PILOT-SCALE PRODUCTION OF *LACTOBACILLUS RHAMNOSUS* ATCC 7469

By

LIEW SIEW LING

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman: **Professor Arbakariya Ariff, Ph.D.**

Faculty: **Institute of Bioscience**

The present study was undertaken in view of the demand for probiotic products as a result of health awareness in consumers and the lack of information pertaining to industrial probiotic production processes. Response surface methodology (RSM) was used to optimize the culture medium for the growth of a probiotic bacterium, *Lactobacillus rhamnosus* ATCC 7469. The factors studied were yeast extract, glucose, vitamins concentrations and initial culture pH. A polynomial regression model with cubic and quartic terms was used for analysis of the experimental data. RSM was found to be effective in developing an analysis model, finding the optimum point of the factors and assessing the effects of the factors. It was found that the effects involving yeast extract, glucose, vitamins and pH on the growth of *L. rhamnosus* were significant, and the strongest effect was given by the yeast extract concentration. Estimated optimum conditions of the factors for the growth of *L. rhamnosus* were as follows : pH = 6.9; vitamin solution = 1.28% (v/v); glucose = 5.01% (w/v) and yeast extract = 6.0% (w/v).

Further improvement of cell production was made by using a process optimization approach. The fermentation parameters investigated were aeration, mixing, pH, inoculum size and temperature. Cell production and viability were greatly influenced by the culture pH and temperature compared to other parameters such as agitation speed. The optimal culture conditions for the cultivation of *L. rhamnosus* in the 2-L stirred tank fermenter were as follows : mixing speed, 0.69 ms^{-1}; pH, 6.9; temperature, 37°C and inoculum size of 5% (v/v) in facultative condition. Under this condition, final cell viability obtained was 1.61 x 10^{10} CFU mL^{-1}, viable cell yield and productivity were 3.20 x 10^{11} CFU $g_{glucose}^{-1}$ and 1.33 x 10^{9} CFU mL^{-1} h^{-1}, respectively.

Unstructured models based on Monod and Luedeking-Piret equations were developed and found to be suitable to describe the cell growth, lactic acid production and substrate consumption by *L. rhamnosus* in batch cultivation in a shake flask, 2-L, 10-L and 100-L stirred tank fermenters. Lactic acid production was a growth-associated and non-growth-associated (mixed) process. Scaling-up on the basis of constant impeller tip speed resulted in increasing mixing time as fermenter working volumes increased, but the mixing times were still within the critical acceptable range as fermentation performance was not significantly affected.

Continuous cultivation was used in an attempt to further improve biomass production of *L. rhamnosus*. The maximum specific growth rate, μ_{max}, and the Monod cell growth saturation coefficient, K_s, were estimated at 0.4 h^{-1} and 0.25 g L^{-1}. Maximum cell viability (1.29 x 10^{10} CFU mL^{-1}) was achieved in the dilution rate (D) range of D = 0.28 h^{-1} to 0.35 h^{-1}, while both maximum viable cell yield and

productivity were achieved at D = 0.35 h^{-1}. Continuous cultivation of *L. rhamnosus* at D = 0.35 h^{-1} gave 267% improvement in viable cell count productivity as compared to batch cultivations. Results obtained from exponentially fed-batch cultivation of *L. rhamnosus* at a D = 0.4 h^{-1} indicated that this mode of cultivation might be a good alternative for *L. rhamnosus* production as higher cell concentration and lower lactic acid production could be achieved when compared to batch and continuous cultivations.

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

PENGHASILAN *LACTOBACILLUS RHAMNOSUS* ATCC 7469 SECARA SKALA BERPANDU

Oleh

LIEW SIEW LING

Disember 2004

Pengerusi: **Profesor Arbakariya Ariff, Ph.D.**

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Kajian ini telah dijalankan memandang permintaan yang semakin meningkat bagi produk probiotik akibat kesedaran kesihatan di kalangan konsumer serta kekurangan maklumat yang berkaitan dengan proses penghasilan probiotik secara industri. Kaedah Respons Permukaan (KRP) telah digunakan untuk mengoptimumkan medium kultur bagi pertumbuhan bakterium probiotik, *Lactobacillus rhamnosus* ATCC 7469. Faktor-faktor medium yang dikaji ialah jumlah ekstrak yis, glukosa, kepekatan vitamin dan pH permulaan medium kultur. Model regresi polinomial dengan sebutan kubik dan kuartik digunakan bagi analisis data ekperimen. KRP didapati berkesan untuk memperkembangkan model analisis, menentukan titik optimum bagi faktor-faktor yang dikaji serta menilai kesan-kesan factor-faktor berkenaan. Jumlah ekstrak yis, glukosa, vitamin dan pH medium kultur didapati memainkan peranan yang signifikan dalam pertumbuhan *L. rhamnosus*, dan ekstrak yis memainkan peranan yang paling ketara. Keadaan optimum faktor-faktor berkenaan bagi pertumbuhan *L. rhamnosus* adalah seperti berikut : pH = 6.9; larutan vitamin = 1.28% (v/v); glukosa = 5.01% (w/v) and ekstrak yis = 6.0% (w/v).

Penghasilan sel seterusnya dipertingkatkan dengan menggunakan pendekatan pengoptimuman proses. Parameter fermentasi yang dikaji adalah keadaan pengudaraan, pengadukan, pH, saiz inokulum dan suhu. pH dan suhu kultur mempunyai pengaruh yang lebih kuat ke atas penghasilan sel berbanding dengan parameter lain seperti pengadukan. Keadaan kultur yang optimum bagi penghasilan sel *L. rhamnosus* dalam fermenter berpengaduk 2 L adalah seperti berikut : kelajuan pengaduk, 0.69 ms^{-1}; pH, 6.9; suhu, 37°C and saiz inokulum 5% (v/v) dalam keadaan fakultatif. Di bawah keadaan tersebut, bilangan sel hidup yang terhasil adalah 1.61 x 10^{10} CFU mL^{-1} serta penghasilan dan produktiviti sel hidup adalah masing-masing 3.20 x 10^{11} CFU $g_{glukosa}^{-1}$ and 1.33 x 10^{9} CFU mL^{-1} j^{-1}.

Model tidak berstruktur yang berdasarkan persamaan-persamaan Monod and Luedeking-Piret telah diperkembangkan dan didapati sesuai untuk menerangkan pertumbuhan sel, penghasilan asid laktik dan penggunaan substrat oleh *L. rhamnosus* dalam sistem sesekelompok di dalam kelalang bergoncang dan fermenter berpengaduk bersaiz 2 L, 10 L and 100 L. Penghasilan asid laktik merupakan proses pertumbuhan berkait dan pertumbuhan tidak berkait (proses bercampur). Peningkatan skala berdasarkan halaju hujung pengaduk yang tetap telah menyebabkan pertambahan masa pengadukan dalam saiz fermenter yang semakin besar, tetapi masa pengadukan masih dapat dikekalkan dalam julat kritikal kerana prestasi fermentasi didapati tidak terjejas.

Fermentasi suapan selanjar telah digunakan untuk meningkatkan lagi penghasilan sel *L. rhamnosus*. Kadar pertumbuhan spesifik maksimum, μ_{maks}, serta koeffisien ketepuan pertumbuhan sel Monod, K_s, telah masing-masing dikira sebagai 0.4 j^{-1} and

0.25 g L^{-1}. Bilangan sel hidup yang maksimum (1.29 x 10^{10} CFU mL^{-1}) diperolehi dalam julat kadar dilusi (D), D = 0.28 j^{-1} hingga 0.35 j^{-1}, manakala penghasilan dan produktiviti sel hidup maksimum dicapai pada D = 0.35 j^{-1}. Fermentasi suapan selanjar *L. rhamnosus* pada D = 0.35 j^{-1} menghasilkan peningkatan sebanyak 267% dalam produktiviti sel hidup jika dibandingkan dengan fermentasi sesekelompok. Keputusan yang diperolehi daripada fermentasi suapan sesekelompok secara eksponen pada D = 0.4 j^{-1} menunjukkan bahawa kaedah fermentasi ini mungkin merupakan pilihan baik bagi penghasilan sel *L. rhamnosus* kerana bilangan sel yang lebih tinggi serta kepekatan asid laktik yang lebih rendah dapat diperolehi jika dibandingkan dengan fermentasi sesekelompok dan fermentasi suapan selanjar.

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I certify that an Examination Committee met on 20 Dec 2004 to conduct the final examination of Liew Siew Ling on her Doctor of Philosophy thesis entitled "Pilot-scale production of *Lactobacillus rhamnosus* (ATCC 7469)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows :

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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LIEW SIEW LING

Date :

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LIST OF ABBREVIATIONS

α	growth-associated rate constant for product formation ($g\ g^{-1}$)
β	non-growth associated rate constant for product formation ($g\ g^{-1}\ h^{-1}$)
Acetyl-P	Acetyl phosphate
ATP	Adenosine triphosphate
CFU	Colony forming units
DCW	Dry cell weight ($g\ L^{-1}$)
Dihydroxyacetone-P	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
DOT	Dissolved oxygen tension
EFBC	Exponentially fed-batch culture
Fructose-1, 6-DP	Fructose-1,6-diphosphate
Fructose-6-P	Fructose-6-phosphate
Glucose-6-P	Glucose-6-phosphate
Glyceraldehyde-3-P	Glyceraldehyde-3-phosphate
GRAS	Generally Recognised as Safe
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
KH_2PO_4	Potassium dihydrogen phosphate
LA	Lactic acid
LDH	Lactate dehydrogenase
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
$MnSO_4.H_2O$	Manganese sulfate monohydrate

NaOH	Sodium hydroxide
Q_b	Biomass productivity (CFU mL^{-1} h^{-1} or g L^{-1} h^{-1})
Q_p	Lactic acid productivity (g L^{-1} h^{-1})
Ribulose-5-P	Ribulose-5-phosphate
RNA	Ribonucleic acid
rpm	Rotation per minute
RSM	Response surface methodology
S	Substrate concentration (g L^{-1})
t	Time (h)
v/v	Volume/volume
vvm	Volumetric airflow rate/liquid volume
w/v	Weight/volume
Xylulose-5-P	Xylulose-5-phosphate
$Y_{p/s}$	Yield of lactic acid based on glucose consumed (g g^{-1})
$Y_{x/s}$	Yield of biomass based on glucose consumed (g g^{-1})

CHAPTER 1

INTRODUCTION

The existence of probiotics has been known for over a century and the relationship between certain foods and health has been investigated for many years. A number of definitions have been proposed to describe probiotics and an appropriate one was suggested by Havenaar & Veld (1992) who defined probiotics as "mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora". The most frequently used probiotics belong to the genera *Bifidobacterium* and *Lactobacillus* (Isolauri, 2004). There is general agreement on the important role of the gastrointestinal microflora in the health status of not only humans, but also animals. Thus, it is not surprising that there has been tremendous interest in the probiotic industry as probiotics have been classified as functional food ingredients (Roberfroid, 2003). In several universities and research centers overseas and in Malaysia, there are currently many ongoing studies on various aspects of probiotics i.e. health effects, sensorial and textural effects on food products and others. In fact, the Fermentation Technology Unit, Laboratory of Enzyme and Microbial Technology, Institute of Bioscience, University Putra Malaysia, where this study was conducted, has been approached by several local companies to help them in producing several probiotic species for human and livestock consumption. One of the species requested was *Lactobacillus rhamnosus*, which was the focus of investigation of the present study.