DEVELOPMENT OF A NEW RECOMBINANT STARTER CULTURE
BACTERIUM FOR MILK COAGULATION

MOHAMMAD RAFTARI

FSTM 2014 1
DEVELOPMENT OF A NEW RECOMBINANT STARTER CULTURE BACTERIUM FOR MILK COAGULATION

By

MOHAMMAD RAFTARI

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

November 2013
COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright© Universiti Putra Malaysia
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

By

MOHAMMAD RAFTARI

November 2013

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.
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 diberikah 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 unggkapan 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 menunjukkan 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.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. Fatimah Abu Bakar, for the patient guidance, encouragement, immense knowledge and advice she has provided throughout my time as her student. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly. I could not have imagined having a better advisor and mentor for my Ph.D study. I would also like to thank all the supervisory committee members, Prof. Raha, Prof. Nazamid and Prof. Zamberi for their encouragement, insightful comments, and hard questions who helped me in my supervisor’s absence.

I would like to thank my parents for everything they have done to nurture me and cultivate my interests. With the affection and inspiration from my parents, I feel it is a blessing to be their son. Since I was a child, I have seen my father as a role model. Therefore, first, I would like to dedicate my achievement to my beloved father, who inspired me to achieve my academic goals. I would like to thank my mother, who has provided me with endless love and support. My mother has always taught me to try to be a virtuous and modest man and to have an enthusiastic and dependable learning spirit. The success of this journey also belongs to her.

Last but not least, I thank those of my family, friends, and colleagues who always had faith in me and never let me give up on my dream, no matter how many obstacles came my way. I am blessed for having such strong pillars of support.
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:

**Name of Examiner 1, PhD**  
Professor Son Radu  
Faculty of Food Science and Technology  
Universiti Putra Malaysia  
(Internal Examiner)

**Name of Examiner 2, PhD**  
Associate Professor Abdul Karim Sabu Mohamed  
Faculty of Food Science and Technology  
Universiti Putra Malaysia  
(Internal Examiner)

**Name of External Examiner, PhD**  
Professor Emeritus Athanasios A. Koutinas  
Department of Food Biotechnology  
University of Patras  
Greece  
(External Examiner)

Seow Heng Fong, PhD  
Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia  
Date:
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:

**Fatimah Abu Bakar, PhD**
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

**Nazamid Saari, PhD**
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

**Raha Abdul Rahim, PhD**
Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Member)

**Zamberi Sekawi, PhD**
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

---

**BUJANG BIN KIM HUAT, PhD**
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:
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
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td></td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td></td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td></td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td></td>
<td>ix</td>
</tr>
<tr>
<td>APPROVAL</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>DECLARATION</td>
<td></td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td></td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td></td>
<td>xviii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td></td>
<td>xix</td>
</tr>
</tbody>
</table>

## CHAPTER 1

1 INTRODUCTION | 1 |

## CHAPTER 2

2 LITERATURE REVIEW | 5 |
2.1 Milk Clotting Enzymes | 5 |
2.1.1 Animal Milk Clotting Enzyme | 6 |
2.1.2 Microbial Milk Clotting Enzymes | 9 |
2.1.3 Plant Milk Clotting Enzyme | 11 |
2.1.4 Genetically Engineered Milk Clotting Enzymes | 13 |
2.2 Lactic Acid Bacteria (LAB) | 16 |
2.2.1 Homofermentative Metabolism | 17 |
2.2.2 Heterofermentative Metabolism | 18 |
2.2.3 Acid Production | 19 |
2.2.4 Genetically Modified Lactic Acid Bacteria | 20 |
2.3 Starter Culture Bacteria | 21 |
2.3.1 Types of Starter Cultures | 22 |
2.3.1.1 Mesophilic Starter Cultures | 23 |
2.3.1.2 Thermophilic Starter Cultures | 24 |
2.4 *Lactococcus lactis* | 26 |
2.5 Protein expression systems in *L. lactis* | 28 |
2.5.1 Constitutive Promoters | 29 |
2.5.2 Inducible Promoters | 29 |
2.6 Rhizomucor pusillus | 31 |

## CHAPTER 3

3 MATERIALS AND METHODS | 34 |
3.1 Fungi and Bacterial Strains and Growth Conditions | 34 |
3.2 Plasmid Vectors | 34 |
3.3 Gene Manipulation | 34 |
3.3.1 Genomic DNA Isolation from *Rhizomucor Pusillus* | 34 |
3.3.2 Spectrophotometric Analysis of DNA | 36 |
3.3.3 Evaluation of DNA Quality | 36 |
3.4 Polymerase Chain Reaction (PCR) Amplification of
Rhizomucor pusillus Rennin (MPR) Gene
3.4.1 Design of PCR Amplification Primers 36
3.4.2 PCR Amplification 37
3.4.3 Purification of MPR gene from Agarose Gel 38
3.5 Cloning of PCR Amplified MPR Gene 39
3.6 Transformation of Recombinant Plasmid into Competent Cells
3.6.1 Preparation of Competent Escherichia coli Strain 39
3.6.2 Transformation 40
3.7 Identification of Recombinant Plasmids 41
3.7.1 Screening on Agar Plate Method 41
3.7.2 Plasmid Extraction Method 41
3.7.3 Single and Double Digestion of Recombinant Plasmids 42
3.7.4 DNA Sequencing 42
3.8 Expression of MPR Enzyme by Lactococcus lactis 43
3.8.1 Construction of Food Grade Expression System 43
3.9 Growth Rate Optimization of Recombinant L. lactis Carrying MPR Gene 60
3.10 Protein Assay of Recombinant MPR Enzyme Expressed by L. lactis Carrying MPR Gene 60
3.10.1 Protein Purification 60
3.10.2 MPR Protein Assay 62
3.11 Detection of MPR Protein Expression of Recombinant L. lactis Using Real-Time PCR (RT-qPCR)
3.11.1 RNA Extraction 62
3.11.2 cDNA Synthesis 63
3.11.3 Standard Curve Preparation 64
3.11.4 Real Time PCR (RT-qPCR) 64
3.12 Milk Clotting Activity of Recombinant MPR 65
3.13 Proteolytic Activity Assay of Recombinant MPR 66
3.14 pH and Thermal Stability of Recombinant MPR 66
3.15 Detection of Milk Clotting Activity of Recombinant L. lactis Carrying MPR Gene 67
3.16 Curd Yield 68
3.17 Recombinant Plasmid Stability 68
3.18 Statistical Analysis 69

4 RESULTS AND DISCUSSION
4.1 Gene Manipulation 70
4.1.1 Genomic DNA Extraction of Rhizomucor pusillus 70
4.1.2 PCR Amplification and Cloning of PCR Amplified Gene
4.1.2.1 Restriction Maps of R. pusillus Rennin (MPR) Gene 71
4.1.2.2 Cloning of PCR Amplified MPR
4.1.2.3 Selection of Recombinant Colonies on Agar Plate
4.1.2.4 Plasmid Extraction and Vector Restriction Digestion Analysis

4.2 Expression of MPR Protein by Lactococcus lactis
4.2.1 Construction of Food Grade Expression Vector
4.2.1.1 PCR Amplifications and Cloning of PCR Amplified Fragment
4.2.1.2 Selection of Recombinant Colonies on Agar Plate
4.2.1.3 Plasmid Extraction and Vector Restriction Digestion Analysis
4.2.2 Sub-cloning LacF Gene in pAMJ399 Expression Vector
4.2.2.1 Selection of Recombinant Colonies
4.2.2.2 Plasmid Extraction and Vector Restriction Digestion Analysis
4.2.3 Sub-cloning MPR Gene in Expression Vector
4.2.3.1 PCR Amplification of MPR Gene with mpr3/mpr4 Primer Set
4.2.3.2 Sub-cloning MPR Gene in Food Grade pAMJ-LacF Expression Vector
4.2.4 MPR Protein Expression of Recombinant L. lactis Carrying MPR Gene

4.3 Growth Rate Optimization of Recombinant L. lactis Carrying MPR Gene
4.4 MPR Protein Assay Expressed by Recombinant L. lactis Carrying MPR Gene
4.5 Detection of MPR Protein Expression of Recombinant L. lactis Using Real-Time PCR (RT-qPCR)
4.6 Milk Clotting Activity of Recombinant MPR
4.7 Proteolytic Activity Assay of Recombinant MPR
4.8 pH and Thermal Stability of Recombinant MPR
4.9 Detection of Milk Clotting Activity of Recombinant L. lactis Carrying MPR gene
4.10 Curd Yield
4.11 Recombinant Plasmid Stability

5 CONCLUSION AND RECOMMENDATION