IMPROVEMENT OF PHYTASE BIOSYNTHESIS BY NEW BACTERIAL ISOLATE, Pediococcus pentosaceus C4/1A VIA CONTINUOUS CULTIVATION

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Chairman : Assoc. Prof. Rosfarizan Mohamad, PhD
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Phytase enzyme is widely distributed in animals, plants and microorganisms which help in the degradation of phytate. Among the microorganisms, members of lactic acid bacteria (LAB) such as *Pediococcus pentosaceus* were found to produce phytase enzyme. The production of phytase in this study was identified by qualitative and quantitative enzyme assay. Qualitative assay was done by cultivation of the strains on modified MRS agar which contains MOPS and sodium phytate to observe the presence of clear halo zone, which indicates the enzyme production. Quantitative test was carried out to identify the highest phytase producer by cultivation of all the five isolates in modified MRS broth in shake-flask. The highest phytase producer was then selected and identified as *Pediococcus pentosaceus* C4/1A for conventional screening process based on medium formulation and culture conditions using Erlenmeyer shake-flask.

Different nitrogen sources (peptone with casein, meat extract, yeast extract), carbon sources (glucose, maltose, lactose), different concentration of sodium phytate and inoculum size were determined on phytase production. From the study, yeast extract (20 g/L), glucose (15 g/L), sodium phytate (5 g/L) and inoculum size (5% (v/v)) gave effect on the phytase production by *P. pentosaceus* C4/1A. Further optimization by using Response Surface Methodology (RSM) was carried out on phytase production and cell biomass. The standardized protocol for phytase production was carried out in large scale using 2L stirred tank bioreactor. Formulated medium with glucose (16.2 g/L), yeast extract (17.2 g/L), sodium phytate (11.8 g/L) and inoculum size (10% (v/v)) were used to obtain high phytase activity. Continuous cultivation was then conducted for mass production and to determine the productivity of microbial biomass and production of phytase. Different range of dilution rate (D) was applied for 48 h to study the productivity of the cell and phytase. Secondary data based on phytase production, cell concentration and substrate consumption in small scale and large scale cultivation was developed and compared.

Generally, all the chosen strains grew well on the modified MRS broth, however only one best phytase producer (*P. pentosaceus* C4/1A) exhibits highest enzyme activity of 21.25 U/mL. Further improvement through approach of RSM, 42.3 U/mL of phytase production was achieved with cell concentration of 6.46 g/L. Production of phytase then applied in large scale fermentation using 2L stirred tank bioreactor and 40.2 U/mL of phytase was synthesized by *P. pentosaceus* C4/1A, which comparable to the shake-flask cultivation (42.3 U/mL) under the optimized conditions. Continuous cultivation was carried out for mass production at dilution rate ranging from 0.1 h^{-1} to 0.4 h^{-1} and the steady state of *P. pentosaceus* C4/1A was achieved after five generation and three residence times. The whole process of cultivation was carried out for 48 h and the highest productivity of phytase and cell concentration was obtained at dilution rate 0.3 h^{-1} which resulted 8.65 U/mL/h and 0.894 g/L/h, respectively. In conclusion, throughout the cultivation process, production of phytase was improved 89.3% from shake-flask experiment to 2L bioreactor. In future work, scaling-up study for mass production at large scale should be performed as the application of phytase is of great interest in many industries.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENAMBAHBAIKAN BIOSINTESIS PHYTASE OLEH BAKTERIA, *Pediococcus pentosaceus* C4/1A MELALUI KULTURAN SAMPINGAN

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Phytase enzim boleh didapati secara meluas pada haiwan, tumbuh-tumbuhan dan mikroorganisma yang membantu dalam degradasi phytate. Antara mikroorganisma, ahli-ahli bakteria asid laktik (LAB) seperti *Pediococcus pentosaceus* didapati menghasilkan phytase enzim. Pengeluaran pytase telah dikenalpasti dengan kaedah enzim kualitatif dan kuantitatif. Kaedah kualitatif telah dilakukan melalui penanaman mikrob pada agar MRS yang diubahsuai dimana ia mengandungi MOPS dan natrium phytate untuk memerhatikan kehadiran zon halo jelas, yang menunjukkan pengeluaran enzim. Ujian kuantitatif dijalankan untuk mengenalpasti pengeluar phytase terbaik melalui penkulturan kesemua lima pencilan dalam MRS didalam kelalang kon. Pengeluar phytase terbaik kemudian dipilih dan dikenalpasti sebagai *Pediococcus pentosaceus* C4/1A untuk proses saringan konvensional berdasarkan pengoptimuman medium.

Sumber nitrogen yang berbeza (pepton dengan kasein, ekstrak daging , ekstrak yis), sumber karbon (glukos, maltos, laktos) dan kepekatan natrium phytate dan inoculum saiz yang berbeza ditentukan dengan perngeluaran pytase. Berdasarkan kajian, ekstrak yis (20 g/L), glukos (15 g/L), natrium phyte (5 g/L) dan saiz inoculum (5 % (v/v)) memberikan kesan keatas pengeluaran phytase oleh *P. pentosaseus* C4/1A. Pengoptimuman lebih lanjut telah dijalankan dengan menggunakan kaedah rangsangan permukaan ke atas pengeluaran phytase dan biomas sel dalam 250 mL kelalang kon Erlenmeyer. Piawaian protokol untuk pengeluaran pytase telah dijalankan dalam skala yang besar dengan menggunakan bioreactor tangki berpangaduk 2L. Dirumuskan bahawa dengan menggunakan glukos (16.2 g/L), ekstrak yis (17.2 g/L), natrium phytate (11.8 g/L) dan saiz inoculum (10% (v/v)) dapat menghasilkan aktiviti phytase yang tinggi. Penanaman berterusan kemudianya dijalankan untuk pengeluaran besar-besaran untuk menentukan produktiviti mikrob dan proses pertumbuhan berkaitan yang lain. Kepekatan yang berbeza bagi kadar pencairan (D) telah digunakan selama 48 jam untuk mengkaji produktiviti sel dan enzim. Data sekunder berasaskan pengeluaran

phytase, kepekatan sel dan penggunaan subtract dalam skala kecil dan penanaman berskala besar telah dibandingkan.

Semua jenis mikrob yang telah dipilih berkembang dengan baik pada MRS yang diubah suai, bagaimanapun hanya satu phytate pengeluar terbaik (*P. pentosaceus* C 4/1A) mempamerkan aktiviti enzim tertinggi 21.25 U/ ml. Penambahbaikan melalui pendekatan RSM, 42.3 U/ml pengeluaran phytase dicapai dengan kepekatan sel 6.46 g/L. Pengeluaran phytase kemudian digunakan dalam penapaian berskala besar dengan menggunakan bioreaktor tangki berpengaduk 2L dan 40.2 U/ml phytase telah disintesis oleh *P. pentasaceus* C4/1A, berbanding dengan penanaman dalam kelalang kon (42.3 U/ml) dibawah syarat-syarat yang dioptimumkan.

Penanaman berterusan telah dijalankan pada kadar pencairan antara 0.1 h^{-1} kepada 0.4 h^{-1} dalam keadaan mantap. *P. pentasaceus* C4/1A dicapai selepas lima generasi. Keseluruhan proses penapaian telah dijalankan selama 48 jam, dan produktiiti tertinggi phytase dan sel kepekatan diperoleh pada kadar pencairan 0.3 h^{-1} yang menghasilkan 8.65 U/ml/h dan 0.894 g/L/h, masing-masing. Kesimpulannya, sepanjang proses penapaian, pengeluaran phytase telah meningkat 89.3 % daripada ekperimen didalam kelalang kon ke 2L bioreaktor. Dalam kerja-kerja masa depan, kajian pada skala besar perlu dilakukan untuk meningkatkan hasil produk dalam kadar yang banyak disebabkan permohonan phytase berada pada kadar permintaan yang tinggi dalam kebanyakkan industri.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

LAB	Lactic acid bacteria
MRS	de Man Rogosa Sharpe
rpm	Rotation per minutes
g/ L	Gram per liter
GRAS	Generally Regarded As Safe
RSM	Response surface methodology
CCD	Central composite design
Conc.	Concentration
h	Hour
t	Time at maximum cell concentration
X_{max}	Maximum cell concentration (g cell/L)
P_{max}	Maximum phytase concentration (U phytase/mL)
S_i-S_f:	Substrate consumed (g substrate/L)
S_i:	Initial substrate concentration (g substrate/L)
S_f:	Final substrate concentration (g substrate/L)
μ	Specific growth rate (h^{-1})
$Y_{x/s}$	Growth yield coefficient (g cell/g substrate)
$Y_{p/s}$	Phytase yield per substrate utilized (U phytase/g substrate)
$Y_{p/x}$	Phytase biosynthesis per cell (U phytase/g cell)
P_r	Phytase productivity (U phytase/mL/h)

CHAPTER 1

INTRODUCTION

Phytate known as myo-inositol hexakisphosphate, is one of the major storage form of phosphate in plants seeds (Suhairin et al., 2010). Phytate acts as anti-nutritional factor and reduces the bioavailability of phosphorus to monogastric animals (Lopez et al., 2002). Feeds given to poultry animals were initially supplemented with inorganic phosphate, due to deficiency of phytase in gastrointestinal tract (Feil, 2001). Many studies have been conducted on hydrolysis of phosphorus and reduce the excretion of excess phytate-phosphorus into the manure. Several bacterial strains were studied for the production of phytase to reduce the content of phosphorus in the environment. Those bacterial strains were *Lactobacillus, Enterobacter, Bacillus sp., Escherichia coli* and *Mitsuokella jalaludinii* (Quan et al., 2001).

In the year 1962, International Minerals and Chemicals (IMC) made an effort to develop commercial phytase. Thousands of microorganisms were tested for their ability to produce phytase. However, after many attempt made by IMC, finally an isolate was identified to produce phytase and able to hydrolyse phytate (Lei et al., 2013). The valuable isolate was *Aspergillus niger* PhyA and phytase was first commercialized in 1990s after characterization by Irving & Cosgrove (1971). However, fungus was no longer applied in animal feed as it contains toxin which might be harmful to animals. In this research, *Pediococcus pentosaceus* was chosen since it is probiotic and able to produce different types of enzymes such as phytase (Zamudio et al., 2001), and β-galactosidase (Semjonovs & Zikmanis, 2008). Phytase producing microorganisms are found mostly from soil especially bacteria which has fast growth (Tseng et al., 2000) and the rumen (Yanke et al., 1998).

Nowadays the use of enzyme in feeds and the demand for phytase are increasing rapidly in the global market. Improvement in the digestibility of nutrient leads to higher needs of feed utilization. India is in high demand of phytase usage since they have the largest population of cattle in the world which was estimated around hundred million. The demand for phytase was expected to increase in the coming years. Besides that, the usage of phytase as feed enzyme was rapidly growing in China after the high increase in the year of 2004 (Sareen Sarah, 2015). Researches nowadays were being carried out on LAB which is also known as probiotics where it has the ability to improve the growth of poultry and beneficial to the host organisms. Hence, LAB has the potential to eliminate pathogens and prevent from infections (Mozzi et al., 2010). It is known as "Generally Regarded as Safe" (GRAS) microorganisms that can be applied in animal feed.

In literature, extracellular production of phytase by native *Bacillus subtilis* was assayed using chemical assay and plate assay. Plate assay was performed on specific medium and chemical assay was carried out using ferrous sulphate molybdenum blue method (Shamna et al., 2012). In spite, most of the studies conducted have used these two methods to analyze and quantify the production of phytase. Optimization is the most

important step in fermentation of industrial bioprocess to enhance the enzyme production by the desired bacterial strain (S. Y. Lee & Kim, 2015). Nowadays, conventional method is not only being used in many studies but also statistical optimization using Response Surface Methodology (RSM) which was introduced by Box & Wilson (1951) is used and helps the researcher to design the experiment, study the effect of parameters used towards the responses in the experiment. Highest yield production can also be achieved through RSM (Kharel et al., 2002; Raissi, 2009). It provides several experimental runs to conduct the fermentation in correct order and helps to improve the production of desired product.

Continuous cultivation is a large scale production of desired product. It is a technique used by most of the researchers and being applied in biotechnology industries. Continuous cultivation process has potential to increase the productivity of the desired product compared to other methods especially microbial enzymes. Culture in continuous cultivation retains at steady state throughout the fermentation period, hence, maximum cell biomass and products can be achieved at this state. Most of the industrial enzymes such as single cell protein and vinegar production are produced by continuous cultivation which is up to 100 m^3 capacity and there are also enzymes which produced in a scale of hundred litres or less (Waites et al., 2009).

The focus of this study was on improvement of medium formulation and culture conditions for mass production of phytase by *Pediococcus pentosaceus* C4/1A in submerged fermentation. Hence, the specific objectives of the study were;

1) To identify and select the highest phytase producing LAB
2) To optimize medium formulation and culture conditions for phytase production by the selected strain of LAB using conventional and statistical approach of response surface methodology.
3) To evaluate continuous cultivation of the selected strain of LAB for mass production of phytase using 2L stirred tank bioreactor.

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