Faculty of Resource Science and Technology

Investigating the effect of media supplements and different urea concentration on the amylase production via Solid State Fermentation by *Aspergillus flavus*

Mary Molly Anak Bihos

(42013)

Bachelor of Science with Honours
(Resource Biotechnology)
2016

Investigating the effect of media supplements and different urea concentration on the amylase production via Solid State Fermentation by *Aspergillus flavus*

Mary Molly Anak Bihos (42013)

A dissertation submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours in Resource Biotechnology

Bachelor of Science with Honours
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2016

UNIVERSITI MALAYSIA SARAWAK

Grade: ____________

Please tick (√)

Final Year Project Report ☑

Masters ☐

PhD ☐

DECLARATION OF ORIGINAL WORK

This declaration is made on the ....17....day of....June....2016.

Student's Declaration:

I MARY MOLLY ANAK BITTOS, 42013, FACULTY OF RESOURCE SCIENCE and TECHNOLOGY (PLEASE INDICATE STUDENT'S NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled, Investigating the effect of media supplements and different urea concentration on the amylase production via Solid State Fermentation by Aspergillus Flavus is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

17 June 2016

Date submitted

MARY MOLLY ANAK BITTOS (42013)

Name of the student (Matric No.)

Supervisor's Declaration:

I ROSMAWATI BINTI SAAT (SUPERVISOR'S NAME) hereby certifies that the work entitled, Investigating the effect of media supplements and different urea concentration on the amylase production via Solid State Fermentation by Aspergillus flavus (TITLE) was prepared by the above named student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment of Bachelors of Science with Honours (Resource Biotechnology) (PLEASE INDICATE THE DEGREE), and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: ROSMAWATI BINTI SAAT (Name of the supervisor)

Date: 17/6/2016

I declare this Project/Thesis is classified as (Please tick (√)):

☐ **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*
☐ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*
☑ **OPEN ACCESS**

Validation of Project/Thesis

I therefore duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies for the purpose of academic and research only and not for other purpose.
- The Centre for Academic Information Services has the lawful right to digitise the content to for the Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic exchange between Higher Learning Institute.
- No dispute or any claim shall arise from the student itself neither third party on this Project/Thesis once it becomes sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student except with UNIMAS permission.

Student's signature ________ 17/6/2016
(Date)

Supervisor's signature: ________
(Date) 17/6/2016

Current Address:
NO 104 J, LORONG YEN-YEN 1A, TAMAN YEN-YEN, 93050 JALAN MATANG, KUCHING, SARAWAK.

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the period and reasons of confidentiality and restriction.

[The instrument was duly prepared by The Centre for Academic Information Services]

ACKNOWLEDGEMENTS

First and foremost, I would like to express my utmost thankfulness to the Lord Almighty for all the blessings, guidance, and gifts which He had bestowed unto me during the period of this research.

Secondly, I would also like to acknowledge my supervisor, Miss Rosmawati binti Saat who has been giving me the chance to do this study under her supervision. I really thankful for her constant support, advices, guidance and patience in assisting me from beginning to the completion of this thesis. I would also like to thank my co-supervisor, Associate Professor Awang Ahmad Sallehin Awang Husaini for allowing me to borrow some equipments in his laboratory. I would like to take this opportunity also to thank the postgraduate students in our laboratory who willingly to lend their hand in sharing information and guide me throughout this research.

Next, I would like to express my deepest gratitude to my family members for their continuous encouragement, moral and financial support, and love in helping me overcome all the difficulties throughout this project.

Last but not least, sincere thanks to all my friends, course mates and laboratory mates for their kindness and moral support during the completion of this research project. Thank you for the friendship and for the whole awesome experiences and memories studying in UNIMAS. Not to be forgotten to the individual who indirectly had contributed in this research project, your kindness means a lot to me. Thank you very much.

Dedicated to beloved my father, my lovely mother, my awesome siblings, my cheerful friends and my kind-hearted lecturers. May this work can provide some useful informations to those who are interested in this field of study in the future.

DECLARATION

I, **Mary Molly Anak Bihos, 42013,** from the Faculty of Resource Science and Technology, hereby declare that the work entitled, **INVESTIGATING THE EFFECT OF MEDIA SUPPLEMENTS AND DIFFERENT UREA CONCENTRATION ON THE AMYLASE PRODUCTION VIA SOLID STATE FERMENTATION BY *ASPERGILLUS FLAVUS*,** to the best of knowledge is my original work. I have not copied from any other students' work or from any other sources except where due references and acknowledgements are made clearly in the text. My work has been submitted and shall not be submitted to other university or institute of higher learning.

Date submitted: 17th June 2016

(MARY MOLLY ANAK BIHOS)

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A. flavus	*Aspergillus flavus*
A. fumigatus	*Aspergillus fumigatus*
A. niger	*Aspergillus niger*
PDA	Potato Dextrose Agar
KH_2PO_4	Potassium dihydrogen phosphate
mL	millilitre
µL	microliter
mg/µL	milligram/microliter
SSF	Solid state fermentation
rpm	revolutions per minute
mg/l	milligram per liter
min	minutes
°C	degree Celsius
w/v	weight per volume
%	percentage
U	milligram/min/ml

LIST OF TABLES

LIST OF FIGURES

Investigating the effect of media supplements and different urea concentration on the amylase production via Solid State Fermentation by *Aspergillus flavus*

Mary Molly Anak Bihos

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

In this study, amylases were produced through solid state fermentation by using *Aspergillus flavus* as the producer microorganism and rice husk as the substrate. Effect of different media supplements on the enzyme activity was investigated. Peptone, ammonium sulphate, potassium dihydrogen phosphate, and urea were used as the media supplements. There were three ways on how the media supplement had been added. Among the three parameters that had been conducted, potassium dihydrogen phosphate and ammonium sulphate showed the highest amylase activity (1.164 U/mL and 0.983 U/mL respectively) in parameter 3 in which media supplements were added to both PDA and SSF substrate. Meanwhile for peptone and urea, the best parameter to produce higber enzyme activity of amylase was parameter where media supplements were added to the SSF substrate. Lastly, tbe suitable concentrations of urea in producing enzyme with higher activity were 0.2% and 0.3%.

Keywords: media supplements, amylase, *Aspergillus flavus*, rice husk, solid state fermentation.

ABSTRAK

Dalam kajian ini, enzim amilase dihasilkan melalui kaedah fermentasi berkeadaan pepejal dengan menggunakan Aspergillus flavus sebagai mikroorganisma pengeluar dan hampas padi sebagai substrat. Kesan penggunaan suplemen media yang berbeza terhadap aktiviti enzim telah dikaji seperti pepton, ammonium sulfat, fosfat kalium dihydrogen, dan urea. Terdapat tiga cara bagaimana suplemen media ditambah untuk membantu di dalam penghasilan enzim. Antara tiga parameter yang telah dijalankan, kalium fosfat dihydrogen dan ammonium sulfat menujukkan aktiviti enzim yang maksima (1.164 U / mL dan 0.983 U/mL masing-masing) dalam parameter 3 di mana suplemen media ditambah pada kedua-dua PDA dan subtrat SSF. Walau bagaimanapun, bagi suplinen media pepton dan urea, parameter yang terbaik untuk menghasilkan amilase yang lebih tinggi adalah melalui parameter 2 di mana suplemen media ditambah pada substrat SSF sahaja . Akhir sekali, kepekatan urea yang bersesuaian dalam menghasilkan enzim yang mempunyai aktiviti enzim yang lebih tinggi tinggi adalah 0.2% dan 0.3%.

Kata kunci: suplemen media, amilase, Aspergillus flavus, hampas padi, fermentasi berkeadaann pepejal.

1.0 INTRODUCTION

Aspergillus species belongs to the Deuteromycota division of the fungi kingdom. *Aspergillus* species is known for their ability to produce various enzymes that are useful in today's industries such as food, agricultural, pharmaceutical, cosmetics and many more (Gurung et al., 2013). Example of enzymes that are produced by *Aspergillus* species are amylase, cellulase, protease and xylanase. It is assumed that the higher the growth rates of *Aspergillus* species, the higher the production of enzymes (Pau & Omar, 2004). In this study, *A. flavus* is selected because it is closely related to *Aspergillus oryzae*, a fungal species that often used for fermentation of Asian food (Chang & Ehrlich, 2010). In addition, *A. flavus* is stable at both high and low pH as well as high temperature, in which makes it suitable for industrial scaled enzyme production (Oyeleke et al., 2010).

Amylases are significant enzymes in biotechnology and are commercially important in various industries. Amylase comprises 30% of the world enzyme production (Maarel van der et al., 2002). Amylase is useful in various applications such as in the clinical, medicinal and analytical chemistry sectors. In addition, amylase is useful in baking, detergent, textile, paper and distilling industry. Commercial amylases are traditionally produced via submerged fermentation (Gangadharan et al., 2006). Solid state fermentation (SSF) is widely used in the industrial process to produce a large scale of useful enzymes due to the advantages of the technology used. Rice husk, the outermost layer of paddy grain was used as the substrate in this study due to its composition which is suitable for enzyme production in which the fungi utilise the rice husk as the carbon source in SSF (Ang, 2013).

Media supplements can be in different form of sources such as carbon sources, nitrogen source, mineral salts, metal ions, and amino acids. It is usually added into the growth media to provide nutrients in cultivating fungi. In addition, media supplement is crucial in helping the biosynthesis of fungi's secondary metabolism (Calvo et al., 2002), which means it is part of the factor that controls the production of the fungal enzyme. In the SSF process, media supplement is usually added to the substrate to enable the fungi to enhance their enzyme production. This is because limited availability of the nutrients in the substrate influences the poor growth of fungus in which later will cause in low production of the degrading enzymes (Kumar & Kanwar, 2012). Concentration of media supplement applied into the SSF also influences the fungal enzyme production (Esfahanibolandbalaie et al., 2008). Based on the previous research, media supplements are only used on the substrate SSF to study their effects on the amylase production by fungi. Therefore, the present work is undertaken to investigate which media supplements that can induce higher amylase activity of *A. flavus* under three different parameters by using rice husk as the substrate.

The objectives of this study are:

i. To determine the effect of different media supplements on the amylase production when added to the PDA.

ii. To determine the effect of different media supplements on the amylase production when added to SSF substrate.

iii. To determine the effect of different media supplements on the amylase production when added to both PDA and SSF substrate.

iv. To determine the suitable urea concentration on the amylase production by *A. flavus* via SSF.

2.0 LITERATURE REVIEW

2.1 Amylase

Amylase is one of the beneficial enzymes that is involved in the food, fermentation, textile, paper, as well as in the medicine production industries. Amylases contributed about 25% of industrial enzymes in the world market (Rajagopalan & Krishnan, 2008; Reddy et al., 2003). Amylases act as digestive enzymes that function to break down glycosidic bonds in starch into glucose, maltose, maltotriose and dextrin (Souza & Magalhaes, 2010).

Amylases can be found in animals and plants but mostly in microorganisms. The amylases produced from microorganisms have a wide range of industrial applications as they are more stable compared to animal and plant amylases (Maarel van der et al., 2002). The major advantage of using microorganisms for the production of amylases is the higher productivity in a short growth period (Mishra and Behera, 2008). In addition, microorganisms are easy to manipulate to obtain desired by-products (Souza & Mugalhaes, 2010).

Three major classes of amylases have been identified in microorganisms, namely α-amylase, β-amylase and γ-amylase. α-Amylases (E.C. 3.2.1.1) which specifically known as endo-1, 4-α-D-glucan glucohydrolase, are extracellular enzymes that randomly split the 1, 4-α-D-glucosidic linkages between neighbouring glucose units in the linear amylose chain (Souza & Magalhaes, 2010). β-amylase (E.C. 3.2.1.2) catalyses the hydrolysis of the second α-1, 4 glycosidic bond, working from the non-reducing end, cleaving off two glucose units (maltose) at a time (Shirpra et al., 2011). Lastly, γ-amylase (E.C. 3.2.1.3) breakdown γ-amylase split α(1-6)glycosidic bond, and cleave last α(1-4)glycosidic linkages at the non-reducing ends of amylose and amylopectin which produced glucose (Konsoula & Liakopoulou-Kyriakides, 2007).

2.2 *Aspergillus flavus*

Aspergillus flavus is a saprophyte that grows on the dead plant and animal tissue (decaying matter) in the soil. This fungus is useful in nutrient recycling (Scheidegger & Payne, 2003). *A. flavus* has the ability to withstand a high temperature environment and can survive at a low water volume compared to the other types of filamentous fungi.

A. flavus grows rapidly at optimum temperature of 37 °C and can grow between the temperatures of 12 to 48 °C (Hedayati et al., 2007). *A. flavus* also has an optimum enzymatic production ranging from pH 3 to pH 7 (Hedayati et al., 2007). It grows by producing network of hyphae, known as the mycelium that secretes enzymes for the breakdown of food. The simpler units of food sources are absorbed by the mycelium to enhance fungal growth.

Amylase produced from fungi had gained much attention because of the availability and high productivity of fungi, which provides a good alternative for the costly enzyme production process (Prasad & Sekhar, 2013). According to Kathiresan & Manivannan (2006), amylases produced from fungi had been used in a variety of applications ranging from chemical, detergent, food, textile, and paper industries. This is due to their affordable cost, consistency, less time and space required for the enzyme production as well as the modification and optimization of the process (Burhan et al., 2003). A study done by Geetha et al. (2011) found that *A. flavus* that had been subjected to solid state fermentation produced higher amylases than submerged fermentation. They also stated that optimum moisture content for maximum enzyme yield by *A. flavus* is 62% (Geetha et al., 2011).

2.3 Solid state fermentation

Solid State Fermentation (SSF) has been used in the production of feed and food for human and animals. This technology obtained the support and carbon supply from the usage of agro-industrial waste for production of various value added products, such as industrial enzymes, secondary metabolites, single cell protein, and biofuel (Couto & Sanoroman, 2006). A number agro-waste residue had been employed for the cultivation of microorganisms to produce desired enzymes. SSF involves the growth of microorganism on moist solid substrate in the absence or near-absence of free water, whereby there is a continuous gas phase between spaces of the solid material (Mitchell et al., 2006; Pandey et al., 2001). In the SSF process, the solid substrate also served as an anchorage for the microbial cells instead of supplying nutrients to the culture. According to Baysal et al. (2003), moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities, thus optimum moisture level of the substrate is a crucial factor for enzyme production.

The main advantage of this technology is that nutrient-rich waste materials can be easily recycled and used as substrates in this method. Besides, SSF allows the substrates to utilize very slowly and steadily. Hence, the same substrate can be used for a longer interval, thereby reducing the necessity to constantly supply substrate to the process (Kunamneni et al., 2005). Other advantages of SSF are requirement of simpler equipment, higher yields in a shorter period of time, better oxygen circulation and lesser effluent generation (Couto & Sanromán, 2006). Filamentous fungi are one of the favourite microbes used in SSF as they can grow and penetrate into the depth of the solid substrate (Pandey, 2003). In addition, SSF provides a medium that resembles the natural habitat of fungal species (Sundarram & Murthy, 2014).

2.3.1 Rice husk as the substrate in SSF

Rice husk is an agro-waste product that is usually produced from rice milling activity which can be used for solid state fermentation of *Aspergillus* species. Rice husk is the outermost layer of paddy grain which accounts of 25% to 35% of its weight. According to the Food and Agriculture Organization (2001), 20% of rice husk is produced every year from 649.7 million tons of rice yielded worldwide. Rice husk has been used as energy generator in some countries (Ahiduzzaman, 2007) as it contained high fibre, low digestibility and low bulk density and high ash/silica content (Saha and Cotta, 2008). Rice husk is lignocellulosic substrates which contained of 25% to 35% of cellulose, 18% to 21% of hemicellulose, 26% to 31% of lignin, 15% to 17% of silica, and 2% to 5% of soluble solids (Luduena et al., 2011). The compositions of rice husk make it suitable for industrial use such as bioconversion and enzyme production. The ability of fungi in bioconversion is attributed to their capability in utilizing lignocellulosic biomass as energy source.

Moisture content of solid substrate in solid-state fermentation is an important parameter for biosynthesis and secretion of enzymes which corresponds with the characteristics of the lignocellulosic materials (Ellaiah et al., 2002; Ramachandran et al., 2004). Moisture level is determined by the type of substrate, type of desired product and the requirement of the microorganism (Mienda et al., 2011). This may be due to the fact that substrates with a good water holding capacity needs lower moisture content for production of the enzyme ideally. In fungal solid-state fermentation, the suitable moisture content of solid substrate used is between 20% to 70% (Perez-Guerra et al., 2003; Rodriguez-Leon et al., 2008).

2.4 Media supplements

Media supplements are added to the media agar not only to enhance the growth of fungi but also to increase secondary metabolites production (Calvo et al., 2002). Examples of media supplement that can be added are glucose, sucrose, fructose (carbon sources), peptone, yeast extract, beef extract (amino acids), proline, methionine, and tryptophan (nitrogen sources), mineral salts and others. Selection of best supplement is one of the most critical phases in determining the efficiency of any growth process of the fungi and their enzyme activity. Media supplements can also be applied to the substrate in SSF and moistened with sterilised distilled water to obtained desired initial moisture content (Mrudula & Murugammal, 2011). The amount of media supplement can be added by using weight per volume of water or weight per weight of substrate (Irfan et al., 2012).

2.4.1 Peptone

Peptones are hydrolysed proteins derived from casein, meat and plants. Peptone can be found in a few numbers of agar media such as Malt Extract Agar and Sabaraud agar. Peptone act as the best nitrogen source for α-amylase production which had been reported earlier by Kaur et al. (2003) and Hernadnez et al. (2006).

2.4.2 Ammonium sulphate

Ammonium sulphate is an inorganic nitrogen source that usually involved in nutritional factor for enzyme production for microbial species such as *Aspergillus* spp., *Bacillus* spp., *Trichoderma* spp., and others. This might due to the fact that ammonium sulphate favoured microorganism growth and their enzyme secretion (Narang & Satyanarayana, 2001).

2.4.3 Potassium dihydrogen phosphate (KH_2PO_4)

KH_2PO_4 is one of mineral salt supplement that can be found in a high salt medium to grow fungi. A study done by Mahmood et al. in 1978 had mentioned that potassium dihydrogen phosphate used in the based medium as phosphorus source favoured amylase production by *Aspergillus niger*.

2.4.4 Urea

Urea belongs to the organic nitrogen family. Based on the research conducted by Geetha and colleagues (2011) urea shows the lowest result in amylase production as compared to other nitrogen source such as beef extract, peptone, and casein. However, a study done by Kumari et al. (2012), enzyme production is higher in medium containing organic nitrogen sources via SSF, especially using urea.

3.2 Cultivation of *A. flavus using* different media supplements

The fungus was grown on PDA media containing different media supplements. A percentage of 1% (w/v) of each media supplements peptone, ammonium sulphate, potassium dihydrogen phosphate, and urea (0.1% to 0.5%) were added into the PDA, separately. Each mixture was dissolved using ultrapure water and autoclaved at 121°C for 15 mins. Then, the mixture was poured into the petri dishes. After cooling and solidification, one plug of *A. flavus* from the fungus culture was placed in each of the petri dishes. PDA media without any media supplement was also prepared and one plug of *A. flavus* was inoculated on it. Each preparation was duplicated. All the plates were incubated at room temperature for 5 to 6 days.