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ISOLATION AND CHARACTERIZATION OF NEW THERMOPHILIC AMINOACYLASE FROM *Geobacillus* sp. STRAIN SZN

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PEMENCILAN DAN PENCIRIAN AMINOASILASE TERMOSTABIL BARU DARIPADA *Geobacillus* sp. STRAIN SZN

ABSTRAK

Kajian ini bertujuan untuk memencilkan dan mencirikan aminoasilase termostabil daripada bakterium termofilik. Dalam pemencilan bakterium, sampel air dan sedimen telah diperolehi dari Air Panas Ulu Slim, Perak. Aminoasilase SZN termostabil diekstrak secara intrasellular dan ditulenkan sehingga homogen dengan menggunakan kromatografi pertukaran ion dan pengasingan saiz dalam eksperimen seterusnya. Aminoasilase SZN yang tulen telah dicirikan pada pelbagai suhu, pH, ion logam dan perencat. Kajian struktur lebih lanjut menggunakan spektrokopi sirkular dikroisma dilakukan bagi menentukan kestabilan α-heliks dan lembaran β dalam pelbagai suhu. Penghasil aminoasilase termofilik yang dipencilkan itu dikenalpasti sebagai *Geobacillus* sp. strain SZN. Aminoasilase SZN dicirikan sebagai enzim termostabil dengan aktiviti optimum pada 60°C, pH 7.5, tempoh separuh hayat 16 jam dengan peningkatan aktiviti dan kestabilan dalam beberapa ion logam dan perencat yang diuji. Keputusan daripada penentuan struktur menunjukkan pengurangan α-heliks secara beransur-ansur daripada 36 hingga 27.6%, diikuti dengan disorientasi mendadak struktur tersebut pada peralihan suhu daripada 60 hingga 70°C (27.6 hingga 19.5%). Sebaliknya, peratusan lembaran β, telah meningkat secara stabil pada suhu yang diuji. Struktur α-heliks yang juga merupakan lokasi residu-residu pengikat logam dan pemangkin yang penting, lemah sepenuhnya pada suhu melebihi 70°C lalu mengakibatkan kehilangan aktiviti. Kesimpulannya, aminoasilase SZN telah dicirikan sebagai enzim termostabil berdasarkan kepada integriti struktur α-heliks dan kestabilan fungsinya pada suhu tinggi. Implikasi kajian ini menunjukkan bahawa aminoasilase SZN mampu menjadi enzim alternatif kepada bioindustri berdasarkan kepada peningkatan aktiviti enzim dalam suhu tinggi dan kestabilan dalam pelbagai perencat yang diuji.

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ABSTRACT

This study aims to isolate and characterize a thermostable aminoacylase from a thermophilic bacterium. For isolation of the bacterium, water and sediment samples were collected from Ulu Slim Hot Spring, Perak. The thermostable aminoacylase was extracted intracellularly and purified to homogeneity by using ion exchange and size exclusion chromatography in subsequent experiment. The purified aminoacylase SZN was characterized at various temperatures, pHs, metal ions and inhibitors. Further structural study using circular dichroism spectropolarimeter was conducted to determine stability of α-helix and β-sheet in various temperatures. The isolated thermophilic aminoacylase producer was identified as *Geobacillus* sp. strain SZN. The aminoacylase SZN was characterized as a thermostable enzyme with optimum activity at 60°C, pH 7.5, half-life of 16 hours with activity increment and stability in several metal ions and tested inhibitors. Results from structural determination have indicated a gradual decrease of α-helix from 36 to 27.6%, followed by a tremendous disorientation of the structure at transition of temperatures from 60 to 70°C (27.6 to 19.5%). In contrast, the percentage of β-sheet has increased steadily over the tested temperatures. The α-helix, where notable metal binding and catalytic residues are located, were totally weakened at temperatures above 70°C, thus resulted in loss of activity. As a conclusion, the aminoacylase SZN was characterized as a thermostable enzyme based on the α-helical structure integrity and functional stability in high temperatures. The implication of this study indicates that the aminoacylase SZN can serve as an alternative enzyme for bioindustries in view from its activity enhancement in high temperatures and stability in various tested inhibitors.

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LIST OF ABBREVIATIONS

A	Ampere
A_{595}	absorbance at 595 nm
A_{570}	absorbance at 570 nm
AMA	Aminoacylase
APIs	Active Pharmaceutical Ingredients
BGSC	Bacillus Genetic Stock Center
bp	base pair
BSA	bovine serum albumin
D-	dextrorotatory
Da	Dalton
DEAE	diethylaminoethyl
dH_2O	distilled water
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
g	gram
g/L	gram per litre
h	hour
IPTG	isopropyl-β-D-galactoside
kb	kilo base pair

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kDa	kiloDalton
kPa	kiloPascal
L	litre
L-	levorotatory
LB	Luria Bertani
M	molar
mM	millimolar
mg	milligram
mL	millilitre
min	minute
MW	molecular weight
mg/mL	milligram per millilitre
NAMET	*N*-acetyl-$_L$-Methionine
nm	nanometer
OD_{600}	Optical Density at 600 nm
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PMSF	phenylmethylsulfonyl fluoride
rpm	rotation per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	N,N,N,N-tetramethyllenediamine
T_m	thermal denaturation
U	unit

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U/mL	unit per millilitre
U/mg	unit per milligram
µg	microgram
µL	microlitre
µm	micrometer
µmoles	micromoles
V	volt
v/v	volume per volume
W	watt
w/v	weight per volume
%	percent
°C	degree Celcius
16S rRNA	16 small Sub-unit Ribosomal Ribonucleic Acid

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Global amino acids market demand was 7.5 million tons in 2016, and was expected to reach a volume of more than 10 million tons in 2020 (Research & Market, 2015). The market of amino acids is anticipated to reach USD 30.8 Billion by the end of 2024. This can be attributed to various factors such as growing consumer awareness and rising demand for healthy and functional food. Furthermore, increasing consumption of dietary supplements is expected to positively impact the growth of global amino acids market, Researchnester.com (2018). All amino acids excluding glycine are chiral molecules, because they have a chiral carbon atom adjacent to the carboxyl group (CO_2^-). This chiral centre allows for stereoisomerism. The amino acids form two stereoisomers that are mirror images of each other, they exist in left and right-handed forms (L- or D-) of enantiomers. The production of single enantiomers of

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chiral intermediates has become increasingly important in the pharmaceutical industry. Single enantiomers can be produced by chemical or chemo-enzymatic synthesis. The advantage of biocatalysis over chemical synthesis is that enzyme-catalyzed reactions are often highly enantioselective and regioselective. They can be carried out at ambient temperature and atmospheric pressure, thus avoiding the use of more extreme conditions that can cause problems with isomerization, racemization, epimerization and rearrangement (Patel, 2013). Microbial cells and enzymes derived from them can be immobilized and reused for many cycles, and enzymes can be over-expressed to make biocatalytic processes economically efficient.

Enzyme catalysis reactions that performed at relatively high temperature have numbers of advantages, such as decreased viscosity of reaction solutions, increased diffusability of substrates and products and extended availability of less water-soluble substrates (Tanimoto, Higashi, Nishioka, Ishikawa, & Taya, 2008). According to Kramers (1940) theory, solvent viscosity results in friction against proteins in solution, and this should result in decreased motion, inhibiting catalysis in motile enzymes (Uribe & Sampedro, 2003). The search for new local thermostable aminoacylases is essential needs in global industry especially in amino acids productions industry. Enzymes from extremophiles, has becoming greater to the traditional catalysts because they can perform industrial processes even under harsh conditions, under which common proteins are completely denatured. Basically, industrial use of enzymes for production of L-amino acids has started since more than 40 years ago in Japan with the resolution of N-acetyl D, L-amino acids by immobilized aminoacylase (Ivanov, Stoimenova, Obreshkova, & Saso, 2014) as an alternative to production of amino acids by fermentation and chemical synthesis

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which is faster and more specific in producing amino acids product. The industrial production of L-methionine is being practiced by immobilization of aminoacylase on to DEAE Sephadex in a packed bed reactor. Nowadays, the thermostable aminoacylases from thermophiles have been further modified for industrial use. Aminoacylase from *Thermococcus litoralis* have been using by Chirotech, the company of Dr Reddys for commercial production of L-amino acids (Holt, 2004).

1.2 Problem Statements

Normally, in common cells, the enzyme will denature and unfold when the temperature increased beyond a specific point. As the temperature is raised, the rate of molecular movement and hence the rate of reaction increases, but at the same time there is a progressive inactivation caused by denaturation of the enzyme protein (Robinson, 2015). The consequences happened because the three-dimensional structure of proteins is damaged due to loss of hydrogen and disulfide bonds which responsible to maintain the structure. Protein unfolding may cause the enzyme to no longer be able to catalyse the reactions appropriately because it had lost its fundamental shape which is associated with its specific affinity and its substrate binding site. Vieille and Zeikus (2001) reported that enzymes from extreme environments may be more suitable in some biotransformations because they offer highly desirable traits of activity and stability for application under various manipulated of temperature process conditions. In industrial processes, enzyme will be exposed to different environment compared to their natural environments. Most of enzymes derived from hyperthermophiles are active (optimally) at temperatures that

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close to the host's optimal growth temperature (Vieille & Zeikus, 2001). Another advantage of application of thermostable enzyme in industry is that, at high temperatures, the thermostable enzyme able to react at higher substrate concentrations, lower viscosity, with higher reaction rates and most importantly can reduce risks of microbial contamination (Otieno, 2010).

Excellent characteristics has been reported on capability of hyperthermostability aminoacylase from *Pyrococcus horikoshii* (Tanimoto et al., 2008) and *Pyrococcus furiosus* (Story, Grunden, & Adams, 2001) that can withstand the catalytic activity at 90 and 100 ^{o}C, respectively. Through this study, newly local thermostable aminoacylase was expected to be isolated with greater characteristics with stability at high temperatures and have the capability to withstand the activity at several tested reducing agents, detergents and denaturing agents which will be an added advantage as the criteria which is needed by the excellent enzymes in order to be applied in industries. In addition, to understand the intrinsic values though molecular investigation of gene encoding thermophilic aminoacylase and analysis of the sequence and database similarity using Basic Local Alignment Tools (Altschul et al., 1997), Biology Workbench (Subramaniam, 1998) and ExPaSy Tools (Gasteiger et al., 2003), this study also will emphasize more about secondary structure stability and its distortion at high temperatures. Thus, the focus of this study is to provide a better understanding on adaptation of thermostable aminoacylase at high temperatures, from structural point of view.

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1.3 Research Questions

1. How to isolate and identify thermostable aminoacylase producer?
2. How to characterize purified thermostable aminoacylase?
3. How to determine structural adaptation of thermostable aminoacylase in various temperatures?

1.4 Objectives of Study

1. To isolate and identify thermostable aminoacylase producer.
2. To characterize purified thermostable aminoacylase.
3. To determine structural adaptation of thermostable aminoacylase in various temperatures.

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1.5 Research Limitations

There are few limitations needed to be addressed throughout this study. First of all, about two main instruments which are not available in this university, such as Circular Dichroism Spectropolarimeter (CD) that is only accessible in Universiti Putra Malaysia (UPM) and Genome Institute Malaysia (GIM). The second major instrument that is lacking here is AKTA Purifier Chromatography System (GE Healthcare, USA) which is vital for protein purification in this research. Through research collaboration

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with UPM, the purification was managed to be done utilizing AKTA Purifier Chromatography System (GE Healthcare, USA) there.

Secondly, regarding information availability through online literature research, there is lacking of latest research information, especially for aminoacylase from bacteria. The greatest finding from archaea aminoacylase in 2008 and several species that has been industrially developed for amino acids productions had proved that this enzyme was worth to explore. Last but not least, the complex gene expression system for aminoacylase has leaded this research to continue with aminoacylase extracted from wildtype even though gene expression approach was attempted at the first place but meet failure.

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1.6 Conclusions

There are three focus areas that will be emphasized in this study, firstly, isolation and identification, second is characterization of the purified aminoacylase and lastly, secondary structure adaptation analysis when aminoacylase was exposed to high temperature. Lacking of research data on secondary structure of aminoacylase from microorganism especially from high temperatures brought this novelty research project to reveal more information about factors involved in structure rigidity and the secondary structure stability and distortion of newly isolated thermostable aminoacylase exposed at high temperatures.

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