BIOPROCESSING DEVELOPMENT FOR ANAEROBIC CULTIVATION OF PROBIOTIC BACTERIA *Bifidobacterium longum* FOR HIGH CELL MASS

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A thesis submitted in fulfillment of the
requirements for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical Engineering
UniversitiTeknologi Malaysia

APRIL 2015

ACKNOWLEDGEMENT

In the Name of Allah, The Most Gracious, and Merciful. Firstly I would like to express my deepest appreciation to all those who provided me the possibility to complete the experiments as well as the thesis. A special gratitude to my supervisor, Prof Dr. Rer .Nat. Hesham Ali El-Enshasy for making this research possible. His support, guidance, advice throughout the research project, as well as his pain-staking effort in reading the last minutes drafts, and helping in compiling the data are greatly appreciated. Indeed, without his guidance, I would not be able to put the topic together. I would also thanks My Co-Supervisor Dr. Hassan Mohamed for his support, suggestions, and knowledge sharing even though we only had one year time together. I am also very thankful to the research officers at IBD especially to Puan Nor Zalina Othman, En. Soleh and Puan Roslinda Malek for their advices, and effort in guiding the students in experiments works. I would also like to thank my fellow postgraduate friends in IBD for their continuous support and my lab mates in Bioprocesing Group, for the discussions and for all the fun that we had together.in my previous 3 years.

Sincere appreciation also extends to all my colleagues and others who have provided assistance at various occasions. Their ideas and support are highly appreciated. Furthermore I would also like to acknowledge with much appreciation the crucial role of the technician staff who always gave the technical support during my study. Last but not least, I would like to thank my parents for their unconditional support, both financially and emotionally throughout my study. Their everyday prayer was the premium strength for me to finish what I've started. In particular, the patience and understanding shown by my family during this long 'journey' is greatly appreciated.

ABSTRACT

Bifidobacteria are used as probiotic mainly in the dairy industry as cell suspensions or as freeze-dried additives. *Bifidobacterium longum* is important in maintaining general health. Anaerobic growth of *B. longum* and its organic acids byproduct give some restriction to their growth. Therefore, the goal of this research is to select suitable optimized medium as production media of *B. longum* and its growth characteristics on this medium. Several media were tested for the potential effect to the cell growth. The best medium gave the cell mass of 2.58 g L^{-1}. In shake flasks, further optimization by using classical and statistical method gave the cell mass of 6.03 g L^{-1} and 5.95 g L^{-1}, respectively. The medium optimized using classical method was selected and used in the bioreactor. Cultivation in bioreactor was carried out with controlled and un-controlled pH. High cell mass was observed in controlled pH with bioreactor which yielded 11.97 g L^{-1}, compared with uncontrolled pH which gave only 7.5 g L^{-1}. Fed-batch cultivation was carried out by constant supplementation of glucose or complete media. Feeding with complete medium gave the highest cell mass comparing with glucose feeding, the cells obtained were 18.5 g L^{-1} and 17.7 g L^{-1}, respectively. Study on its growth characteristics revealed that *B. longum* has good resistance to bile salt concentration with 79 % cell survival at concentration of 0.3 % of bile salts. However, the resistance to bile salt was found to be affected by pH value. Following two-hour treatment, no cells survived at pH 1.0 while about 41.7 % of cells survived at pH 4. Surface adherence of the cells was tested by using hydrophobicity assays. High degree of hydrophobicity was observed during exposure to chloroform which gave the value of 86.1 %. Susceptibility of the cells was also tested with antimicrobial and antibiotics. *B. longum* was found resistant to all the pathogenic microorganism tested and susceptible to rifampicin, gentamycin, erythromycin, and ampicillin.

ABSTRAK

Bifidobakteria digunakan sebagai probiotik terutamanya dalam industri tenusu dalam bentuk ampaian atau bahan tambahan kering sejuk beku. *Bifidobakterium longum* adalah penting dalam mengekalkan kesihatan umum. Pertumbuhan anaerobik *B. longum* dengan pengeluaran asid organik menyekat pertumbuhan sel. Oleh itu, tujuan kajian ini adalah untuk memilih media yang sesuai untuk dioptimumkan sebagai media pengeluaran dan ciri-ciri berfungsi sebagai probiotik turut dikaji. Beberapa media telah diuji untuk mengkaji kesan potensi terhadap pertumbuhan sel. Medium terbaik memberikan ketumpatan sel sebanyak 2.58 g L^{-1}. Medium dioptimumkan lagi dengan menggunakan kaedah klasik dan statistik dan ketumpatan sel yang diperolehi adalah 6.03 g L^{-1} dan 5.95 g L^{-1}. Media yang dioptimumkan dengan kaedah klasik telah dipilih dan diaplikasi di dalam bioreactor samada dengan atau tanpa kawalan pH. Ketumpatan sel yang tinggi dapat diperhatikan dalam kawalan pH dengan bioreaktor yang menghasilkan 11.97 g L^{-1} berbanding dengan pH yang tidak dikawal yang memberikan hanya 7.5 g L^{-1} ketumpatan sel. Pengkulturan suap kelompok dilakukan dengan membekalkan glukosa dan media lengkap. Suapan menggunakan media lengkap memberikan ketumpatan sel terbaik iaitu 18.5 g L^{-1} berbanding dengan suapan glukosa iaitu 17.7 g L^{-1}. Kajian terhadap ciri berfungsi probiotik mendedahkan bahawa *B. longum* mempunyai tahap kehidupan sel sehingga 79 % pada kepekatan 0.3 % garam hempedu. Akan tetapi, rintangan terhadap garam hempedu dipengaruhi oleh nilai pH. Selepas 2 jam, tiada sel yang hidup pada pH 1.0 manakala kira-kira 41.7 % sel hidup pada pH 4. Tahap pelekatan permukaan sel diuji terhadap pelbagai jenis pelarut. Didapati pendedahan sel terhadap klorofom memberikan peratusan sebanyak 86.1 %. Sel juga mempunyai rintangan terhadap semua jenis mikroorganisma yang diuji, manakala di dalam ujian kepekatan antibiotik yang berbeza, tiada rintangan diperhatikan terhadap rifampicin, gentamycin, erythromycin, dan ampicillin.

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LIST OF ABBREVIATION

B. longum	-	*Bifidobacterium longum*
CDW	-	Cell dry weight
OD	-	Optical density
OD 540	-	Optical density at 540 nm
OD 600	-	Optical density at 600 nm
Sp	-	Species
USD	-	US Dollar
C	-	Carbon
DNS	-	3, 5-dinitro-salicylic acid
H_2	-	Dihydrogen
H_3BO	-	Boric acid
HCl	-	Hydrochloric acid
K_2HPO_4	-	dipotassium hydrogen phosphate
KH_2PO_4	-	Potassium dihydrogen phosphate
$MgSO_4.7H_2O$	-	Magnesium sulfate heptahydrate
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
P	-	Phosphate

LIST OF SYMBOLS

%	-	Percent
>	-	Greater than
µ	-	Specific growth rate (h -1) µmax
t_0	-	Initial time (h)
V	-	Volume of reactor liquid (L)
v/v	-	Volume per volume
v/v/m	-	Volume per volume per minute
X	-	Biomass concentration (g L -1)
α	-	Alpha
β	-	Beta
° C	-	Degrees Celsius
µl	-	Micro liter
µm	-	Micrometer
g	-	Gram
h	-	Hour
kg	-	Kilogram
L	-	Liter
M	-	Molarity
min	-	Minute
ml	-	Milliliter
rpm	-	Rotations per minutes

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nowadays, the demand on the functional food with the increase in properties such as low calorie value, low fat content and give benefit to the host. Within this gastrointestinal tract that consists of stomach, small intestine and large intestine can be described as a complex microbial ecosystem that comprises hundreds of bacterial species. According to Gibson and Roberfroid (1995), there are more than 500 kinds of bacteria with the number of 10^{12} for every gram of faeces exist in the human colon and vary with individuals. Major genus of human microflora includes *Bacteroides*, *Bifidobacteria*, *Coliform*, *Enterobacteriace*, *Fusobacteria*, and *Streptococci* (Fanaro *et al.,* 2003). Microflora brings health benefits to the host as the habitat was provided by the host in the symbiotic way (Shanahan, 2002).

Microorganism of the genus *Bifidobacterium* constituting one of the predominant microorganisms in the colon during the early stages of life (Harmsen *et al.,* 2000; Lay *et al.,* 2005), at a time when the immune system is not fully developed and the observed antagonistic has stimulated a great deal of interest in the role of *Bifidobacteria* in human health. Increasing health concerns and knowledge of intestinal microflora has led to the interest in using probiotic bacteria especially from genus *Bifidobacterium* as supplements in various types of foods products.

Bifidobacteria considered as a probiotic as they contribute in desirable changes and produces protective effect in intestinal tract. *Bifidobacteria* plays a role in inhibition growth of pathogenic bacteria by producing organic acids as it will reduce the pH of the colon (Ballongue, 1998). The ability of adherence to colon mucosa also helps to prevent the pathogen adherence as well as colon cancer induction (Ballongue, 1998). Apart from that, *Bifidobacteria* also important in the prevention and treatment of diarrhoea by participating in competitive exclusion against diarrheagenic bacteria (Bernet *et al.*, 1993).

A lot of media have been proposed for the detection of these bacteria however none can give complete selective effect on the media proposed (Rada and Petr, 2002). Media for *B. longum* which are simple and cost-effective have been established, but the influence of each medium composition and the interaction between each of media component still unknown. Furthermore, little are known about the behaviour and biomass production of this bacteria in bioreactor level.

Growth of *B. longum* influenced much by the composition of the medium used such as carbon sources, nitrogen sources, micronutrients, and macronutrients. Each medium composition plays a major part in terms of cost especially in large scale production process. Screening method was carried out before getting an optimized medium composition. During screening, each composition with best concentration will be select for further optimization process.

Thus, in this study, the effect of each media component on the growth of *B. longum* was evaluated by using one factor at a time method (OFAT) and statistical model approach by response surface methodology (RSM). This approach is important to accurately predict the influence of each component to get optimum cell mass production at shake flask level (Azaola *et al.*, 1999). Furthermore, application of the optimum medium and cultivation conditions were applied for bacterial biomass production in semi-large scale bioreactor 16-L.

Optimized medium composition was carried out further in pilot scale 16-L bioreactor under controlled and uncontrolled pH batch fermentations. Then, fermentation by fed-batch strategy with constant feeding was carried out under controlled pH. In fed-batch strategy, the cultivation media was fed constantly with full media and the best carbon source (glucose) that was screening before. Pilot scale fermentations is an important approach to study the kinetic growth of the probiotic bacteria studied.

Despite of medium optimization study, there are some others important factors such as acidity, bile, hydrophobicity, antimicrobial sensitivity, and antibiotics susceptibility which can affect the probiotic cells to remain and exert their potential functionalities in a host. Thus, a study on the growth of *B. longum* towards acidity and bile environment was carried out, followed by antimicrobial test with various pathogenic bacteria, antibiotics susceptibility, and hydrophobicity test with different types of solvent. Acidity and bile test was prepared by subjecting the cells in synthetic gastric juice at pH ranges from 1 to 4 and in media containing ox gall at different concentration for 0 min, 30 min, 60 min, 90 min, and 120 min. The tolerance of probiotic bacteria toward acid and bile salt was determined by the study growth of the cells in extreme environment.

1.2 Problem Statement

The demand for using *B .longum* as probiotic products has increase due to the health benefit that it can give to the host. The challenge that is faced when dealing with this anaerobic microbe is the preparation of this probiotic microbe for high biomass production and low cost and suitable media composition for industrial purposes. This research is aimed by designing efficient experimental design for optimization of medium composition. Hence, suitable medium composition that contribute to high biomass and produce low lactic acid as a byproduct could be obtained. Moreover, their fastidious character with little information about their behaviour, and delivery efficiency of live cultures at their site of action represents major challenges in probiotic product development.

1.3 Objectives

To develop and select optimized production medium composition that can give maximum biomass with minimal production of lactic acid and evaluate its functional characteristics as probiotic bacteria.

1.4 Scope of Research

i. This study is conducted to design and screen the best medium for the growing of high cell mass of *B. longum* for probiotic applications.

ii. To investigate the effect of controlled and uncontrolled pH condition and fed-batch strategies in 16-L bioreactor towards biomass production.

iii. Fed-batch cultivation of *B. longum* in a 16-L semi industrial scale for high cell mass production.

iv. Growth characteristics of *B. longum* will be evaluated in different concentrations of bile salts, gastric juices, acidity, and antimicrobial susceptibility.

1.5 Research Significant

Since *Bifidobacteria* has been extensively used for probiotic application, the demand of food products contained living microorganism has increase due to their beneficial effect that it can give associated with the consumption. However, production of probiotic foods and the supply of sufficient amount of probiotic cells for consumer demand still become limitations plus the beneficial effect of the probiotic can only be achieved in sufficient amounts of living microorganism. Thus,

this research is conducted to optimize the production of *B. longum* for high yield density of biomass by using experimental design (factor by factor and response surface methodology) and furthermore reduce the production cost by selecting the best cheapest media composition.

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