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EXPRESSION OF RECOMBINANT GEOBACILLU SP α-AMYLASE IN PICHIA PASTORIS GS115

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EXPRESSION OF RECOMBINANT *GEOBACILLU* SP α-AMYLASE IN *PICHIA PASTORIS* GS115

By

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

EXPRESSION OF RECOMBINANT *GEOBACILLUS* SP. *α*-AMYLASE IN *PICHIA PASTORIS* GS115

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Amylases are a group of enzymes which are significantly applied in food and beverages, detergent, paper and textiles industries. A thermophilic microorganism producing α-amylase for sustainable high temperature operation offer many advantages such as lowering the risk of microbial contamination during substrate degradation and increasing substrate solubility. Previously, production of α-amylase SR74 in *Escherichia coli* expression system under regulation of T7 *lac* promoter in pET-32b significantly enhanced the amylase activity to 15.3 U/ml as compared to the native strain. However, current intracellular expression level in *Escherichia coli* was low and not that suitable for commercialization. Therefore, increased production and commercialization of thermostable α-amylase strongly warrants the need of a suitable expression system. *Pichia pastoris* is a currently well established methanol utilizing yeast expression system. The aim of the studies was to produce a recombinant *Geobacillus* sp. α-amylase SR74 in *P. pastoris* GS115 and characterize the biochemical properties of the purified recombinant α-amylase. In this study, the gene encoding the thermostable *α*-amylase SR74 in *Geobacillus* sp. was amplified by PCR, sequenced and sub-cloned into *P. pastoris* GS115 strain under the control of a methanol inducible promoter namely, alcohol oxidase I (*AOX1*). The recombinant plasmid was subsequently transformed into *P. pastoris* via electroporation method. Methanol induced recombinant expression and secretion of the protein resulted in high levels of extracellular amylase production. YPTM medium supplemented with methanol 1% (v/v) was found to be the best medium with the highest expression level (28.6 U/mL) after 120 hours of post induction. The recombinant *α*-amylase SR74 was purified by using affinity chromatography technique. The purified *α*-amylase SR74 was found to be 1.9 fold higher with a product yield of 52.6% and a specific activity of 151.8 U/mg. Purified α-amylase SR74 was found to be stable between pH 6.0-8.0 with an optimum pH of 7.0. The enzyme was found to be thermostable and thermoactive at 65°C with a half-life ($t_{1/2}$) at 60°C for 88 min. Such thermostable and thermoactive characteristic of the *α*-amylase SR74 would be beneficial for industrial applications, especially in liquefying saccrification.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Science

PENGEKSPRESAN REKOMBINAN α-AMILASE DARIPADA *GEOBACILLUS* SP. DI DALAM *PICHIA PASTORIS* GS115

Oleh

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Amilase ialah kumpulan enzim yang memainkan peranan yang penting dalam pelbagai aplikasi di dalam industri seperti makanan dan minuman, bahan pencuci, kertas dan tekstil. Mikroorganisma termofilik yang menghasilkan α-amilase yang boleh beroperasi pada suhu yang tinggi boleh memberikan faedah seperti menurunkan risiko pencemaran mikrob semasa degradasi substrat dan meningkatkan keterlarutan substrat. Pengeluaran α-amilase SR74 dalam sistem pengekspresan *Escherichia coli* di bawah pengawalaturan promoter T7 lac dalam vektor pET-32b telah meningkatkan aktiviti α-amilase dengan ketara kepada 15.3 U/ml berbanding dengan perumah asal. Walau bagaimanapun, tahap ekspresi intraselular semasa dalam *Escherichia coli* adalah rendah dan tidak sebegitu sesuai untuk dikomersialkan. Oleh itu, sistem pengekspresan yang sesuai adalah diperlukan untuk meningkatan pengeluaran dan pengkomersilan termostabil α-amilase. *Pichia pastoris* adalah antara sistem pengekspresan yis yang menggunakan metanol yang terbukti berkesan. Tujuan kajian ini adalah untuk menghasilkan rekombinan α-amilase SR74 daripada *Geobacillus* sp. di dalam *P. pastoris* GS115 dan mencirikan rekombinan α-amilase yang tulen untuk beberapa aspek biokimia. Dalam kajian ini, pengekodan gen α-amilase tahan panas SR74 daripada *Geobacillus* sp. telah diamplifikasi dengan PCR, disusun dan sub-klon ke dalam *P. pastoris* strain GS115 di bawah kawalan alkohol oxidase I (*AOX1*) iaitu menggunakan metanol sebagai pengaruh. Plasmid rekombinan telah dikekalkan dan dimasukkan ke dalam *P. pastoris* melalui kaedah elektroporasi. Metanol menyebabkan pengekspresan dan perembesan protein rekombinan ke dalam media dan menyebabkan tahap pengeluaran α-amilase secara ekstraselular adalah tinggi. YPTM yang ditambah dengan 1% (v/v) metanol merupakan medium terbaik dengan tahap ekspresi tertinggi (28.6 U/mL) selepas 120 jam induksi. Rekombinan α-amilase SR74 telah ditulenkan dengan menggunakan teknik kromatografi. α-amilase SR74 yang tulen didapati 1.9 kali ganda lebih tinggi daripada yang asal dengan hasil produk sebanyak 52.6% dan aktiviti spesifik adalah 151.8 U/mg. α-amilase SR74 yang tulen didapati stabil antara pH 6.0-8.0 dengan pH optima adalah 7.0. Enzim ini didapati termostabil dan termoaktif pada 65°C dengan separuh hayat ($t_{1/2}$) pada 60°C selama 88 min. Dengan ciri-ciri termostabil dan termoaktif SR74 α-amilase, ia akan memberi manfaat untuk aplikasi industri, terutama dalam sakarafikasi pencairan kanji.

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This thesis was submitted to the Senat 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 follow:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

CV- column volume

PCR- Polymerase Chain Reaction

α : Alpha

β : Beta

CAZy : Carbohydrate active enzymes

Da : Daltons

dH_2O : distilled water

DNA : Deoxyribonucleic acid

DNSA : Dinitrosalicylic acid

g : Gram

GH : Glycosyl hydrolases

h : Hour

IUB-MB : International union for biochemistry and molecular biology

kDa : Kilo Dalton

L : Litre

LB : Luria Bertani

M : Molar

µg : Microgram

µl : Microliter

mg : Miligram

YPD : Yeast Peptone Dextrose

YPDS : Yeast Peptone Dextrose Sorbitol

U : Unit

w/v: Weight per volume

v/v: Volume per volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

In industrial biotechnology, biocatalysts deemed to be an important tool. Its abilities are used in the synthesis of chemicals, as intermediates for agrochemical and pharmaceutical, active pharmaceuticals and ingredients of food. Unfortunately, such applications are modest in terms of number and diversity. This is mainly due to the limited availability of enzyme, its substrate scope and operational stability. However, these limitations could be overcome via exploitation of biodiversity, directed enzyme evolution and genomics (Fernandes, 2010). By modifying or engineering the biocatalyst production rate, stability and efficiency, these demands are met.

Amylase is a starch degrading enzyme which has garnered a lot of attention due to its economic benefits and its perceived technology significance. Some of the sources of amylases are microorganisms, plants and animals. Generally, amylases from microorganisms are the best in meeting industrial demands. The main reason is its short growth period and productivity (Omar, 2008). In starch processing industry, amylases from microorganisms have successfully replaced the chemical hydrolysis of starch. Due to a great demand, the indigenous production of α-amylase had to be increased extensively. α-amylase is used in industries like food, textile, starch liquefaction, detergent, distilling, pulp, saccrification and brewing industries (Gupta *et al*., 2003). Currently, the scope of amylase application has widened its range to other fields like, clinical, medical and analytical chemistry, thanks to the arrival of new frontiers in biotechnology especially biofuels, bioenergy and pharmaceuticals.

Most of these industries use the extracellular enzymes extracted from microorganisms. In terms of commercial exploitation, α-amylase is ranked first among various extracellular enzymes (Divakaran *et al*., 2011). Extracellular digestion is carried out when amylases are secreted by bacteria and fungi to the exterior of the cells. When starch is disintegrated, it produces soluble products like glucose or maltose which is absorbed into their cells. Factors such as, less energy consumption when compared to the conventional chemical methods, requirement of mild conditions for the reaction and specificity of reaction has led to an increase in demand for microbial amylase.

Despite vast availability of α-amylase, there is a pressing need for thermostable and thermoactive α-amylase. This is mainly due to its demand in the industry to save time and space required and also for easier proses modification and optimization in industrial production (Burhan *et al*., 2003).

Geobacillus sp. is a thermostable α-amylase producer which had been isolated from a hot spring near Slim River of Perak state in Malaysia by Elias, (2009). The isolated α-amylase SR74 was expressed in a recombinant *Escherichia coli* intracellularly by Elias (2009). In order to commercialize this enzyme, an improved expression system is required as well as a proper characterization of the enzyme. Therefore, in this study molecular cloning of α-amylase and utilization of *Pichia pastoris* expression system to produce the enzyme extracellularly were attempted. Purification and characterization of the recombinant α-amylase SR74 was also carried out. It is important to note that the expression of many methylotrophic yeasts, especially *P. pastoris* has been successfully used for the expression and purification of foreign proteins (Mattanovich *et al*., 2012). This system has offered many advantages which includes the usage of alcohol oxidase I (*AOX1*) promoter for integration of recombinant plasmids into the genome of *P. pastois*. In addition, *P. pastoris* is able to grow at high cell-densities thus making it beneficial for industrial application (Marx *et al*., 2009).

P. pastoris also has the ability to carry out post-translational modifications to foreign proteins and as a result, it usually produces correct folding, disulphide bond formation, O- and N- linked glycosylation and processing of signal sequences. Another benefit of using *P. pastoris* expression system is that the desired protein (or) the protein of interest can be secreted into the production medium. Another major advantage of *P. pastoris* is its ability to release the protein of interest at high levels than native protein into the production medium, thus making the purification procedure less tedious.

Therefore, this project revolves around using the *P. pastoris* expression system to meet the industrial demand through these specific objectives:-

- to produce a recombinant *Geobacillus* sp. α-amylase in *P. pastoris* GS115
- to optimize the production of the recombinant α-amylase in *P. pastoris* GS115
- to characterize the biochemical properties of the purified recombinant α-amylase

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