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LYSINE AND METHIONINE PRODUCTION FROM AGRO-WASTE SUBMERGED FERMENTATION USING LOCALLY ISOLATED LACTIC ACID BACTERIA

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By

NORFARINA MUHAMAD NOR

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

December 2016

….DEDICATION….

"I dedicate this thesis to my beloved parents,
Husband,
&
My 3 lovely kids
……
….
..

"...No matter how tough this story,

How thousands of miles I drove to be right here,

I am striving for triumph and gratification…"

……"Where there's a will, there's always a way"……

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in the fulfillment of the requirement of the Degree of Doctor of Philosophy

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Chairman : Assoc. Prof. Rosfarizan Mohamad, PhD
Faculty : Biotechnology and Biomolecular Sciences

Animal diets are essential to be supplemented with essential amino acids that are imperative for health and good growth. Intensive animal system and environmental constraints required new feeding strategies for the industry to be viable and sustainable. Improving animal productivity can be obtained through the maximum expression of the genetic potential of animals by nutritional approaches. Essential amino acid especially lysine and methionine are widely used as a feed additive in animal's diet to meet the requirements. However, limited sources and food grade of the essential amino acid supplied in the animal feed formulation has drawn the interest of many researchers to search lactic acid bacteria producing amino acids from the cheap substrate. Lysine and methionine play a major role in improving the efficiency of animal protein production during the early stage of growth. The production of lysine and methionine is possible through a judicious selection of microbial species, and the strains of lactic acid bacteria (LAB) are the potential lysine-methionine producers. Therefore, this study concerns on identifying high-potential isolate from LAB and also focusing on formulating the medium from agro-waste by using various optimization approaches.

In this study, isolation of indigenous LAB from different food sources was conducted to isolate superior lysine-methionine producer. A total of 18 isolates from 40 isolates were successfully identified then compared for their lysine and methionine productions. The superior LAB isolate was known as *Pediococcus pentosaceus* RF-1 which was identified fundamentally using 16S rRNA and scanning electron microscopy. Productions of lysine and methionine by *P. pentosaceus* RF-1 was further investigated using unstructured kinetic models (Logistic and Luedeking-Piret) by comparing two substrates (MRS and agro-wastes) used. The models were found suited to describe lysine-methionine

productions as a growth-associated process where the values of the non-growth-associated rate constant (β) for lysine and methionine productions were shown as zero (0). For the subsequent study of optimization, five environmental factors (molasses, nitrogen source, fish meal, glutamic acid and initial medium pH) were investigated in the shake-flask experiment. It showed that the molasses (5 g/L), fish meal (5 g/L), glutamic acid (0.5 g/L) and initial medium pH 7 gave significant effects on the growth of *P. pentosaceus* RF-1, and lysine methionine productions. Comparisons on the optimization study were conducted between the predictive RSM and ANN models. The RSM using central composite design (CCD) demonstrated 30 experiments of four factors. The RSM suggested that molasses (9.86 g/L), fish meal (10.06 g/L), glutamic acid (0.91 g/L) and initial medium pH 5.3 could enhance the productions of lysine and methionine. Data gathered from the RSM model were then applied in ANN study. The optimal configuration of the ANN model was found to be 4-5-2 with the explanation of incremental back propagation (IBP) algorithm in a combination of a sigmoidal transfer function (output) and linear hidden layer. Prediction of ANN models indicated that using molasses (10.02 g/L), fish meal (18 g/L), glutamic acid (1.17 g/L) and initial medium pH (4.26) was the greatest combination.

The cultivation of *P. pentosaceus* RF-1 for lysine-methionine productions was carried out in 2 L stirred-tank bioreactor using batch and continuous mode of operations. The maximum specific growth rate (μ_{max}) of 0.306 h^{-1} was obtained during the batch cultivation process. The effects of dilution rates (D) ranging from 0.2 to 0.4 h^{-1} were performed in continuous operation. The cultivation of *P. pentosaceus* RF-1 in continuous operation was prolonged to 40 h to attain a steady-state condition. This result implied that the optimum dilution rate was at 0.30 h^{-1} for the lysine and methionine productivity of 2.09 g/L/h and 0.879 g/L/h, respectively.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENGHASILAN LISIN DAN METHIONIN DARIPADA SISA PERTANIAN FERMENTASI TENGGELAM MENGGUNAKAN BAKTERIA ASID LAKTIK PENCILAN TEMPATAN

Oleh

NORFARINA BT MUHAMAD NOR

Disember 2016

Pengerusi : Profesor Madya Rosfarizan Mohamad, PhD
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Diet haiwan adalah penting untuk ditambah dengan asid amino yang terpenting untuk kesihatan dan pertumbuhan yang baik. Sistem haiwan yang intensif dan kekangan alam sekitar memerlukan strategi pemakanan baru bagi industri untuk menjadi berdaya maju dan mampan. Peningkatan produktiviti haiwan boleh diperolehi melalui ekspresi maksimum genetik haiwan dengan pendekatan pemakanan yang berpotensi. Asid amino penting, terutamanya lisin dan methionin digunakan secara meluas sebagai bahan tambahan makanan dalam diet haiwan untuk memenuhi keperluan. Walau bagaimanapun, sumber-sumber terhad dan makanan gred asid amino penting yang dibekalkan dalam penggubalan makanan haiwan telah menarik minat ramai penyelidik untuk mencari bakteria asid laktik menghasilkan asid amino daripada substrat murah. Lisin dan methionin memainkan peranan utama dalam meningkatkan kecekapan pengeluaran protein haiwan pada peringkat awal pertumbuhan. Pengeluaran lisin dan methionine mungkin melalui pilihan yang bijak untuk spesies mikrob, dan strain bakteria asid laktik (LAB) adalah potensi pengeluar lisin-methionine. Oleh itu, kajian ini mengenal pasti pencilan yang berpotensi tinggi dari LAB dan juga memberi tumpuan kepada merangka sederhana dari agro-sisa dengan menggunakan pelbagai pendekatan pengoptimuman.

Dalam kajian ini, pencilan LAB asli daripada sumber makanan yang berbeza telah dijalankan untuk mengasingkan pengeluar lisin-methionine yang terunggul. Sebanyak 18 pencilan daripada 40 pencilan telah berjaya dikenal pasti dan kemudian dibandingkan untuk penghasilan lisin dan methionin. Pencilan LAB terunggul dikenal pasti sebagai *Pediococcus pentosaceus* RF-1 dengan menggunakan 16S rRNA dan mikroskop elektron imbasan. Penghasilan lisin dan methionin oleh *P. pentosaceus* RF-1 seterusnya dikaji dengan menggunakan model kinetik tidak berstruktur (Logistik dan Luedeking-

Piret) dengan membandingkan dua substrat (MRS dan agro-bahan buangan) yang digunakan. Model didapati sesuai dan menggambarkan pengeluaran lisin-methionine sebagai satu proses pertumbuhan yang berkaitan di mana nilai-nilai kadar yang berterusan bukan pertumbuhan berkaitan (β) untuk lisin dan penghasilan methionin ditunjukkan sebagai sifar (0). Untuk kajian seterusnya iaitu pengoptimuman, lima faktor persekitaran (sirap pekat, sumber nitrogen, makanan ikan, asid glutamik dan pH medium awal) telah di kaji dalam eksperimen kelalang-goncang. Ini menunjukkan bahawa molasses (5 g / L), makanan ikan (5 g / L), asid glutamik (0.5 g / L) dan medium awal pH 7 memberikan kesan yang penting kepada pertumbuhan *P. pentosaceus* RF-1, dan penghasilan lisin dan methionin. Perbandingan kajian pengoptimuman telah dijalankan antara ramalan RSM dan ANN model. RSM menggunakan pusat reka bentuk komposit (CCD) menunjukkan 30 eksperimen empat faktor. RSM mencadangkan bahawa sirap pekat (9.86 g / L), makanan ikan (10.06 g / L), asid glutamik (0.91 g / L) dan medium awal pH 5.3 boleh meningkatkan produksi lisin dan methionin. Data yang diperolehi daripada model RSM kemudiannya digunakan dalam kajian ANN. Konfigurasi optimum model ANN itu didapati menjadi 4-5-2 dengan penjelasan tambahan algoritma perambatan belakang (IBP) dalam gabungan fungsi sigmoidal pemindahan (output) dan linear lapisan tersembunyi. Ramalan model ANN menunjukkan bahawa menggunakan sirap pekat (10.02 g / L), makanan ikan (18 g / L), asid glutamik (1.17 g / L) dan medium awal pH (4.26) adalah gabungan yang terbaik.

Penanaman *P. pentosaceus* RF-1 untuk penghasilan lisin-methionin telah dijalankan di dalam 2 L bioreactor berpengaduk menggunakan kelompok dan mod berterusan operasi. Kadar maksimum tertentu pertumbuhan (μ_{max}) 0.306 h^{-1} telah diperolehi semasa proses penanaman berkelompok. Kesan kadar pencairan (D) antara 0.2 - 0.4 h^{-1} telah dijalankan dalam operasi yang berterusan. Pertumbuhan *P. pentosaceus* RF-1 dalam operasi berterusan berlarutan sehingga 40 j untuk mencapai keadaan mantap. Keputusan ini menunjukkan bahawa kadar pencairan optimum adalah pada 0.30 h^{-1} untuk lisin dan methionine produktiviti iaitu masing-masing pada 2.09 g / L / h dan 0.879 g / L / h.

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I certify that a Thesis Examination Committee has met on 1 December 2016 to conduct the final examination of Norfarina binti Muhamad Nor on her thesis entitled "Lysine and Methionine Production from Agro-Waste Submerged Fermentation using Locally Isolated Lactic Acid Bacteria" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

LAB	Lactic acid bacteria
Conc.	Concentration
Lys	Lysine
Met	Methionine
MRS	de Man Rogosa Sharpe
PCR	Polymerase chain reaction
RSM	Response surface methodology
ANN	Artificial neural network
rpm	Rotation per minutes
vvm	Volume per volume per minute
v/v	Volume per volume
w/v	Weight per volume
µM	Micromolar
g/L	Gram per liter
PKC	Palm kernel cake
D	Dilution rate (flow rate/volume) (h^{-1})
K_s	Monod cell growth saturation constant (g/L)
P_{max}	Maximum amino acid production (g/L)
X_{max}	Maximum cell concentration (g/L)
S	Substrate
α	Growth-associated constant (g/g)
β	Non-growth associated constant (g/g/h)
µ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})
$Y_{x/s}$	Growth yield coefficient (g cell/g substrate)
$Y_{p/s}$	Product yield per glucose utilized (g amino acid/g glucose)
$Y_{p/x}$	Amino acids per cell (g amino acid/g cell)
$P_{r.}$	Overall productivity (g amino acid/L.h)
m	Maintenance coefficient

CHAPTER 1

INTRODUCTION

Amino acids often called the building blocks of life. They have long played a significant role in both human and animal nutrition and health maintenance (Ivanoc et al., 2014; Leuchtenberger, 2005). They are used as animal feed additives (lysine, methionine, threonine), flavor enhancers (aspartic acid, monosodium glutamate, serine), ingredients in cosmetic and medicinal products and as specialty nutrients (Kranenburg, 2003). Of the 20 standard protein amino acids, the nine essential amino acids occupy a critical position in that the animals cannot synthesize them in quantities sufficient for excellent performances, and they must be supplied in the diet (Ravindran and Bryden, 2007). Lysine and methionine are one of the essential amino acids and well known as first and second limiting amino acids in most poultry diets (Saengkerdsub et al., 2013). The supplementations of lysine and methionine are the most efficient and cost-effective way to get maximum animal performances.

The industrial production of lysine and methionine is generally based on fermentation process by bacteria or fungi (Ivanov et al., 2014). Selections of the microorganisms are important in the fermentation process for producing products at optimal production levels. Strains of lactic acid bacteria (LAB) had been applied in amino acids production. LAB possessed particular physiological activities and regarded as safe (GRAS) organisms which have been extensively utilized in food industries (Li et al., 2010).

Malaysia is a well-known as a major producer of palm oil. The industry produces numerous agricultural by products including palm kernel cake, oil palm empty fruit bunch, rice husk, bagasse, banana peel and etc. These agro-wastes are good sources of sugars, minerals, and proteins (Pandey et al., 2000). Hence, they can be considered as raw materials for industrial processes in the production of products such as amino acids, alcohol and organic acid (El-Aasar, 2006; Sarlin and Philip, 2013).

In the fermentation process, kinetic and modeling for the beneficial microorganisms in food metabolites systems have been used to describe the cell growth, substrate uptake, and product formation. In structured models, the cell growth is defined and includes intracellular components, such as the RNA content, enzymes, reactants and products (Nielson et al., 1991). In unstructured models, the bacterial kinetics could be described in multiple natural substrates. Growth or non-growth models are also applied to propose the variations of other biochemical compounds and physical properties from the overall processes (Leroy et al., 2002).

Optimization of fermentation conditions is an important stage in the development of industrial bioprocess. The traditional of *one-factor-at-a-time* (OFAT) in optimization studies is not only time consuming but involved a number of experiments to estimate the optimum levels. Hence, optimization of the statistical approach such as response surface methodology (RSM) package enables the researcher to design experiments, build the blocks, and evaluate the effect of factor and response throughout the study. The RSM is a combination of good experimental design, regression modeling techniques, and optimization using RSM is a useful tool for the process of improvement. Another advance technique in evolving continuing work from RSM is known as artificial neural network (ANN). The possible used of ANN in many fields of studies are well reported (Basri et al., 2007; Nelofer et al., 2012; Yadav et al., 2013). The ANN is a superior technique when compared to the RSM approach. The simple structure of ANN is comprised of an input layer, a hidden layer, and the output layer. Modeling framework with proven and potential applications across sciences has been reported (Yadav et al., 2013).

The importance of amino acids as a feed additive in poultry diet requires continuous efforts in their production via fermentation processes. As such, efficient methods to enable high substrate conversion are needed. Batch, fed-batch, and continuous cultivation processes are the modes of operation frequently applied in industries. However, each of these processes has some advantages and disadvantages (Hamidreza et al., 2014). Although these methods have been established in the production of valuable metabolites, but the variables involved like substrate and beneficial strains to be used would differ according to availability and efficiency in the amino acid production.

Hence, the scope of the study was to use the statistical approach to optimize lysine and methionine production by LAB using agro-wastes media in submerged fermentation. The specific objectives were:

1. To isolate and identify lysine and methionine producing LAB from local food sources.
2. To compare the kinetic and modeling of LAB isolate grown in MRS and agro-waste media.
3. To optimize the medium formulations and initial medium pH via OFAT method for lysine and methionine productions by LAB isolate.
4. To compare the performances using statistical methods (RSM and ANN) for lysine and methionine productions by LAB isolate.
5. To scale up the productions for lysine and methionine using batch and continuous modes of bioreactor operation.

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