SCREENING AND CHARACTERISATION OF LACTIC ACID BACTERIA AND SUBSTRATES FOR LYSINE PRODUCTION

TOE CUI JIN

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By

TOE CUI JIN

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TOE CUI JIN

June 2017

Chair : Professor Foo Hooi Ling, PhD
Faculty : Biotechnology and Biomolecular Sciences

Every year, large scale of amino acids (AA) production is required to meet the high demand in various industries especially the livestock industry. Most of the AA are produced from genetically engineered *Corynebacterium glutamicum* and *Escherichia coli.* However, there are increasing concern regarding the use of genetically modified microorganisms and products derived from these microorganisms which may have negative impact on human and environment. Therefore, it has urged the search for a safer food-grade producer strain. Previous studies reported that lactic acid bacteria (LAB) have the capability of producing AA owing to their well-established proteolytic system. However, the production of AA by LAB has not been studied extensively. Therefore, this study was conducted to establish the process of producing AA by selected LAB isolates using locally available substrates.

Initially, phenotypic and genotypic characteristics of the selected LAB isolates were determined for the identification of LAB isolates. The results showed that all studied LAB isolates were stained Gram positive with seven LAB isolates were identified as *Pediococci* sp. and one *Lactobacillus* sp. The growth profile of all LAB isolates showed that active cultures were obtained at 10 h of incubation. Furthermore, a standard reference of Log colony forming unit (CFU)/mL versus optical density at 600 nm (OD_{600nm}) was constructed to quantify the cell concentration for each LAB isolate. Subsequently, the detection of active extracellular proteolytic activity towards skim milk and azocasein under three different pH conditions showed that the extracellular proteolytic enzymes produced by LAB isolates were versatile and active over a broad pH range.

The AA production profile of the eight LAB that were previously isolated from fermented tapioca were determined in MRS medium. Results showed that all LAB isolates were able to produce free isoleucine, glutamate, proline and glycine. The production of AA by LAB isolate was strains specific. Among the studied LAB isolates, the best amino acid producer was *Pediococcus pentosaceus* I-UP2 with a total of 15 different amino acids being produced. Moreover, *P. pentosaceus* I-UP2 also showed the most promising result for lysine production as compared to other LAB isolates. The increment of lysine production by *P. pentosaceus* I-UP2 was suggested to be attributed to the biodegradation mechanism, whereby the increment of proteolytic activity at pH 5 was also detected correspondingly. Hence, the increment of lysine content was most probably due to the enhancement of extracellular proteolytic activity released by *P. pentosaceus* I-UP2.

Proximate analysis and AA determination on different substrates for lysine production was carried out prior to the selection of suitable substrates for factorial design. The results suggested that yeast extract, meat extract, peptone from casein, fish meal, mushroom waste, and fresh PKC were potential substrate for the production of lysine due to the availability of lysine and aspartate in these substrates. Based on the ANOVA results of 2-level fractional factorial design, meat extract, peptone from casein, mushroom waste and fresh PKC were revealed to be significant variables affecting the production of lysine by *P. pentosaceus* I-UP2. Validation of the identified variables showed that media V (meat extract, 8.18 g/L; peptone from casein, 7.41 g/L; mushroom waste, 57.87 g/L; glucose, 20 g/L; dipotassium hydrogen phosphate, 2 g/L; Tween 80, 1 mL; diammonium hydrogen citrate, 2 g/L; sodium acetate, 5g/L; magnesium sulphate heptahydrate, 0.2 g/L; manganases sulphate tetrahydrate, 0.04 g/L) was the best medium combination where up to 122% enhancement of lysine yield (54.75 mg/L) was detected. The present study will provide useful information to allow further attempts to explore the potential of LAB to produce AA and reduce the importation bill of Malaysia.

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

PENYARINGAN DAN PENCIRIAN BAKTERIA ASID LAKTIK DAN SUBSTRAT UNTUK PENGELUARAN LISINE

Oleh

TOE CUI JIN

Jun 2017

Pengerusi : Profesor Foo Hooi Ling, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Asid amino telah dihasilkan secara skala besar setiap tahun untuk memenuhi permintaan yang tinggi dalam pelbagai industri terutamanya industri ternakan. Kebanyakan asid amino yang dihasilkan adalah menggunakan *Corynebacterium glutamicum* dan *Escherichia coli* yang diubahsuai melalui kejuruteraan genetiik. Walau bagaimanapun, peningkatan kebimbangan tentang penggunaan mikroorganisma yang diubahsuai melalui kejuruteraan genetik dan produk yang diperolehi daripada mikroorganisma tersebut mungkin mempunyai kesan negatif terhadap manusia dan alam sekitar. Oleh demikian, ini menggesa pencarian penghasil asid amino yang bergred makanan. Bakteria asid laktik (LAB) telah dilaporkan mempunyai sistem proteolitik yang mantap dan boleh digunakan untuk menghidroliskan molekul protin kepada peptida dan asid amino bebas. Namun, penghasilan asid amino oleh LAB belum dikaji secara meluas. Oleh itu, objektif umum kajian ini adalah untuk menghasilan asid amino secara tempatan oleh LAB yang dipencilkan daripada Tapai ubi.

Ciri-ciri fenotip dan genotip daripada LAB telah ditentukan untuk menetapkan pengenalan setiap LAB. Hasil kajian menunjukkan bahawa semua isolate LAB adalah Gram positif dengan 7 isolat LAB ditetapkan sebagai *Pediococci* sp dan 1 *Lactobacillus* sp. Profil pertumbuhan semua isolat LAB menunjukkan bahawa LAB aktif telah diperolehi pada 10 h pengkulturan. Selain itu, keluk piawai Log CFU/mL berbanding OD_{600nm} telah diplotkan untuk setiap LAB untuk mengukur populasi sel. Tambahan pula, pengesanan aktiviti proteolitik ekstrasel yang aktif terhadap susu skim dan azocasein bawah 3 keadaan pH yang berbeza menunjukkan bahawa enzim proteolitik ekstrasel dihasilkan oleh isolat LAB adalah serba boleh dan aktif dalam julat pH yang luas.

Penghasilan asid amino daripada sebanyak 8 isolat LAB telah dijalankan di MRS media. Hasil kajian menunjukkan bahawa semua isolat LAB dapat

menghasilkan isoleucine, glutamat, proline dan glycine. Penghasilan asid amino oleh setiap isolat LAB adalah bergantung pada strain. Antara isolat LAB yang diuji, *P. pentosaceus* I-UP2 merupakan penghasil asid amino yang terbaik dengan sejumlah 15 asid amino berbeza yang dihasilkan. Selain itu, *P. pentosaceus* I-UP2 juga menunjukkan keputusan yang paling berpotensi dalam penghasilan lisin berbanding dengan isolat LAB lain yang diuji. Penghasilan lisin oleh *P. pentosaceus* I-UP2 telah dicadangkan melalui biodegradasi kerana peningkatan aktiviti proteolitik pH 5 mengakibatkan peningkatan kandungan lisin.

Komposisi proksimat dan profil asid amino bahan-bio telah dijalankan untuk memilih bahan-bio yang sesuai bagi penghasilan lisin. Keputusan mencadangkan bahawa ekstrak yis, ekstrak daging, pepton dari kasein, serbuk ikan, sisa cendawan, dan PKC segar adalah substrat yang berpotensi untuk penghasilan lisin disebabkan oleh kandungan lisin dan aspartik dalam bahan-bio. Seterusnya, kesan bahan-bio bagi penghasilan lysine oleh *P. pentosaceus* I-UP2 telah dikaji dengan menggunakan reka bentuk factorial. Berdasarkan ANOVA, faktor penting yang menjejaskan penghasilan lisin oleh *P. pentosaceus* I-UP2 telah dikenal pasti. Pengesahan faktor mengenal pasti bahawa media V adalah kombinasi yang terbaik dengan penghasilan lisin tertinggi. Lisin dihasilkan di media V adalah 54.57 mg/L dan adalah lebih tinggi berbanding dengan yang lisin dihasilkan di MRS tanpa perbezaan yang signifikan. Kajian ini akan memberikan maklumat yang berguna untuk membolehkan lagi percubaan untuk menerokai potensi LAB untuk menghasilkan asid amino dan mengurangkan bil import di Malaysia.

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This thesis was submitted to the Senate 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 follows:

Foo Hooi Ling, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Norhani Abdullah, PhD
Research Fellow
Institute of Tropical Agriculture and Food Secrurity
Universiti Putra Malaysia
(Member)

Rosfarizan Mohamad, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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Signature: ______________________________
Name of Chairman of Supervisory Committee: Professor Dr. Foo Hooi Ling

Signature: ______________________________
Name of Member of Supervisory Committee: Professor Dr. Norhani Abdullah

Signature: ______________________________
Name of Member of Supervisory Committee: Professor Dr. Rosfarizan Mohamad

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%	Percentage
% (v/v)	percent volume/volume
% (w/v)	percent weight/volume
°C	degree Celsius
µL	Microliter
µm	Micrometer
µm	micrometer
ANOVA	analysis of variance
BSA	bovine serum albumin
CFS	cell free supernatant
DAD	diode array detector
DAP	diaminopimelic acid
DNS	dinitrosalicylic acid reagent
FDA	food and drug administrtion
FMOC	9- fluorenylmethyl chloroformate
g	gram
GRAS	generally regarded as safe
h	hour(s)
H_2O_2	hydrogen peroxide
H_2SO_4	sulfuric acid
HBr	hydrogen Bromide
HCl	hydrochloride Acid
ISTD	internal standard
kg	Kilogram
L	liter
LAB	lactic acid bacteria
Log CFU/mL	colony forming unit
M	molar
mg	milligram
mg	milligram
min	minute(s)
mL	milliliter
mm	millimetre
mM	millimolar
MPOB	Malaysia Palm Oil Board
MRS	deMann, Rogosa and Sharpe
MSG	monosodium glutamate
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NaH_2PO_4	sodium dihydrogen phosphate monohydrate
NaOH	sodium hydroxide
nm	nanometre
OD	optical density
OPA	o-phthalaldehyde
PCR	polymerase chain reaction
PKC	palm kernel cake
pmol	picomole

rDNA	ribosomal deoxyribonucleic acid
sec	second(s)
SEM	standard error of the mean
TCA	trichloroacetic acid
U	unit of proteolytic activity
UV	ultra Violet
V	volt
× *g*	number of times the gravitational force

CHAPTER 1

INTRODUCTION

Amino acids (AA) are the monomers for proteins synthesis, which are vital in living organisms. Apart from acting as the substrate for protein synthesis, AA are also important in metabolic functions. There are a total of 20 different types of biological importance AA, known as the standard AA (Jakubke and Sewald, 2008). The standard AA are divided into essential and non-essential AA based on the ability of living organisms to synthesis the particular AA.

AA have been applied in various industries, including livestock industries, food industries and health care industries, as well as in many other sectors. (Leuchtenberger, 2005). In livestock industries, the major protein source use in animal feed is soybean meal. However, to reach the maximum growth and meat productivity, the amount of soybean meal used often caused some of the AA to be in excess. The excess AA resulted in excretion of excess ammonia from the animal which may pollute the environment (Gulinski *et al.,* 2016; Kerr and Easter, 1995). On the other hand, a decrease in the amount of protein use may result in deficiency of some AA. Deficiency in essential AA could halt the protein biosynthesis of animal and lead to severe growth impairment (Dersjant-Li and Peisker, 2011). Therefore, the usage of appropriate level and composition of AA as feed supplement to compensate the protein amount in animal feed could improve the performance and well-being of the animals (Emmert and Baker, 1997; Nurhazirah et al., 2013). In Malaysia, the livestock industry relies heavily on importation of high cost AA. Therefore, it is critical to produce AA locally to meet the aggressive development of livestock industry.

Lysine is an industrially important AA and it is heavily used as a supplement in various livestock industries. L-lysine is one of the essential AA that cannot be synthesised internally by the animals. A study carried out by Berri *et al.* (2008) showed that supplementation of lysine in broiler diet could improve the growth and breast meat yield of the broiler chicken. Although AA can be obtained from animals and plants by extraction methods, most AA are produced via fermentation by microorganisms owing to the rapid growth of microorganisms and mass production (Ramakrishnan *et al.,* 2013). Industrial production of AA is commonly conducted via submerged fermentation with agitated tank fermenter equipped with different controller to maintain the controlled environment. Besides, the use of cheap substrates from agro-wastes has been strongly suggested, whereby agro-wastes are converted into useful products by microorganisms via fermentation (Banuelos *et al.,* 2000; Benhabiles *et al.,* 2012; Gopinath *et al.,* 2011; Hermann 2003; Khan *et al.,* 2006). Additionally, optimisation studies on media and cultivation strategies for different products have been carried out to further enhance the productivity (Coello *et al*., 2002; Hadj *et al*., Li *et al*., 2010; 1988; Shah *et al*., 2002b). Optimisation can be

achieved via statistical approach and conventional approach. Current trend focuses more on statistical approach since it can explain interation effects with minimum experimental run (Elibol, 2004; Mander *et al*., 2013).
Currently, genetically modified *Corynebacterium glutamicum* and *Escherichia coli* are the most dominant microorganisms used for the production of AA (Hermann 2003; Leuchtenberger *et al.,* 2005; Razak and Viswanath, 2015; Wittmann and Heinzle, 2001). However, the use of genetically engineered microorganisms which are not food grade has been a major concern in food and feed industries. This has led to the search of a safer food grade producer strain. Recent studies showed that wild type lactic acid bacteria (LAB) are potential candidate for the production of AA either by biosynthesis or biodegradation pathways (El-Nemr and Mostafa, 2010; Simova *et al.,* 2006). Moreover, the use of LAB for AA production may simplify the downstream processing in food and feed industry because of the generally recognised as safe (GRAS) reputation of LAB.

Most of the LAB possess a well-established proteolytic system with complex combinations of proteinases and peptidases to obtain AA from complex peptides (Kok, 1990; Liu *et al.,* 2010; Mierau *et al.,* 1997). By exploiting LAB, protein molecules could be hydrolysed into peptides and free AA *in vitro* (Juillard *et al.,* 1995). Futhermore, Kleerebezem *et al.* (2003) showed that the genome of *Lactobacillus plantarum* encodes complete biosynthesis pathway for most AA. This showed that LAB have the ability to biosynthesis AA. A study conducted by Simova *et al.* (2006) showed that the proteolytic activity of LAB isolates has the capability to release free AA. In addition, El-Nemr and Mostafa (2010) have also concluded that *Lactobacillus* strains have potential to produce various AA. However, the production ability of AA by LAB has not been studied extensively.
The reports for LAB on AA production are limited and the search for LAB on AA production should be intensively pursued. Therefore, this study was conducted to investigate the production of AA by LAB via submerged fermentation. The specific objectives of this study were:

1. to identify and characterise the selected LAB isolated from fermented tapioca, *Tapai Ubi*.
2. to determine the AA production profile by selected LAB in MRS medium.
3. to characterise and determine the effect of locally available substrates on lysine production by selected LAB.

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