



UNIVERSITI PUTRA MALAYSIA

**CHARACTERIZATION OF PROBIOTIC MICROORGANISMS
IN EQUOL BIOTRANSFORMATION**

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**CHARACTERIZATION OF PROBIOTIC MICROORGANISMS IN EQUOL
BIOTRANSFORMATION**

By

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CHARACTERIZATION OF PROBIOTIC MICROORGANISMS IN EQUOL BIOTRANSFORMATION

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Metabolism of daidzin within human subject produces daidzein and equol, the metabolites which have potential health benefits against hormone-dependent diseases in humans. This study aimed to identify bacterial species for equol production from daidzin and daidzein, determine isoflavones, sugars and short chain fatty acids contents in soymilk during fermentation with selected bacteria and optimize soymilk as a medium for equol production.

Seven bacteria species, namely *Lactobacillus casei* - 01, *Lactobacillus plantarum* FTCC 0350, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium longum* ATCC BB536, *Bifidobacterium infantis* ATCC 15697, *Bifidobacterium pseudocatenulatum* G4 and *Eggerthella lenta* ATCC 43055 were screened for equol production from daidzin and daidzein. Each of the pre-cultured bacterial strains was inoculated into

Brain Heart Infusion (BHI) broth containing 100 μ l of daidzin and incubated anaerobically at 37° C for 48 h. In addition, *B. breve* ATCC 15700, *B. longum* ATCC BB536 and *E. lenta* ATCC 43055 were incubated up to 96 hours by 200 μ l daidzein in similar condition. Daidzin, daidzein and equol were determined using high performance liquid chromatography. In addition, bacterial growth and formation of short chain fatty acids (lactic and acetic acid) were investigated.

Daidzin was metabolized to daidzein and equol with *B. breve* ATCC 15700, *B. longum* ATCC BB536, *B. infantis* ATCC 15697 and *L. plantarum* FTCC 0350. *B. pseudocatenulatum* G4 and *L. casei* -01 were incapable to produce equol from either daidzin or daidzein, but they can metabolize daidzin to daidzein. Both of daidzein and equol were not produced when daidzin was incubated with *E. lenta* ATCC 43055. Since both of *B. breve* ATCC 15700 and *B. longum* BB536 ATCC provided the highest amount of equol; their daidzein metabolism was further investigated. They can produce equol within 6 hours incubation. Bacterial growth was not affected by daidzein content in the culture medium. However, incubation of the bacteria with daidzein produced more lactic and acetic acids compared to incubation without daidzein. After 96 h of incubation, the amounts of lactic and acetic acids for *B. breve* ATCC 15700 were at 5.53 and 8.83 mmol l⁻¹, respectively and for *B. longum* BB536 were 6.86 and 7.20 mmol l⁻¹, respectively.

Subsequently, to estimate β -glucosidase enzyme activity for each of *B. breve* ATCC 15700, *B. longum* BB536, *B. pseudocatenulatum* G4, *B. infantis* ATCC 15697, *L. casei* -01 *L. plantarum* FTCC 0350 and *E. lenta* ATCC 43055, a single culture of the strains was inoculated in sterile soymilk for 48 h at 37° C. *B. breve* ATCC 15700 and

B. longum BB536 offered the highest equol level and β -glucosidase activity. Therefore, fermentation of soymilk reduced its daidzin level and increased daidzein that later decreased accompanying equol production. Moreover, the heights amount of equol was obtained when soymilk was enriched with inulin and inoculated with co-culture of *B. breve* ATCC 15700 and *B. longum* BB536. Thus, fermentation of soymilk supplemented with inulin and inoculated with co-culture of *B. breve* ATCC 15700 and *B. longum* BB536 was optimized for equol production using bioreactor BIOSTAT Q-DCU3, and utilizing response surface methodology (RSM) approach. Effects of three independent variables, namely pH (4-8, X_1), temperature (30-40 °C, X_2) and inulin amount (0.5-1.5%, X_3) on transformation of soymilk isoflavones (daidzin, Y_1 and daidzein, Y_2) to equol, Y_3 were studied. All responses significantly ($p < 0.05$) fitted into quadratic models with coefficient of determination (R^2) close to 1 (0.935 - 0.989). Daidzin reduction was influenced by factors in both 24 h and 48 h fermentation. Daidzein level was affected by all factors in 24 h fermentation but only by temperature in 48 h fermentation. Equol production was influenced by both pH and temperature in 24 h fermentation and by all factors in 48 h fermentation.

The optimum incubation conditions for highest equol production were at pH 8, 30° C and 0.5% inulin. Model validation demonstrated there was no significant ($p > 0.05$) difference between the experimental and predicted values, suggested the suitability of established models in explaining the daidzin and daidzein transformation to equol as function of the pH, temperature and inulin.

Besides, to study the effect of the fermentation process in soymilk oligosaccharides content, the levels of oligosaccharides in fermented soymilk were investigated. It was

found that, the concentrations of oligosaccharides (stachyose and raffinose) and disaccharide (sucrose) in soymilk medium decreased and followed by increment on the levels of monosaccharides (glucose, galactose and fructose) and formation of short chain fatty acids (lactic acid and acetic acid) as by-products.



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KARASTERISTIK MIKROORGANISMA PROBIOTIK DALAM BIOTRANSFORMATION EQUOL

Oleh

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Metabolisma daidzin dalam manusia menghasilkan daidzein dan equol, metabolit yang bersifat estrogenic dan mempunyai potensi faedah kesihatan melawan penyakit bersandarkan hormon. Kajian ini bertujuan untuk menyaring spesis bakteria berlainan untuk penghasilan equol daripada daidzin dan daidzein, menentukan kandungan isoflavon, gula dan asid lemak rantai pendek semasa penapaian susu soya. Tujuh spesis bakteria iaitu *Bifidobacterium breve* ATCC 15700, *Bifidobacterium longum* BB536, *Bifidobacterium infantis* ATCC 15697, *Bifidobacterium pseudocatenulatum* G4, *Lactobacillus casei* 01, *Lactobacillus plantarum* FTCC 0350 dan *Eggerthella lenta* ATCC 43055 telah disaring untuk penghasilan equol dari isoflavon. Setiap strain bakteria yang telah dipra-kultur diinokulasikan ke dalam kaldu Brain Heart Infusion (BHI) yang mengandungi 100 µl daidzin dan diinkubasi secara anaerob pada 37 °C selama 48 jam. Di samping itu, *B. breve*, *B. longum* dan *E. lenta* diinkubasikan sehingga 96 jam dengan 200µl daidzein dan dengan keadaan serupa. Daidzin, daidzein dan equol telah ditentukan dengan

menggunakan kromatografi cecair berprestasi tinggi (HPLC). Di samping itu, pertumbuhan bakteria dan pembentukan asid lemak rantai pendek (asid laktik dan asetik) telah di kaji.

Daidzin dimetabolismekan kepada daidzein dan equol oleh *B. breve*, *B. longum*, *B. infantis* dan *L. plantarum*. *B. pseudocatenulatum* dan *L. casei* tidak mampu untuk menghasilkan equol dari sama ada daidzin atau daidzein, tetapi mereka boleh memetabolismekan daidzin kepada daidzein. Daidzein dan equol tidak dihasilkan oleh *E. lenta*, kerana kedua-dua *B. breve* dan *B. longum* menghasil equol terbanyak; metabolisme daidzein dikaji selanjutnya. Mereka menghasilkan equol dalam masa 6 jam inkubasi manakala bagi *E. lenta*, equol dikesan hanya selepas 18 jam penggeraman. Pertumbuhan bakteria tidak terjejas oleh kandungan daidzein dalam medium kultur. Walau bagaimanapun, penggeraman bakteria dengan daidzein menghasilkan asid laktik dan asetik lebih tinggi berbanding penggeraman tanpa daidzein, dengan jumlah asid laktik dan asetik sebanyak 5.53 dan 8.83 mmol l⁻¹ bagi *B. breve*, dan 6.86 dan 7.20 mmol l⁻¹ bagi *B. longum* selepas 96 jam penggeraman.

Seterusnya, aktiviti enzim β -glucosidase dan metabolisme isoflavon *B. breve*, *B. longum*, *B. pseudocatenulatum*, *B. infantis*, *L. casei*, *L. plantarum* dan *E. lenta* diinokulasi dalam kultur tunggal dalam penggeraman susu soya yang disteril pada 37 °C selama 48 jam, telah ditentukan. Penapaian susu soya mengurangkan kandungan daidzin dan meningkat kandungan daidzein yang kemudiannya menurun kerana penghasilan equol. *B. breve* dan *B. longum* menghasilkan equol dan aktiviti β -glucosidase paling tinggi. Kepekatan oligosakarida (stachyosa, raffinosa dan sukrosa) dalam medium susu soya menurun semasa penapaian dan meningkatkan

kandungan monosakarida (glukosa, galaktosa dan fruktosa) dan pembentukan asid lemak rantai pendek (asid laktik, asid asetik, asid propionik dan asid butirik) sebagai hasil sampingan penapaian.

Penapaian susu soya yang ditambah inulin dan diinokulat dengan kultur bersama *B. breve* dan *B. longum* telah dioptimumkan untuk penghasilan equol menggunakan bioreaktor BIOSTAT Q-DCU3, dan menggunakan pendekatan kaedah respons permukaan (RSM). Kesan tiga pembolehubah bebas, iaitu pH (4-8, X_1), suhu (30-40 °C, X_2) dan amaun inulin (0.5-1.5%, X_3) terhadap transformasi isoflavon soya (daidzin, Y_1 dan daidzein, Y_2) kepada equol, Y_3 telah dikaji. Semua respon dapat dicu secara signifikan ($p < 0.05$) kepada model kuadratik dengan pekali penentuan (R^2) menghampiri 1 (0.935-0.989). Pengurangan daidzin dipengaruhi oleh semua faktor bagi 24 jam dan 48 jam penapaian. Kandungan daidzein dipengaruhi oleh semua faktor bagi 24 jam penapaian tetapi hanya dipengaruhi oleh suhu dalam 48 jam penapaian. Penghasilan equol dipengaruhi oleh pH dan suhu bagi 24 jam penapaian dan oleh semua faktor-faktor dalam 48 jam penapaian. Kesimpulan pengoptimuman untuk penghasilan equol terbanyak ialah pada pH 8, 30 °C dan 0.5% inulin. Pengesahan model menunjukkan tiada perbezaan signifikan ($p > 0.05$) antara nilai eksperimen dan ramalan, mencadangkan kesesuaian model yang dihasilkan dalam menjelaskan transformasi daidzin dan daidzein ke equol sebagai fungsi pH, suhu dan inulin.

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I certify that an Examination Committee has met on **3rd December 2012** to conduct the final examination of Salma Elghali Mustafa on her Doctor of Philosophy thesis entitled "CHARACTERIZATION OF PROBIOTIC MICROORGANISMS IN EQUOL BIOTRANSFORMATION" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981.

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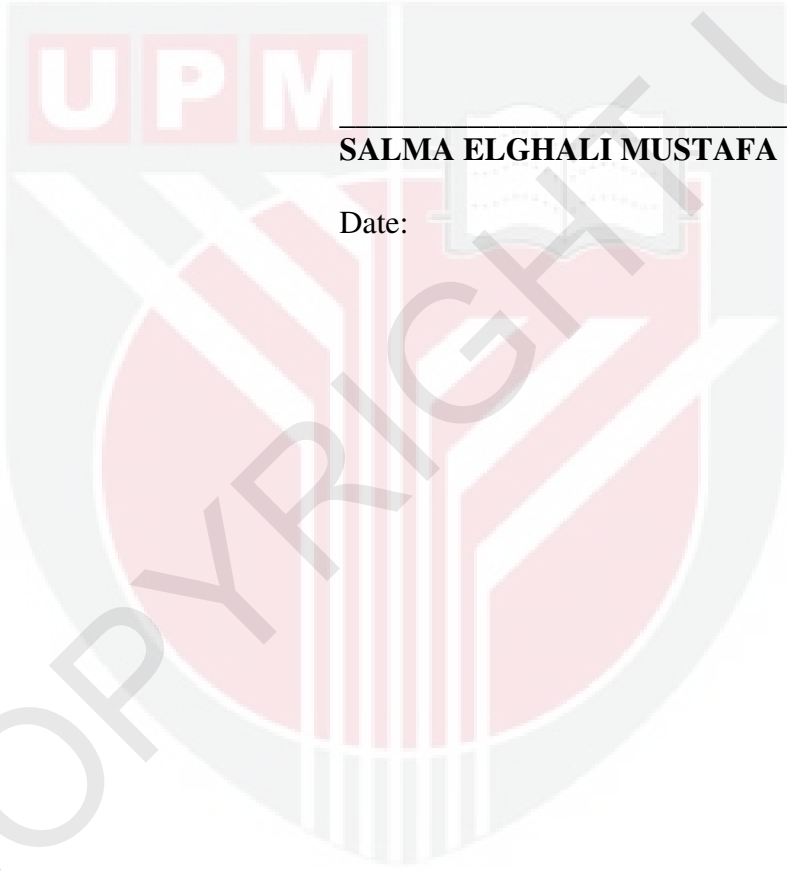
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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.



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TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	viii
APPROVAL	ix
DECLARATION	xi
LIST OF TABLES	xvii
LIST OF FIGURES	xx
LIST OF ABBREVIATIONS	xxii

CHAPTER

1	INTRODUCTION	
	1.1 Research background	1
	1.2 Problem of statements	4
	1.3 Significant of the study	5
	1.4 Research objectives	6
	1.4.1 General objective	6
	1.4.2 Specific objectives	6
2	LITERATURE REVIEW	
	2.1 Phytoestrogen	8
	2.1.1 Non-flavonoids phytoestrogens	8
	2.1.2 Flavonoids phytoestrogens	9
	2.1.2.1 Isoflavones structure, nomenclature, and occurrence	9
	2.1.2.2 Resources of isoflavonoids in the human diet	10
	2.1.2.3 Deglycosylation of isoflavones	13
	2.1.2.4 Absorption of isoflavones	14
	2.1.2.5 Metabolism and bioavailability of isoflavones in humans	15
	2.1.2.6 Metabolism of isoflavones in the gut	17
	2.2 Microbiota of human gastrointestinal tract (GIT)	18
	2.2.1 Probiotics bacteria	19
	2.2.2 Role and functions of the microorganisms of the gut	21
	2.2.3 Role of gut microflora in isoflavones metabolism	23
	2.2.4 Role of gut microflora in daidzin and daidzein metabolism	23
	2.3 Factors that affect isoflavone metabolism in the gut	26
	2.3.1 Effect of diet	26
	2.3.2 Effect of antibiotics	27
	2.3.3 Effect of prebiotics	27
	2.3.4 Effect of isoflavones structure	28
	2.4 Equol	29

2.4.1	Structure of equol	30
2.4.2	Biological properties of equol	31
2.4.2.1	Estrogenicity properties	31
2.4.2.2	Antioxidant properties	32
2.4.2.3	Antiandrogenic	33
2.4.3	Production of equol in humans	34
2.4.4	Factors affecting equol production in humans	35
2.4.4.1	Effect of diet	35
2.4.4.2	Effect of genetic factors	37
2.4.4.3	Effect of the composition of the gut bacteria	37
2.4.4.4	Effect of gender	38
2.5	Bacterial species involved in equol production	39
2.5.1	<i>Bifidobacterium spp</i>	39
2.5.2	<i>Lactobacillus sp.</i>	40
2.5.3	<i>Eggerthella sp.</i>	40
2.5.4	<i>Eubacterium spp</i>	41
2.5.5	<i>Clostridium spp.</i> and <i>E. coli</i>	41
2.6	Functional foods	42
2.7	Soymilk	42
2.8	Fermentation process	44
2.8.1	Batch Fermentation	45
3	IDENTIFICATION OF SELECTED BACTERIAL STRAINS FOR CONVERSION OF DAIDZIN AND DAIDAZEIN TO EQUOL	47
3.1	Introduction	47
3.2	Materials and methods	49
3.2.1	Chemicals and media	49
3.2.2	Preparation of media and solutions	49
3.2.2.1	Preparation of Brain Heart Infusion (BHI) agar plates	49
3.2.2.2	Preparation of Trypticase Phytone Yeast (TPY) agar plates	50
3.2.2.3	Preparation of BHI Broth	50
3.2.2.4	Preparation of Rehydrated de Mann, Rogosa and Sharpe (MRS) Broth	50
3.2.2.5	Preparation of peptone saline	51
3.2.2.6	Preparation of daidzein standard	51
3.2.2.7	Preparation of equol standard	51
3.2.2.8	Preparation of daidzin standard	52
3.2.3	Bacterial species and storage condition	52
3.2.4	Preparation of the inocula	52
3.2.4.1	Evaluation of daidzin metabolism for equol production by bacterial strains	53
3.2.4.2	Bioconversion of daidzein to equol	53
3.2.5	HPLC analysis	54

3.2.5.1	Sample preparation for HPLC analysis	54
3.2.5.2	HPLC analysis conditions	54
3.2.5.3	Liquid chromatography mass spectrometry (LC/MS) analysis	55
3.2.6	Enumeration of viable bacteria	56
3.2.7	Determination of pH during incubation of bacterial strains with daidzein	56
3.2.8	Analysis of organic acids formation during incubation of bacterial strains with daidzein	56
3.2.8.1	Supernatant preparation and HPLC analysis	56
3.3	Statistical analysis	57
3.4	Results and discussion	58
3.4.1	Evaluation of daidzin metabolism for equol production by bacterial strains	58
3.4.2	Liquid chromatography mass spectrometry analysis (LC/MS)	62
3.4.3	Bioconversion of daidzein to equol	65
3.4.4	Enumeration of viable bacteria	69
3.4.5	Determination of pH during incubation of bacterial strains with daidzein	71
3.4.6	Analysis of organic acids formation during incubation of bacterial strains with daidzein	73
3.5	Conclusions	76
	OPTIMIZATION OF CULTURE CONDITIONS OF SOYMILK FOR EQUOL PRODUCTION BY BIFIDOBACTERIUM SPECIES	78
4.1	Introduction	78
4.2	Materials and methods	79
4.2.1	Bacterial species and storage conditions	80
4.2.2	Bacterial growth media	80
4.2.3	Commercial prebiotics	81
4.2.4	Preparation of soymilk	81
4.2.5	Screening for β - glucosidase activity	82
4.2.5.1	Assay for β glucosidase activity	82
4.3	Fermentation of soymilk	83
4.3.1	Preparation of the inoculum	83
4.3.2	Batch Fermentation conditions	84
4.3.3	Preparation of sample for isoflavones analysis by High Performance Liquid Chromatography	84
4.4	Screening of prebiotics for high equol production	85
4.5	Optimization of culture conditions of soymilk designed for equol production	85
4.5.1	Experimental design and statistical analysis	86
4.5.2	Optimization and confirmation procedures	88
4.6	Results and discussion	89
4.6.1	β -Glucosidase activity of different bacterial species in fermented soymilk	89

4.6.2	Screening of prebiotics for high equol production by different bacterial species	93
4.6.3	Fitting the response surface models	102
4.6.3.1	Daidzin concentration (Y_1)	104
4.6.3.2	Daidzein concentration (Y_2)	107
4.6.3.3	Equol concentration (Y_3)	110
4.6.4	Validation of the final reduced models	114
4.7	Conclusions	118

5

ANALYSIS OF OLIGOSACCHARIDES AND SHORT CHAIN FATTY ACIDS CONTENTS IN SOYMILK FERMENTED WITH SELECTED HUMAN INTESTINAL BACTERIA

5.1	Introduction	119
5.2	Materials and methods	121
5.2.1	Bacterial species and growth media	121
5.2.2	Chemicals and sugar standards	122
5.2.3	Preparation of soymilk	122
5.2.4	Preparation of Inocula	122
5.2.5	Batch fermentation procedure	123
5.2.6	Bacterial growth in soymilk	124
5.2.7	Determination of the pH for the fermentation medium	124
5.2.8	Preparation of sample for analysis of oligosaccharides	125
5.2.9	Preparation of sample for analysis of short chain fatty acids using HPLC	125
5.3	Statistical analysis	126
5.4	Results and discussion	126
5.4.1	Enumeration of bacterial Species in fermented soymilk	126
5.4.2	Concentration of oligosaccharides in fermented soymilk	128
5.4.3	Determination of p H during soymilk fermentation	136
5.4.4	Production of lactic and acetic acids	137
5.5	Conclusions	141

6

SUMMARY, GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1	Summary and general conclusions	143
6.2	Recommendations for future research	145

REFERENCES	147
APPENDICES	167
BIODATA OF STUDENT	171
LIST OF PUBLICATIONS	172