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CHARACTERIZATION OF PROBIOTIC MICROORGANISMS IN EQUOL BIOTRANSFORMATION

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Chairperson: Associate Professor Shuhaimi Mustafa, PhD

Faculty: Faculty of Biotechnology and Biomolecular Sciences

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 µl 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 µl 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 37º 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 independents 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.

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

KARASTERISTIK MIKROORGANISMA PROBIOTIK DALAM BIOTRANSFORMATION EQUOL

Oleh

SALMA ELGHALI MUSTAFA

Disember 2012

Pengerusi: Profesor Madya Shuhaimi Mustafa, PhD

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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 rantaian pendek semasa penapaian susu soya. Tujuh spesies 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 rantaian 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 pengeraman. Pertumbuhan bakteria tidak terjejas oleh kandungan daidzein dalam medium kultur. Walau bagaimanapun, pengeraman bakteria dengan daidzein menghasilkan asid laktik dan asetik lebih tinggi berbanding pengeraman 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 pengeraman.

Seterusnya, aktiviti enzim β-glucosidase dan metabolisma isoflavon *B. breve, B. longum, B. pseudocatenulatum, B. infantis, L. casei, L. plantarum* dan *E. lenta* diinokulasi dalam kultur tunggal dalam pengeraman susu soya yang disteril pada 37 ºC 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 meningkaatkan

kandungan monosakarida (glukosa, galaktosa dan fruktosa) dan pembentukan asid lemak rantaian 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. Kondusi pengeraman optimum 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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This thesis was 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 were as follows:

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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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SALMA ELGHALI MUSTAFA

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