UNIVERSITI PUTRA MALAYSIA

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By

STEPHENIE WONG YOKE WEI

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October 2006

Chairman : Professor Mohd Yazid Abdul Manap, PhD

Faculty : Food Science and Technology

This study was undertaken to optimize the production of probiotic in a milk-based medium and to establish process parameters for the batch cultivation of *Bifidobacterium pseudocatenulatum* G4. The locally isolated strain exhibited high tolerance to pH 1.0-3.0 and fulfilled other probiotic criteria. Identification of the organism was done using polymerase chain reaction (PCR) based method. A defined band at 1.35 kb and 289 bp were produced using genus-specific and species-specific 16S rRNA primers, respectively.

An initial screening of bacteria were done using 2^3 full factorial design in order to identify the effect of medium components consisting of skim milk, yeast extract and glucose towards biomass production. Results showed that yeast extract had a significant positive effect on viable cell count whereas glucose resulted in a negative effect, which was then eliminated from the study. Response surface methodology (RSM) was then applied to optimize the use of skim milk and yeast extract. A quadratic model was

derived using a face-centered central composite design to represent cell mass as a function of the two variables. The optimized medium composition of 2.8% (w/v) skim milk and 2.2% (w/v) yeast extract gave the maximum biomass concentration of 1.3 x 10^9 cfu mL^{-1}, which was 3 log unit higher compared to the commonly used 10.0% (w/v) skim milk (6.3 x 10^5 cfu mL^{-1}). The application of RSM resulted in an improvement in biomass production in a more cost-effective medium, where the skim milk composition was reduced by 71.8%.

Further improvement on the biomass production was carried out in a 2-L stirred tank bioreactor. The highest viable cell count was obtained at pH 6.5, with 0.56 ms^{-1} impeller tip speed. Scaling-up fermentation to a 10-L stirred tank bioreactor based on constant impeller tip speed (0.56 ms^{-1}) successfully yielded reproducible fermentation kinetic values. The results were similar to the smaller-scale reactor. Under this condition, the following were obtained: maximum biomass concentration, X_{max} (1.4 x 10^9 cfu mL^{-1}), maximum specific growth rate, μ_{max} (0.48 h^{-1}), biomass productivity, P_x (7.70 x 10^7 cfu mL^{-1} h^{-1}), and biomass yield, $Y_{x/s}$ (9.46 x 10^{10} cfu $g_{lactose}^{-1}$).

The survival of *B. pseudocatenulatum* G4 during freeze-drying and spray-drying processes was also evaluated. During freeze-drying, the strain exhibited high percentage survival (71.7 - 82.1%) when different combinations of skim milk and sugar solutions (glucose, sucrose and lactose) were used as cryoprotectants. The viable cell counts of 2.1 x 10^9 cfu g^{-1} to 3.1 x 10^9 cfu g^{-1} were obtained after the lyophilization process. Since the addition of sugar did not result in higher percentage survival, 10.0% (w/v) skim milk was suggested as a suitable cryoprotectant. On the other hand, the strain experienced

over 99.0% loss in viability after spray-drying regardless of the spray-drier air outlet temperature and use of heat-adaptation treatments.

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

PENGOPTIMAAN PENGHASILAN *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 SESEKELOMPOK DI DALAM MEDIA BERASASKAN SUSU

Oleh

STEPHENIE WONG YOKE WEI

Oktober 2006

Pengerusi : Profesor Mohd Yazid Abdul Manap, PhD

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Kajian telah dijalankan untuk mengoptimumkan media berasaskan susu dan kawalan proses menggunakan *Bifidobacterium pseudocatenulatum* G4. Strain tempatan ini dipilih kerana menunjukkan toleransi yang tinggi terhadap pH 1.0-3.0 serta memenuhi kriteria probiotik yang lain. Proses identifikasi bacteria telah dijalankan dengan menggunakan teknik 'polymerase chain reaction' (PCR). Jalur DNA dihasilkan pada saiz 1.35 kb dan 289 bp apabila primer untuk gen 16S rRNA yang khusus untuk peringkat genus dan spesis digunakan.

Kajian awal pemilihan bacteria dijalankan dengan menggunakan '2^3 full factorial design' untuk mengenalpasti kesan komposisi media yang terdiri daripada susu skim, ekstrak yis dan glukosa terhadap pertumbuhan bakteria. Daripada analisa statistik, didapati ekstrak yis mempunyai kesan yang signifikan terhadap tumbuhan sel, manakala glukosa pula memaparkan kesan negatif. Oleh itu, glukosa disingkirkan daripada kajian ini. Kaedah respons permukaan telah digunakan untuk pengoptimuman kultur media

yang terdiri daripada susu skim dan ekstrak yis bagi untuk meningkatkan bilangan sel ketika proses fermentasi. Dengan menggunakan 'face-centered central composite design' untuk mewakili sel hidup sebagai fungsi kedua-dua variasi, model kuadratik diperolehi. Komposisi media optima yang diperolehi adalah 2.8% (w/v) susu skim dan 2.2% (w/v) ekstrak yis. Bilangan sel hidup maksima yang dicapai adalah 1.3 x 10^9 cfu mL^{-1}, iaitu sebanyak 3 unit log lebih tinggi daripada kepekatan susu skim 10.0% (w/v) yang biasa digunakan (6.3 x 10^5 cfu mL^{-1}). Aplikasi kaedah respons permukaan ini berjaya meningkatkan pertumbuhan bakteria dalam media susu yang lebih kos efektif, di mana kandungan susu telah dikurangkan sebanyak 71.8%.

Penghasilan sel dipertingkatkan lagi di dalam tangki fermenter berpengaduk 2-L. Bilangan sel hidup yang paling tinggi dicapai pada pH 6.5, dengan halaju hujung pengaduk setinggi 0.56 ms^{-1}. Peningkatan skala fermentasi kepada fermenter berpengaduk 10-L berdasarkan halaju hujung pengaduk yang tetap (0.56 ms^{-1}) berjaya menghasilkan data fermentasi kinetik yang hampir serupa dengan skala fermenter yang lebih kecil. Berdasarkan keadaan tersebut, data fermentasi kinetik berikut didapati: bilangan sel hidup maksima, X_{max} (1.4 x 10^9 cfu mL^{-1}), kadar pertumbuhan spesifik maksima, μ_{max} (0.48 h^{-1}), produktiviti sel, P_x (7.70 x 10^7 cfu mL^{-1} h^{-1}) dan penghasilan sel, $Y_{x/s}$ (9.46 x 10^{10} cfu $g_{lactose}^{-1}$).

Seterusnya, bilangan sel hidup selepas menjalani proses pembekuan kering dan semburan kering dikaji. Selepas proses pembekuan kering, strain ini menunjukkan keupayaan hidup yang tinggi apabila kombinasi susu skim dan gula (glukosa, sukrosa dan laktosa) yang berlainan digunakan sebagai pelindung. Peratus bakteria hidup

sebanyak 71.7 - 82.1%, dengan bilangan sel hidup sebanyak 2.1 x 10^9 cfu g^{-1} to 3.1 x 10^9 cfu g^{-1} didapati apabila bakteria sel di beku keringkan. Memandangkan penambahan gula kepada susu skim tidak memberi kesan lindungan yang ketara, 10.0% susu skim adalah dicadangkan sebagai pelindung semasa pembekuan kering. Apabila strain ini disembur kering, lebih daripada 99.0% bakteria sel mati pada kesemua suhu luaran semburan kering dan suhu adaptasi haba.

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I certify that an Examination Committee met on 19 October 2006 to conduct the final examination of Stephenie Wong Yoke Wei on her Master of Science thesis entitled "Optimization of batch production of *Bifidobacterium pseudocatenulatum* G4 in a milk-based medium**"** 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 Master of Science. The members of the Supervisory Committee are as follows:

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Date: 16 January 2007

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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STEPHENIE WONG YOKE WEI

Date: 30 November 2006

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LIST OF ABBREVIATIONS

μL	Micro liter
μM	Micro molar
μ_{max}	Maximum specific growth rate (h^{-1})
A_{max}	Maximum acetic acid concentration (g L^{-1})
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
bp	Base pair
BSE	Bovine spongiform encephalopathy
BSH	Bile salt hydrolase
CCD	Central composite design
cfu	Colony forming unit
CO_2	Carbon dioxide
$C_5H_8O_2$	Glutaraldehyde
d	Day
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Example gratia* (for example)
et al.	Et cetera (and company)
GIT	Gastrointestinal tract

GRAS	Generally regarded as safe
h	Hour
H^+	Hydrogen ion
H_2SO_4	Sulphuric acid
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
i.e.	*id est* (that is)
JCM	Japan Collection of Microorganism
kb	Kilo base pair
kV	Kilo volt
L	Liter
L_{max}	Maximum lactic acid concentration (g L^{-1})
LAB	Lactic acid bacteria
Log	Logarithm
M	Molar
min	Minute
MIT	Microbial interference therapy
Mg	Magnesium
$MgCl_2$	Magnesium chloride
mL	Milliliter
mM	Millimolar
MRS	de Man Rogosa Sharpe Medium
N	Normality

ng	Nano gram
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
P_A	Acetic acid productivity (g L^{-1} h^{-1})
P_L	Lactic acid productivity (g L^{-1} h^{-1})
P_x	Biomass productivity (cfu mL^{-1} h^{-1})
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
rpm	Revolution per minute
RSM	Response surface methodology
SEM	Scanning electron microscopy
s	Second
S.D.	Standard deviation
spp.	Species
TPY	Trypticase-Phytone-Yeast Extract
WHO	World Health Organization
w/v	Weight per volume
X_{max}	Maximum biomass (log_{10} cfu mL^{-1})
$Y_{A/s}$	Acetic acid yield (g acetic g $lactose^{-1}$)
$Y_{L/s}$	Lactic acid yield (g acetic g $lactose^{-1}$)
$Y_{x/s}$	Biomass yield (cfu g $lactose^{-1}$)

CHAPTER 1

INTRODUCTION

The complex ecosystem of the gut microflora plays a significant role in the gastrointestinal health of humans and animals. This has attracted worldwide interest in conducting intensive researches on issues pertaining to gut health. It is desirable to have gut microbial ecosystem that exists in equilibrium, that contains predominance of beneficial bacteria over harmful ones. However, many factors that include aging, stress, diet and antibiotic therapy may easily upset this balance (Gibson and Fuller, 2000).

This has paved the way to the concept of probiotic. Probiotic are describe as live beneficial microorganisms that when administered orally, helps to promote the growth of "friendly" bacteria in the gut. A good probiotic is able to prevent colonization of pathogens, regulate intestinal motility, reduce risk of carcinogenesis in the intestine and alleviate lactose intolerance (Marks, 2004).

Lately, there is rising concern on the increase of bacterial resistance to antibiotics which has become a major public health problem. A disturbing scenario as quoted by Mainous *et al.* (1997) that 71% of *Enterococcus faecium*, the second most common lethal pathogen isolated in the intensive-care environment are vancomycin-resistant. This give rise to high infection rates and pose a serious medical challenge. As the concern heightened, the World Health Organization (WHO) has urged for immediate reduction in the use of antibiotics in animals and human medicine. Instead, application of

microbial interference therapy (MIT), the use of beneficial bacterial to destroy pathogens, as a natural alternative disease control strategy was strongly recommended (Bengmark, 2000). These developments create the need to explore new potential probiotic strain.

Among the widely used bifidobacteria species as probiotic are *Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium lactis* and *Bifidobacterium longum* (Kopp-Hoolihan, 2001). One of the least studied but commonly found species in local breast-fed infants is *Bifidobacterium pseudocatenulatum (*Kleesen *et al*., 1995; Shuhaimi *et al*., 2002), that remain to be further unexplored for its potential. The Probiotic Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia has carried out extensive study of this species, specifically in the classification of *Bifidobacterium* isolates using PCR-based and 16S rDNA Partial Sequences Analysis methods (Shuhaimi *et al*., 2002), generation of genomic DNA fingerprints for *B*. *pseudocatenulatum* isolates by RAPD (Shuhaimi *et al*., 2001a), deconjugation of bile acids (Mariam *et al.,* 2004), antibacterial activity, antimicrobial susceptibility, and adherence properties (Shuhaimi *et al.,* 1999b). Thus, this project would further explore on the acid tolerance, biomass production and cell preservation of this particular species as a probiotic candidate.

Probiotic is now effectively being applied in the food industry, human and animal medicine (O'Brien *et al*., 1999; Shortt, 1999). The probiotic bacteria that are of commercial interest mainly belong to the genus bifidobacteria and lactic acid bacteria (LAB), where they are commonly incorporated as functional food in yogurt, cultured milk drink, sour cream, buttermilk, cheese and also as pharmaceutical application in