UNIVERSITI PUTRA MALAYSIA

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By

KHALILAH ABDUL KHALIL

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

July 2012

Dedicated to…..

My beloved mother, Rahmah, my dearest husband, Awis Qurni, and my adorable children, Shahira, Ammar, Ammir and Amsyar. As well goes to teachers, researchers, scientists and peoples who contribute the knowledge in this field.

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

MICROENCAPSULATION of *Bifidobacterium pseudocatenulatum* G4 USING NATURAL MATRICES

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Chairman: Associate Professor Shuhaimi Mustafa, PhD

Faculty : Biotechnology and Biomolecular Sciences

Probiotic cultures, nowadays, are widely used in food products for health enhancement. Low survivability of probiotic cultures in acidic environment such as in the stomach region has limited their potential benefits. The possibility of using encapsulation method to improve the survivability of probiotic bacterium, *Bifidobacterium pseudocatenulatum* G4 (G4), during passage through the gastrointestinal tract was investigated in this study. Bovine and fish gelatin with the combination of genipin (a plant extract) and sodium alginate were used as encapsulating matrices. The study was accomplished through the following approaches: 1) formulation of medium based on skim milk and yeast extract for development of active inoculum for G4 cultivation; 2) optimization of inoculum medium prior to subsequent fermentation, 3) optimization of the encapsulation matrices for improvement of encapsulation yield (%) and beads strength (g) before and after being exposed to simulated gastric (SGF) and intestinal fluids (SIF), and 4) determination of cell release activities based on swelling rate (%),

release activity (OD), survival assay (cfumL^{-1}) and beads morphology using scan electron microscope (SEM) during exposures to SGF and SIF.

The use of 2 and 4% (w/v) skim milk as inoculum medium has elevated only 1 log cfumL^{-1} after 24 h of cultivation. Skim milk concentration ranging from 6 to 10% (w/v) greatly enhanced cell growth with more efficient carbon and free amino nitrogen usage as well as higher production of β-galactosidase. Through statistical modeling based on the Face Centered Central Composite Design (FCCD), the optimum concentration of combined skim milk and yeast extract was determined as 7.02 and 1.73% (w/v), respectively. A validation experiment proved that the predicted and experimented values were not significantly different ($p > 0.05$). Substantial improvement in biomass production (11.72 cfumL^{-1}) was achieved in cultivation with optimized medium in 2-L stirred tank bioreactor for 18 h, and this biomass production was not statistically different ($p > 0.05$) as compared to the cultivation using commercial inoculum medium.

FCCD was also employed for the optimization of encapsulating matrices. The optimum concentration for bovine gelatin-genipin-alginate was predicted at 11.21% (w/v), 13.96 mM and 2.60% (w/v), respectively. While, in the case of fish gelatin-genipin-alginate, combined matrices at 12.57% (w/v), 19.12 mM and 5% (w/v) was predicted to generate optimum responses. Upon verification, experimental data of bovine gelatin-genipin-alginate and fish gelatin-genipin-alginate remained close value to the predicted data with low error for all the responses. As compared to porcine-genipin-alginate encapsulating matrices, the optimized bovine and fish gelatin-genipin-alginate have both demonstrated lower strength ($p < 0.05$) after SIF exposure.

The performances of the optimized bovine gelatin-genipin-alginate and fish gelatin-genipin-alginate in protecting G4 and other probiotics were also determined. Eight groups of encapsulating matrices were evaluated: 1) optimized bovine gelatin-genipin-alginate, 2) optimized fish gelatin-genipin-alginate, 3) porcine gelatin-genipin-alginate, 4) optimized bovine gelatin-alginate, 5) optimized fish gelatin-alginate, 6) porcine gelatin-alginate, 7) alginate alone, 8) free cell (unencapsulate). Low encapsulation yield was observed in groups 2 and 5, respectively. Meanwhile group 1 showed highest in encapsulation yield. Slow swelling rate during the SGF exposure was shown by group 1, 3, 4 and 6 while groups 5 was demonstrated progressive swelling and slightly erode at 120 min of exposure. The releases of cells occur when the beads disintegrate and these were observed through the cells release activity analysis. All groups were presented positive performance in releasing cells into the intestinal region with higher optical density and lower survivability of entrapped cells obtained under SIF exposure except for groups 3 and 6, respectively. Factors like gelatin source, bloom strength and the presence of alginate played important roles in stabilizing the chemical cross link of the gelatin especially in acidic environment. Encapsulations of both bovine and fish gelatin with genipin and alginate combinations have successfully improved the survival and cells release and this approach could potentially be useful in replacing porcine gelatin for the delivery of probiotic culture to the target area in the intestinal region.

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

MIKROENKAPSULASI *Bifidobacterium pseudocatenulatum* G4 MENGGUNAKAN MATRIKS ASLI

Oleh

KHALILAH ABDUL KHALIL

Julai 2012

Pengerusi: Profesor Madya Shuhaimi Mustafa, PhD

Fakulti : Bioteknologi and Sains Biomolekul

Pada masa sekarang, kultur probiotik digunakan secara meluas di dalam produk makanan untuk tujuan kesihatan. Akan tetapi, tahap kehidupan probiotik kultur ini adalah rendah di dalam persekitaran berasid seperti di dalam perut. Oleh itu, kaedah enkapsulasi dikaji untuk melihat potensi bagi memperbaiki tahap kehidupan probiotik, *Bifidobacterium pseudocatenulatum* G4, semasa melalui sistem pencernaan. Kombinasi bahan enkapsulasi seperti gelatin lembu dan ikan bersama genipin (ekstrak tumbuhan) dan sodium alginat telah digunakan. Kajian in melibatkan beberapa peringkat seperti: 1) meningkatkan pengaktifan inokula G4 dengan mencari formulasi susu skim and ekstrak yis yang sesuai sebagai persediaan media untuk inokula, 2) mengoptimasikan media tersebut bagi meningkatkan populasi G4 sebelum proses penapaian yang selanjutnya, 3) mengoptimasikan kombinasi bahan enkapsulasi yang digunakan berdasarkan kadar enkapsulasi sel (%) dan kekuatan kapsul yang dihasilkan (g) sebelum dan selepas pendedahan kepada bendalir gastrik (SGF) dan pencernaan (SIF), 4) mengkaji aktiviti pelepasan sel probiotik ketika di dalam SGF dan SIF berdasarkan kadar pembengkakan kapsul (%), analisa pelepasan sel (OD), kadar

tahap kehidupan sel di dalam kapsul ($cfumL^{-1}$) dan perubahan bentuk kapsul dengan menggunakan mikroskop imbasan elektron (SEM).

Penggunaan 2 dan 4% (w/v) susu skim sebagai media pengaktifan inokula hanya memberi peningkatan sel sebanyak 1 $log_{10}cfumL^{-1}$ selepas 24 jam kultivasi. Sebaliknya, kepekatan susu skim dari 6 hingga 10% (w/v), memberi penambahan bilangan sel yang besar dengan penggunaan karbon dan amino nitrogen yang cekap serta penghasilan β-galaktosidase yang tinggi. Model statistik berdasarkan "Face Centered Central Composite Design" (FCCD) memberi titik optima kepekatan bagi susu skim dan ekstrak yis iaitu 7.02 dan 1.73% (w/v). Validasi eksperimen membuktikan yang nilai ramalan dan eksperimen yang dijalankan tiada perbezaan ketara ($p > 0.05$). Peningkatan yang tinggi dalam penghasilan sel (11.72 $cfumL^{-1}$) tercapai di dalam bioreaktor 2-L selepas 18 jam kultivasi dan peningkatan ini tiada perbezaan statistik ($p > 0.05$) dengan sel yang diaktifkan menggunakan media komersial.

FCCD juga digunakan untuk mengoptimasikan bahan enkapsulasi. Titik optima kepekatan untuk gelatin lembu-genipin-alginat adalah pada 11.21% (w/v), 13.96 mM dan 12.57% (w/v). Manakala, gelatin ikan-genipin-alginat ialah pada 12.57% (w/v), 19.12 mM dan 5% (w/v) untuk memberi kesan yang optima. Berdasarkan verifikasi, nilai eksperimen bagi kapsul yang dioptimakan tidak menunjukkan perbezaan besar dengan nilai ramalan. Perbandingan dibuat dengan kapsul dari gelatin khinzir-genipin-alginat dan menunjukkan kapsul gelatin lembu-genipin-alginat dan gelatin ikan-genipin-alginat yang dioptimakan memberi kesan kekuatan yang rendah berbanding dengan kapsul gelatin khinzir-genipin-alginat apabila didedahkan kepada SIF.

Tahap perlindungan bagi G4 dan probiotik lain dengan menggunakan bahan enkapsulasi yang dioptimakan telah dikaji. Lapan formulasi bahan enkapsulasi yang dioptimakan melibatkan: 1) gelatin lembu-genipin-alginat, 2) gelatin ikan-genipin-alginat, 3) gelatin khinzir-genipin-alginat, 4) gelatin lembu-alginat, 5) gelatin ikan-alginat, 6) gelatin khinzir-alginat, 7) alginat sahaja, 8) sel bebas (tidak dienkapsulasikan). Kadar enkapsulasi sel yang rendah telah diperolehi dari kumpulan 2 dan 5. Manakala, kumpulan 1 menunjukkan kadar enkapsulasi sel yang paling tinggi. Kadar pembengkakan yang rendah telah dilihat dari kumpulan 1, 3, 4 dan 6, manakala kumpulan 5 menunjukkan pembengkakan yang cepat setelah 120 minit di dalam SGF. Pembebasan sel berlaku apabila kapsul mula pecah dan ini boleh dilihat menerusi analisa aktiviti pembebasan sel. Kesemua kumpulan kecuali kumpulan 3 dan 6, menunjukkan kesan positif di dalam membebaskan sel ketika dalam SIF dengan menunjukkan nilai ketumpatan optik (OD) yang tinggi dan tahap kehidupan sel tertinggal di dalam kapsul adalah rendah. Faktor seperti jenis gelatin, kekuatan “bloom” dan penggunaan alginat memainkan peranan penting dalam penstabilan gelatin struktur kimianya terutama ketika di dalam persekitaran yang berasid. Enkapsulasi menggunakan gelatin lembu dan ikan dapat memperbaiki tahap kehidupan dan pembebasan sel serta berpotensi untuk mengantikan penggunaan kapsul dari gelatin khinzir untuk penghantaran probiotik kultur ke tempat sasaran di dalam usus.

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APPROVAL

I certify that a Thesis Examination Committee has met on 5th July 2012 to conduct the final examination of Khalilah Abdul Khalil on her thesis entitled "**Enhanced survivability of *Bifidobacterium pseudocatenulatum* G4 by encapsulation using gelatin-genipin-alginate matrices**" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Doctor Philosophy.

Member of the Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment 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 based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that is has not been previously and is not concurrently, submitted for any other degree at Universti Putra Malaysia or at any other institution.

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KHALILAH ABDUL KHALIL

Date: 5 July 2012

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