PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF AMYLASE FROM SPHINGOMONAS SP. ISOLATED FROM ARTIC SOIL

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ABSTRACT

Amylase is an important enzyme that is responsible for hydrolysis of starch based materials into simple sugars. To date, extensive researches had been done on the characteristics of amylase produced by microorganisms isolated from different sources. However, most of the reported amylases do not have a wide range of thermostability. In this project, a total of 14 bacteria isolated from artic regions were screened for their capability of amylase production. The *Sphingomonas* sp. was found to be the best candidate for amylase production among the rest, it had the lowest Hc value (0.44). Production of amylase was subsequently quantified in submerged liquid fermentation (SmF). The *Sphingomonas* sp. showed highest amylase activity of 0.23 U at 18^{th} hour of incubation in amylase production medium, pH 7, containing 0.1% (w/v) of soluble starch. Optimization of amylase production was conducted using One-Factor-at-a-Time (OFAT) method. Medium with pH 9 containing 0.87% (w/v) autoclaved baker's yeast cells, and 3% (w/v) soluble starch was found to have increased the amylase activity by 27.6 fold (6.31U). For partial purification of amylase, three methods: ammonium sulphate precipitation, 60% (w/v), centrifugal concentrator and the combination of both were used. The highest specific activity was obtained in ammonium sulphate precipitation, 60% (w/v) which increased the specific activity by 2.5 fold (23.43 U/mg) as compared to the activity of crude enzyme (9.41 U/mg). Characterization of amylase further revealed that the enzyme was stable at temperature ranging from 4ºC to 95˚C (72.3% relative activity remained) with the optimum temperature of 20˚C. It was most stable at pH8 with optimum activity at pH7. The SDS-PAGE and zymogram analysis revealed that the amylase having molecular weight between 50 to 60 kDa.

ABSTRAK

Amilase merupakan enzim yang penting bagi tujuan komersial dan industri. Walaupun terdapat banyak laporan tentang pengasingan dan pencirian amilase daripada punca-punca yang berbeza, namum amilase yang dihasilkan biasanya tidak dapat mengekalkan kestabilan enzim pada pelbagai suhu-suhu. Dalam projek ini, seramai 14 jenis bakteria yang telah diasingkan dan dikenalpastikan daripada rantau artik telah disemak kemampuan untuk menukar kanji. *Sphingomonas* sp. dengan HC terendah (0.44) telah dipilih. Kemudian, profil pertumbuhan dan aktiviti amilase dalam media yang mangandungi 0.1% kanji terlarut telah dikenalpastikan dengan cara SmF. Aktiviti yang tertinggi didapati pada ke-18 jam (0.23 Unit), pH 7 dengan 0.1% (w/v) kanji terlarut. Bagi pengoptimuman untuk pengasilan amilase, eksperimen OFAT telah dijalankan untuk mangkaji ciri-ciri termasuk punca nitrogen, kepekatan substrak and pH. Aktiviti amilase telah dinaikan sebanyak 27.4 kali dengan penggunaan sel ibu roti sebaigai punca nitrogen, 3% kanji larut, pH 9. Penulenan separa, kecekapan cara-cara termasuk ammonium sulfat, ultrafiltration dan campuran kedua-dua cara telah dibandingkan. Keputusan ammonium sulfat, 60% (w/v) adalah terbaik iaitu 2.5 kali kenaikan aktiviti spesifik (23.43 Unit/mg) apabila dibandingkan dengan enzyme kasar (9.41 Unit/mg). Pencirian amilase telah mendedahkan fakta iaitu enzim ini dapat mengekalkan kestabilan daripada 4 °C sampai 95 °C (72.3% aktiviti dikekal selepas 120 min) dengan suhu optima pada 20 °C. Enzim ini lebih kekal pada pH 8 dan aktivitinya optima pada pH yang sama. SDS-PAGE dan zymogram telah membuktikan enzim ini mempunyai saiz molekul 50-60kDa.

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LIST OF ABBREVATIONS AND SYMBOLS

ABM	-	Antarctic Bacterial Medium
°C	-	Degree Celcius
g	-	Gram
g/ml	-	Gram Per Millilitre
h	-	Hour
HC	-	Coefficient of hydrolysis
H2O	-	Water
L	-	Litre
Min	-	Minutes
mg/ml	-	Miligram per milliliter
ml	-	Mililitres
mM	-	Milimolar
M	-	Molar
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
µmol		micromole
%	-	Percent
rpm	-	Revolutions Per Minute
sec	-	Seconds
µL	-	Microlitre
w/v	-	Weight per volume

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CHAPTER 1

Introduction

1.1 Research background

Organisms that are able to grow in extreme temperature, pH, salinity, low water activity, high pressure, low oxygen concentration and so on are termed as extremophiles. These organisms have adapted, at the molecular level to the extreme condition where it is usually inconducive for the growth of other living organism. On the other hand, around 85% of earth's surface is occupied by cold environment including the depths of ocean, polar and alphine regions. From there, around 70% is covered by oceans which have constant temperature of 4-5°C regardless of latitude. The extremophiles that able to strive in the aforementioned cold environment are called phychrophile (Kuddu and Roohi, 2010).

Enzymes are biocatalysts used to accelerate biochemical reactions of a living organism. Besides metabolic function, enzymes aid in various industries for the catalysis of many processes such as pharmaceutical, sugar, textile and brewing industries (Maryan and Montazer, 2013). In recent years, enzymes such as protease, amylase, lipases and cellulases have become one of the important detergent formulations in laundry industry. Enzyme formulated detergent is more favourable because it could effectively remove stain by degrading the biological component, as

well as environmental friendly in terms of reduction in energy and water consumption through lower washing temperature (Soleimani *et al*., 2012).

Extremozymes are the biocatalyst of extremophiles which have adapted to function in harsh conditions; it plays a vital role in the survival of the host organism in the extreme environment. Among them, cold-active enzyme is one of the examples of extremophile biocatalyst; it is able to work in low temperature. Despite its importance in the in vivo biochemical reaction especially for psychrophiles, its great potential in various biotechnological applications in industries were hidden. In terms of operating temperature, it helps the process to be operated with lower the energy consumption compared to their mesophilic counterparts (Ramtake and Bhatt, 2007).

Amylase is one of the most important enzymes which covers around 30% of total commercial enzyme in the world market (Singh and Gupta, 2014). Formerly, it was mainly used in the bakery and brewing processes, nowadays more and more applications of α-amylase are being explored, and one of them is the used in the detergent formulation. Currently, almost 90% of all liquid detergents contain amylase (Roy *et al*., 2012). This enzyme breakdown starch to produce a diversity of smaller products including dextrins, oligosaccharides and glucose molecules (Mukherjee *et al*., 2009). Besides that, it is very important for the removal of stains which constitute of starch, for example gravy, pasta paste and corn soup. Apart from it, amylases also serve as antistalling agent in bread-baking industry, syrups production catalyst in pharmaceutical industry, desizer in textile industry, viscosity control of starch slurry in paper and pulp industry as well as applied in wastewater treatment and bioremediation processes (Roohi & Kuddus, 2014).

Due to the increasing demand of amylase, more and more works have been done in order to explore new sources for the production of this enzyme. The sources for this enzyme generally include plants, animals and microbes. Among them, microbial is a better host to be used in mass production due to its cost effectiveness,

consistency, and required less time and space for operation. Also, the used of microorganism could ease the modification and the process optimization (Swetha *et al*., 2006).

In this project, *Sphingomonas* sp. was selected from a total of 14 pre-isolated artic bacteria based on its capability in starch hydrolysis. Its growth pattern and amylase activity were determined. Subsequently, the production of amylase was optimized followed by partial purification of the crude amylase. The enzyme was then characterized for its optimum and stability in various temperature and pH while the protein size of amylase was identified through SDS-PAGE and zymogram analysis.

1.2 Problem statement

Enzyme can be the substitution of chemical substances in many of processes in where most of the chemical used can caused harm to human being and one of the examples was detergent for dishwashing. The major drawback in most of the commercial dishwasher was the use of chemical reagent in their formulation. This due to the food poisoning can be happen when it is be ingested (Sundarram and Murthy, 2014). Therefore, there is a need for the use of an alternative which will cause less or no harm to the user while providing desirable cleaning effect. Enzymes are good choice of alternatives, as they do not just remove the undesired substances but they convert them into substances which cause less harm to the human being and the environment.

Nowadays, most of the commercial α-amylase are derived from strains *Bacillus* or *Aspergillus* genera (Hmidet *et al*., 2009), which are mesophiles. Although several extremophiles had been identified (Mesbah & Wiegel, 2014; Michelin *et al*., 2010), however, most of the researchers were focusing on producing

α-amylase with high stability in high temperature, acidic and/or alkaline conditions (Aygan *et al*., 2008; Minod *et al*., 1968; Mikami *et al*., 1987). Enzyme with low optimum temperature had not been studied sufficiently. In the existing method, moderate to high reaction temperature was required to ensure the enzyme to be functioned well. It will impose to the rise of electricity demand which is not environmental friendly; therefore amylase with high activity in lower temperature is needed.

Although many of the amylases with low optimum temperature was isolated and characterized; however many of them do not acquired a wide range of stabilities in various temperature. This characteristic limits their applications as they are unable to take part in the reaction when the temperature is increased beyond their relatively narrow temperature range. Therefore, this project aims to produce an amylase with low optimum temperature and yet able to remain stable in a wide range of temperature.

1.3 Objectives

1. To screen for starch hydrolysis capability and select the bacteria with lowest Hc among artic bacteria
2. To determine the growth pattern and initial amylase activity of the selected bacteria.
3. To optimize the production of amylase by *Sphingomonas* sp.
4. To partially purify the produced amylase.
5. To characterize the partially purified amylase.

1.4 Scope of study

In this project, the scope of study was to optimize the production, partially purify and characterize the amylase produced from Artic bacteria. The starch hydrolysis capability of preisolated bacteria was screened using ABM-starch supplemented agar. Then several parameters including nitrogen source, substrate concentration and pH in amylase production medium was optimized. Subsequently the crude amylase was partially purified and followed by characterization of amylase. This was to understand the optimum and stability of amylase under various temperature and pH. The molecular weight of amylase was then determined through SDS-PAGE and zymogram analysis.

1.5 Significance of study

Cold-active amylase with high enzymatic activity was expected to be produced and characterized as the outcome of this study. Besides, it was expected to increase the yield of amylase production as a result of the medium formulation and culturing conditions optimization.

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