolethanol, EDTA dan SDS. Justeru itu, enzim tersebut telah menunjukan ciri-cirinya selaku enzim bebas Ca^{2+} yang berlainan daripada kebanyakkan amilase maltogenik yang telah dilaporkan.

Dua mutasi titik tunggal pada posesi A550K dan Q294H telah dilaksanakan untuk meningkatkan kadar ketahanan suhu bagi enzim asal. Mutan Q294H telah

menunjukkan suhu optima yang lebih tinggi (60°C), manakala mutan A550K menunjukkan suhu optima yang sama dengan enzim asal. Separuh hayat bagi mutan Q294H adalah 85 minit berbanding 35 minit bagi enzim asal dan mengekalkan 80% dari aktiviti enzim dengan kehadiran 1 mM Mg^{2+}, Ca^{2+}dan Fe^{2+} , dan tidak terkesan dengan kehadiran pencuci, agen penurun dan juga bahan tambah. Yang menariknya, aktiviti enzim asal bertambah dengan adanya 1 mM Mn^{2+} dan 0.1% (v/v) Tween20, justeru itu, ianya telah menunjukan bahawa enzim tersebut mempunyai ciri yang berbeza berbanding kebanyakkan amilase maltogenik yang dilaporkan.

Bagi mengkaji kesan mutasi ke atas kadar stabil suhu terhadap enzim-enzim mutan, simulasi *molecular dynamic (MD)* keatas kedua-dua enzim asal dan enzim mutan telah dijalankan selama 20 ns pada suhu 60°C. *Root mean square deviation (RMSD)* bagi kedua-dua enzim asal dan mutan menunjukkan tiada perubahan struktur yang signifikan telah berlaku. Secara relatif, perubahan yang sedikit pada jejari legaran (R_g) akan memberi kepadatan struktur yang lebih baik kepada mutan-mutan tersebut. Manakala nilai *solvent accessible surface area (SASA)* yang lebih rendah bagi mutan Q294H berbanding dengan mutan A550K dan enzim asal telah menunjukkan bahawa mutan Q294H mempunyai lipatan protein yang lebih baik dan seterusnya menjadi lebih stabil suhu.

Analisa spektra dikroisme bulat (CD) bagi kedua-dua enzim mutan telah menunjukan perbezaan pada ciri-ciri struktur sekunder berbandingkan dengan enzim asal. Peratusan kepengan beta (23.6%) yang lebih tinggi daripada heliks alfa (16.7%) dalam mutan Q294H berbanding dengan mutan A550K dan enzim asal telah memberikan lebih kestabilan kepada lipatan protein. Manakala takat lebur yang lebih tinggi bagi mutan Q294H (79.31°C) berbanding dengan mutan A550K (41.66°C) dan enzim asal (53.3°C) telah menawarkan ciri-ciri yang baik untuk kegunaan industri.

Hasil kajian kedua-dua jenis enzim asal dan mutan Q294H telah menunjukkan bahawa enzim-enzim ini boleh digunakan dalam pelbagai sektor industri termasuk industri makanan dan bahan pencuci yang diperkayakan dengan enzim. Justeru itu, kajian selanjutnya untuk pengeluaran secara skala besar bagi mutan Q294H adalah disyorkan. Selain itu, keunikan amilase-amilase yang dihasilkan oleh empat pencilan yang lain juga perlu diteliti dengan lebih lanjut.

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A	adenine
bp	base pai
r	
C	cytosine
°C	degree centigrade
CCMB	calcium/manganese-based buffer
DNA	deoxyribonucleic acid
dH_2O	distilled water
G	guanine
g	gram
Xg	gravity
h	hour
IPTG	isopropyl-ß-thio-D-galactopyranoside
kb	kilobase pair
kDa	kilo Dalton
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MW	molecular weight
n.a	not available
ng	nanogram
OD	optical density

ORF	open reading frame
PBS	phosphate buffer
PCR	polymerase chain reaction
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED N,N,N,N	tetramethyl-ethylene diamine
T	thymine
U	unit of activity
uv	ultra violet
µM	micro molar
µg	microgram
µl	micro liter
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Enzymes production is one of the promising industries. The global value of the enzyme production had achieved to $ 2.4 billion with an average annual growth rate (AAGR) of 3.3% (Thakore, 2004). Globally, almost 75% of the total enzymes are manufactured by three top enzyme companies namely Novozymes (Denmark), DuPont (USA) and Roche (Switzerland). The market value for technical enzymes alone was estimated about $1 billion in 2010 and to reach $1.5 billion in 2015 at a 6.6% compound annual growth rate (CAGR). These technical enzymes are used as bulk enzymes in various applications including textile, pulp and paper industries, detergents, organic synthesis and biofuels industry (Li *et al*., 2012). Huge amount of enzymes that utilized in various industrial sectors in Malaysia are imported that caused the country to expend millions of ringgit for the imported enzymes. Therefore, it is very significant to locally produce the enzymes that can reduce the expense of imported enzymes and simultaneously to generate the economy of country.

About 60 million tonnes per annum of sago starch is produced in South-East Asia (Wang *et al*., 1996). These sago starches were used for glucose productions in Sarawak (Suryani, 2002) and were considered as the main carbohydrate source for Papua New Guinea nation (Greenhill *et al*., 2009). Hence, starch degrading enzymes such as amylases particularly the thermostable amylases are highly important in wide varieties of starch based food industries.

Maltogenic amylases (EC 3.2.1.133) possess unique characteristics including various catalytic capabilities for hydrolysing and/or synthesizing various glycosidic linkages including α-1,3, α-1,4 and α-1,6 and multi-substrate specificity. Furthermore, maltogenic amylases along with cyclodextrinases (EC 3.2.1.54), neopullulanases (EC 3.2.1.135) and α-amylases type II are grouped under the glycoside hydrolases family 13 (GH-13). These enzymes are nearly indistinguishable (Park *et al*., 2005) and are regularly referred as cyclodextrins hydrolyzing enzymes (Kim *et al*., 2007). Maltogenic amylases are mostly found in many different microbial sources including *Bacillus* (Liu *et al*., 2006; Le *et al*., 2009), *Geobacillus caldoxylosilyticus* TK4 (Kolcuoğlu *et al.,* 2010), *Thermofilum pendens* (Li *et al*., 2011) and plant pathogenic fungi such as *Byssochlamys fulva* (Doyle *et al*., 1998). Other than that, maltogenic amylases from seed of plant such as Fenugreek seeds (*Trigonella foenum graecum)* were also reported (Khemakhem *et al*., 2013).

High catalytic activities of many natural enzymes have been concerns of many enzymologists. Cloning and expression of genes of interest are often considered as an approach for improvement the enzyme catalytic activity. Nowadays, wide varieties of expression systems including prokaryotic and eukaryotic expression systems are available. Among prokaryotic expression systems, a gram-negative bacterium *Escherichia coli* is the expression host of choice for overexpressing of many heterologous proteins (Rosano and Ceccarelli, 2009).

Protein purification is literally defined as a process of separating a protein of interest out of many other unwanted proteins usually known as contaminants. Various purification techniques can be applied in order to purify the protein of interest. These different techniques are selected depending on different characteristics and degree of purity of the protein of interest that is required for downstream applications. Among different purification techniques, different column chromatography techniques have been regularly used including gel filtration using Butyl Sepharose and Sephacryl S-200 (Prakash *et al*., 2009), ion exchange, affinity column, hydrophobic interaction and reverse phase. Other techniques including ammonium sulphate fractionation was also reported (Liu and Xu, 2008).

Comprehensive understand how different enzyme domains act synergistically to hydrolyze multiple and/or versatile substrates is very important for a researcher in order to design a very effective enzyme for industrial application. Protein engineering seems to be a practical approach for designing such desired criteria of the enzymes. Protein engineering has become an effective tool for enhancing such enzyme activity and modifying the enzyme characteristics to the desired one to meet the specific industrial demands which can be done either via direct evolution or rational design. The direct evolution or random mutagenesis seems to be simpler and a straightforward process as compared to rational design. Where in the rational design a three dimensional (3D) structure of an enzyme to be engineered has to be fully elucidated which can be done through protein crystallization, diffraction and three dimensional approaches. Thus structural studies of a protein structure for better understanding of the enzyme behaviours and large scale production of the enzymes to meet the global market demands had been interested by many researchers.

Theoretically, thermostability of a protein correlates with its structure rigidity. It is believed that the local networks of salt bridges and hydrogen bonds that are abundantly found in thermophiles have contributed towards structure stability of many thermostable proteins (Mamonova *et al*., 2013). Rigidifying the most flexible protein region by amino acid substitution into Lys, Arg or Glu could enhance thermostability of many enzymes (Yu and Huang, 2014) due to the fact that large fraction of these amino acid residues were found in the exterior of many thermostable proteins (Mamonova *et al*., 2013). Besides, the consensus amino acid contributed more than average to the stability of proteins than the non-consensus amino acids at a given position in amino acid sequence alignment of the homologous protein (Lehmann and Wyss, 2001).

Since enzymes are environmentally friendly that possess a very neglected effect, therefore, over 75% of the hydrolysis process were conducted by using enzymes rather than by acids (Kandra, 2003). And this can minimize the cost of waste management and subsequently can reduce the overall manufacturing costs (Hasan *et al*., 2006).
The effectiveness and rapidity of an enzyme in fulfilling its catalytic activity is among the crucial parameters that often required by many industries since high enzyme activity and their ability to withstand harsh conditions such as high temperature and extreme pH determines economic feasibility in industrial processes. Many enzymes that naturally produced by various living organisms including bacteria are quite limited in their properties to cater the industrial need. Besides, many microorganisms that

naturally produce enzymes cannot be easily cultured under industrial conditions (Olempska-Beer *et al*., 2006). Therefore, with the advantage of genetic techniques many enzymes with the improved their properties have been developed and manufactured. Hence, this study is mainly aimed to develop a recombinant enzyme with the improved properties. However, in order to achieve this main objective, other objectives are also included. Among other objectives are:

1. to isolate and screen for a thermophilic maltogenic amylase-producing bacteria
2. to express the thermostable maltogenic amylase in prokaryotic expression system.
3. to purify and characterize the recombinant thermostable maltogenic amylase for further downstream applications.
4. to assess the effect of mutation on the recombinant thermostable maltogenic amylase and its variants.

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