UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A NEW RECOMBINANT STARTER CULTURE BACTERIUM FOR MILK COAGULATION

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By

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

DEVELOPMENT OF A NEW RECOMBINANT STARTER CULTURE BACTERIUM FOR MILK COAGULATION

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Chairman: **Professor Fatimah Abu Bakar, PhD**

Faculty: **Food Science and Technology**

Milk clotting enzyme is the key factor for the production of different types of cheese. Hence, calf rennet (chymosin) is traditionally used as a milk coagulant in cheese manufacturing. As the increase in cheese manufacturing globally, coincided with a decline in the supply of calf rennet, it became imperative that substitutes for rennet be found. Recently, the use of fungal mucor rennin, in industrial cheese manufacturing, has become prevalent. *Lactococcus lactis* is a lactic acid bacterium which is generally used as a starter in dairy industries for the production of various types of hard and soft cheeses. Therefore, due to the key role of milk clotting enzyme as well as starter culture bacteria in cheese manufacturing, this study was an attempt to express recombinant mucor rennin (MPR) enzyme by *L. lactis* to produce milk coagulation enzyme. It is a novel milk clotting procedure using recombinant bacterium capable of milk coagulation. To achieve this, the amplified MPR gene was sub-cloned into pAMJ-LacF expression vector. The food grade pAMJ-LacF expression vector was created by sub-cloning the amplified LacF gene in pAMJ399 vector which lost its erythromycin gene. The recombinant pAMJ-LacF-MPR vector

was then electro-transferred into *L. lactis* NZ3900 and plated onto Elliker-L medium. The plasmid extraction and restriction digestion methods were performed to check the presence of insertion; in addition, the SDS-PAGE and western blotting were carried out to detect the MPR protein expression of recombinant *L. lactis* carrying MPR gene. The protein assay, milk clotting activity (MCA) and proteolytic activity (PA) of purified recombinant MPR protein were also studied after optimizing the growth rate, and protein expression of recombinant *L. lactis* carrying MPR gene. Finally, milk coagulation ability of recombinant *L. lactis* carrying MPR gene was tested. Nucleotide sequencing of DNA insertion from the clone revealed that the MPR activity corresponded to an open reading frame consisting of 1218 bp coding for a 43.45-kDa MPR protein. A clear band on 43.45-kDa size on SDS-PAGE and western blotting confirmed the successful expression of MPR protein by recombinant *L. lactis*. Optimizing the growth rate of recombinant *L. lactis* showed the highest cell biomass for the cultures incubated at 33°C. The MPR protein assay results indicated that the highest MPR enzyme, approximately 65.6 µg/ml.h, were obtained for cultures which were incubated at pH 5.5 and 30°C. Statistical analysis of results revealed that there was no significant difference ($P<0.05$) between MPR protein expression at 30 and 33°C but a significant difference was noted with the expressed MPR protein at 27 and 36°C. Analysis of the mean of the results of milk clotting activity and MCA/PA of purified recombinant MPR protein for the highest purified levels of expressed protein at pH 5.5 and temperatures 30, 33, 27 and 36°C and control showed 870.54, 809.86, 491.85, 358.54 and 651.38 SU/ml for milk clotting activity and 7914, 7362.36, 4471.36, 3259.45 and 5664.17 SU/OD for MCA/PA, respectively. The thermal and pH stability results of purified recombinant MPR protein showed that the recombinant MPR protein is stable at the pH range 3.5–7.5

and thermal stability range 20-50°C. Interestingly, milk coagulation was observed after inoculating milk with recombinant *L. lactis* carrying MPR gene due to the high expression rate of MPR enzyme by recombinant *L. lactis*. The mean of the results indicated that the milk coagulated after 220 and 205 min when inoculated milk were incubated at 33°C, under static and agitation conditions, respectively. The curd yield results showed 14.35 g/100ml compared to 13.86 g/100ml solid curd for milk added recombinant *L. lactis* carrying MPR gene and commercial rennet, respectively. The plasmid stability results also showed that the recombinant pAMJ-LacF-MPR vector has high stability around 88.9% after 200 generations in *L. lactis.* This study presents novel findings, as the *L. lactis* was used for the first time as a cell factory for the production of recombinant rennin. In addition, this study introduced a novel milk clotting procedure using recombinant bacterium capable of milk coagulation. The recombinant *L. lactis* carrying MPR gene, created in this study, has the ability to function as starter culture for acidifying and subsequently coagulating milk by producing mucor rennin as the milk coagulant agent. Thus, these findings would have a significant impact on the cheese industry.

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

PENGHASILAN KULTURE BAKTERIA PEMULA REKOMBINAN BAHARU UNTUK MEMBEKUKAN SUSU

Oleh

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Pengerusi: **Profesor Fatimah Abu Bakar, PhD**

Fakulti: **Sains dan Teknologi Makanan**

Enzim pembekuan susu adalah faktor utama untuk pengeluaran pelbagai jenis keju. Oleh itu, rennet anak lembu (chymosin) secara tradisinya digunakan sebagai koagulan susu dalam pembuatan keju. Memandangkan peningkatan dalam pembuatan keju di peringkat global penurunan dalam bekalan pada masa yang sama rennet lembu, ia menjadi penting bahawa gantian untuk rennet hasilkan. Baru-baru ini, penggunaan kulat rennin mucor, dalam pembuatan keju perindustrian semakin meluas. *Lactococcus lactis* adalah asid bakteria laktik yang biasanya digunakan sebagai permulaan dalam industri tenusu untuk pengeluaran pelbagai jenis keju keras dan lembut. Oleh itu, kerana peranan utama enzim pembekuan susu serta pengkulturan pemula dalam pembuatan keju, kajian ini adalah satu usaha untuk menyatakan rekombinan enzim rennin mucor (MPR) oleh *L. lactis* untuk menghasilkan enzim pembekuan. Ia adalah prosedur pembekuan baru menggunakan bakteria rekombinan hidup untuk pembekuan susu. Untuk mencapai matlamat ini, gen MPR dikuatkan adalah sub-klon ke pAMJ-LacF ekspresi vektor. Gred makanan pAMJ-LacF campuran vektor dicipta oleh sub-pengklonan dikuatkan LacF gen

dalam pAMJ399 vektor yang hilang gen erythromycin. Rekombinan pAMJ-LacF-MPR vektor kemudian aya dielektro-pindahkan ke *L. lactis* NZ3900 disalut ke medium Elliker-L. Kaedah pencernaan pengeluaran dan sekatan plasmid telah dilakukan untuk memeriksa kehadiran kemasukan; di samping itu, SDS-PAGE dan serap barat telah dijalankan untuk mengesan ekspresi protein MPR rekombinan *L. lactis* membawa gen MPR. Cerakin protein, aktiviti pembekuan susu (MCA) dan aktiviti proteolitik (PA) yang ditapis protein rekombinan MPR juga dikaji selepas mengoptimumkan kadar pertumbuhan dan ungkapan protein rekombinan *L. lactis* membawa gen MPR. Akhirnya, keupayaan pembekuan susu rekombinan *L. lactis* membawa gen MPR telah diuji. Urutan nukleotida kemasukan DNA daripada klon mendedahkan bahawa aktiviti MPR selaras dengan bingkai bacaan terbuka yang terdiri daripada 1218 bp kod untuk protein MPR 43.45-kDa. Sebuah band yang jelas bersaiz 43.45-kDa pada SDS-PAGE dan serapan barat mengesahkan protein rekombinan MPR oleh *L. lactis*. Mengoptimumkan kadar pertumbuhan rekombinan *L. lactis* menunjukkan biomas sel tertinggi bagi pengkulturan yang dieram pada 33°C. Keputusan MPR assay protein menunjukkan bahawa enzim MPR tertinggi, kira-kira 65.6 μg/ml.h, telah diperolehi bagi pengkulturan yang dieram pada pH 5.5 dan 30°C. Analisis statistik keputusan menunjukkan bahawa terdapat perbezaan yang signifikan ($P < 0.05$) di antara ungkapan protein MPR pada 30 dan 33°C tetapi perbezaan yang ketara telah diperhatikan dengan protein MPR dinyatakan pada 27 dan 36°C. Analisis keputusan aktiviti pembekuan susu dan MCA/PA protein rekombinan MPR yang dihasilkan pada tahap tertinggi daripada protein dinyatakan pada pH 5.5 dan suhu 30, 33, 27, 36°C dan kawalan menunjukkan masing-masing 870.54, 809.86, 491.85 , 358.54 dan 651.38 SU/ml untuk aktiviti susu beku dan 7914, 7362.36, 4471.36, 3259.45 dan 5664.17 SU/OD untuk MCA/PA. Keputusan

kestabilan terma dan pH protein rekombinan MPR yang dibersihkan menunjukkan bahawa protein rekombinan MPR adalah stabil pada julat pH 3.5-7.5 dan pelbagai kestabilan haba 20-50°C. Menariknya diperhatikan pembekuan susu selepas susu diinokulat dengan rekombinan *L. lactis* membawa gen MPR disebabkan oleh kadar ungkapan yang tinggi enzim MPR oleh rekombinan *L. lactis*. Hasil keputusan menunjukkan bahawa susu beku selepas disuntik telah dieram pada 33°C masing-masing selama 220 dan 205 min, di bawah keadaan statik dan agitat. Keputusan hasil curd menunjukkan 14.35 g/100ml berbanding 13.86 g/100ml pepejal susu ditambah rekombinan *L. lactis* membawa gen MPR dan rennet komersial. Keputusan kestabilan plasmid juga menunjukkan bahawa rekombinan pAMJ-LacF-MPR vektor mempunyai kestabilan yang tinggi di sekitar 88.9% selepas 200 generasi *L. lactis*. Kajian ini menunjukhan penemuan baru, memandangkan *L. lactis* telah digunakan buat pertama kali sebagai sebuah kilang sel untuk pengeluaran rennin rekombinan. Di samping itu, kajian ini memperkenalkan prosedur pembekuan susu menggunakan bakteria rekombinan hidup yang mampu membekukan susu. Rekombinan *L. lactis* membawa gen MPR, yang dicipta dalam kajian ini, mempunyai keupayaan untuk berfungsi sebagai pengkulturan permulaan untuk pengasidan dan seterusnya membekukan susu dengan menghasilkan rennin Mucor sebagai agen koagulan susu. Oleh itu, hasil kajian ini akan memberi kesan yang tinggi pada industri keju.

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I certify that an Examination Committee has met on 27/11/2013 to conduct the final examination of Mohammad Raftari on his Doctor of Philosophy thesis entitled "Development of a New Recombinant Starter Culture Bacterium Capable of Milk Coagulation" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Doctor of Philosophy degree.

Members 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 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.

MOHAMMAD RAFTARI

Date: 27/11/2013

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