DEVELOPMENT OF SECRETORY LACTOCOCCUS LACTIS VECTORS WITH CHARACTERIZED HETEROLOGOUS SIGNAL PEPTIDE FROM PEDIOCOCCUS PENTOSACEUS

ALI BARADARAN

FBSB 2010 12
DEVELOPMENT OF SECRETORY *LACTOCOCCUS LACTIS* VECTORS
WITH CHARACTERIZED HETEROLOGOUS SIGNAL PEPTIDE FROM
*PEDIIOCOCCUS PENTOSACEUS*

BY

ALI BARADARAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Master of Science

December 2010
This thesis is dedicated to my wife, parents, parents-in-law, my brothers and sister who have been stressing the importance of academic excellence and always been the fountain of my strength.
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

DEVELOPMENT OF SECRETORY *LACTOCOCCUS LACTIS* VECTORS WITH CHARACTERIZED HETEROLOGOUS SIGNAL PEPTIDE FROM *PEDIOCOCCUS PENTOSACEUS*

By

ALI BARADARAN

December 2010

Chairperson: Raha Abdul Rahim, PhD

Faculty: Biotechnology and Biomolecular Sciences

*Lactococcus lactis*, the model of lactic acid bacteria (LAB), is a generally regarded as safe (GRAS) organism and one of the most widely used LAB in the food industry. The potential application of *Lactococcus lactis* as a live vehicle for the production and delivery of heterologous protein for industrial and medical applications are on the rise. Investigation of heterologous protein production in different location of *L. lactis* revealed that secretion is preferable to cytoplasmic production. Although considerable attentions have been given to the development of efficient gene expression and protein secretion systems, however, there is still an acute lack of system to secrete heterologous proteins in *L. lactis*. The Gram-positive low GC content bacterium, *Pediococcus pentosaceus* was isolated from a local herbal plant *Polygonum minus* and identified by biochemical and 16S rRNA sequencing. The nucleotide sequence of the
Cell wall binding protein from *P. pentosaceus* was amplified by polymerase chain reaction (PCR), cloned into Zero Blunt® TOPO® plasmid and transformed into *Escherichia coli*. The coding region of signal peptides (SP) SPK1 and SPK3 were amplified from the cell wall binding proteins of *P. pentosaceus* and studied by *in silico* analysis. The *in silico* analysis of signal peptide revealed that SPK1 has higher hydrophobicity, GRAVY index, aliphatic index and more stability compared to SPK3 and USP45. The gene coding region of green fluorescent protein (GFP) and *L. lactis* signal peptide USP45 were then amplified by using *Pfu* DNA polymerase. Secretion cassettes were constructed using GFP as the reporter protein and USP45 as the control. Then, the SP-GFP cassette was cloned into *L. lactis* expression vectors pNZ8084 and pMG36e (inducible and constitutive) resulting in pNZK801, pNZK803, pNZU801 and pMGK36e1, pMGK36e3, pMGU36e1, respectively. The constructed plasmids were electro-transformed into *L. lactis* strain MG1363 and NZ9000 as host. Recombinant plasmids were identified by restriction enzyme digestion and sequence analyses. Western blot and ELISA analysis of transformants indicated the potential of the signal peptides SPK1 and SPK3 from *P. pentosaceus* to be used as a secretory signal for heterologous protein secretion in *L. lactis*. 
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBANGUNAN VECTOR REMBESAN LACTOCOCCUS LACTIS DENGAN PEPTIDA ISYARAT HETEROLOG DARIPADA PEDIOCOCCUS PENTOSACEUS

Oleh

ALI BARADARAN

Desember 2010

Pengerusi: Raha Abdul Rahim, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Lactococcus lactis iaitu model bakteria asid laktik (LAB) merupakan organisma yang dianggap selamat secara umumnya (GRAS), dan salah satu LAB yang digunakan dengan meluas dalam industri makanan. Potensi Lactococcus lactis untuk digunakan sebagai “kenderaan hidup” bagi penghasilan dan penghantaran protein heterolog dalam aplikasi-aplikasi industri dan perubatan sedang meningkat. Kajian penghasilan protein heterolog di lokasi yang berbeza dalam L. lactis mendedahkan rembesan cenderung ke arah sitoplasmik. Walaupun perhatian yang secukupnya telah digunakan bagi membina sistem penzahiran gen dan sistem rembesan protein yang cekap, tetapi masih ada kekurangan dalam sistem berkenaan untuk merembeskan protein heterolog dalam L. lactis. Gram-positif bakteria yang rendah kandungan GC, Pediococcus pentosaceus telah dipencilkan daripada tumbuhan herba tempatan Polygonum minus dan dikenalpasti dengan menggunakan kaedah biokimia dan 16S rRNA. Jujukan nukleotida v
daripada protein dinding sel bakteria *Pediococcus pentosaceus* telah digandakan dengan menggunakan tindakbalas berantai polimerase (PCR), dikeluarkan ke dalam plasmid Zero Blunt®TOPO® dan ditransformasikan ke dalam *Escherichia coli*. Peptida isyarat (SP) iaitu SPK1 dan SPK3 telah digandakan daripada protein dinding sel *Pediococcus pentosaceus* dan diuji dengan analisis *in silico*. Analisis *in silico* pada peptida isyarat mendedahkan bahawa SPK1 mempunyai sifat hidrofobik, indeks GRAVY, indeks alifatik yang tinggi dan lebih stabil berbanding dengan SPK3 dan USP45. Protein pendaflour hijau (GFP) dan peptida isyarat USP45 *L. lactis* kemudiannya digandakan menggunakan *Pfu* polimerase DNA. Kaset rembesan telah dibina menggunakan GFP sebagai gen pelapor dan USP45 sebagai kawalan. Kemudian, kaset-kaset SP-GFP itu telah dikeluarkan ke dalam vektor penzahiran *L. lactis* pNZ804 dan pMG36e masing-masing menghasilkan pNZK801, pNZK803, pNZU801 dan pMGK36e1, pMGK36e3, pMGU36e1. Plasmid yang dibina telah di elektrotransformasikan ke dalam *L. lactis* MG1363 dan NZ9000. Transforman positif telah dikenalpasti dengan menggunakan cernaan enzim pembatas dan analisis-analisis jujukan. Analisis ELISA dan blot Western terhadap transforman menunjukkan peptida isyarat SPK1 dan SPk3 dari *Pediococcus pentosaceus* berpotensi untuk digunakan sebagai isyarat perembes bagi rembesan protein heterolog dalam *L. lactis*. 

vi
ACKNOWLEDGEMENTS

Thanks God for His mercy and guidance that helped me in conducting this study and in the preparation of this thesis.

I would like to express my appreciation and gratitude to the chairperson of my Supervisory Committee, Prof. Dr. RAHA ABDUL RAHIM for exquisite guidance, suggestion and encouragement throughout this project and my co-supervisors Assoc. Prof. Dr. Foo Hooi Ling and Dr. Sieo Chin Chin for their valuable advice. All their invaluable help is greatly appreciated.

I would like to convey warm thanks to my labmates especially Vithya, Shamsia, Noreen, Kak Yan, Erni, Adlin, Morteza, Sadegh, Bakhtiar, Azmi, Farzaneh, Yee, Fong, Tannaz, Omid and Hassan for their help.

A special thanks to my wife, parents, parents-in-law, my brothers and sister for their endless support and encouragement.
APPROVAL

I certify that an Examination Committee has met on date of viva voce to conduct the final examination of Ali Baradaran on his degree of Master of Science thesis entitled “Development OF Secretory Lactococcus lactis Vectors with Characterized Heterologous Signal Peptide From Pediococcus pentosaceus” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

Shuhaimi Mostafa, PhD
Assoc. Prof. Dr.
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Tan Wen Siang, PhD
Prof. Dr.
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Adam Leow Thean Chor, PhD
Dr.
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Rostli Md. Illias, PhD
Prof. Dr.
Faculty of Chemical and Natural Resources Engineering
Universiti Teknologi Malaysia
(External Examiner)

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia
Date:

viii
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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

**Foo Hooi Ling, PhD**  
Associate Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Member)

**Sieo Chin Chin, PhD**  
Lecturer  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Member)

HASANAH MOHD GHAZALI, PhD  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia  
Date:
DECLARATION

I declare that the thesis is based on my original work except for quotations and citation 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 other institutions.

ALI BARADARAN

Date: 24 December 2010
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>viii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xviii</td>
</tr>
</tbody>
</table>

## CHAPTER

1 INTRODUCTION

1.1 Overview
1.2 Hypothesis
1.3 Objectives

2 LITERATURE REVIEW

2.1 Lactic acid bacteria
2.1.1 The role of lactic acid bacteria in food industry
2.1.2 The role of lactic acid bacteria in human health
2.2 *Lactococcus lactis*
2.2.1 Genomic study of *Lactococcus*
2.3 Production of recombinant protein
2.3.1 Choosing and designing the optimal promoter
2.3.2 Transcription regulation
2.3.3 Translation regulation
2.3.4 Optimizing codon usage
2.4 *Lactococcus lactis* as a host 16

2.5 Introduction to protein secretion in Gram-positive bacteria 19

- 2.5.1 Signal peptide and processing 24
- 2.5.2 Translocation 29
- 2.5.3 Signal peptide cleavage 33
- 2.5.4 Extracytoplasmic folding 33
- 2.5.5 Passage through the cell wall 36
- 2.5.6 Quality control factors 38

2.6 Green fluorescent protein 39

2.7 *Pediococcus pentosaceus* 40

2.8 *Polygonum minus* 42

3 ISOLATION, IDENTIFICATION AND PRELIMINARY CHARACTERIZATION OF LACTIC ACID BACTERIA FROM POLYGONUM MINUS 43

3.1 Introduction 43

3.2 Materials and Methods 46

- 3.2.1 Sample collection 46
- 3.2.2 Morphological, physiological, and biochemical tests 46
- 3.2.3 Bile tolerance analysis 49
- 3.2.4 API 50 CHL biochemical test 49
- 3.2.5 Genomic DNA extraction 50
- 3.2.6 Agarose gel electrophoresis 51
- 3.2.7 PCR amplification of 16S rRNA sequencing 51
- 3.2.8 Sequence determination and analysis 52
- 3.2.9 Purification of PCR Products and Plasmid 52

3.3 Results 53

- 3.3.1 Morphological and biochemical characterization of isolates 53
- 3.3.2 Characterization of isolated bacteria by API 50 CHL kit 54
- 3.3.3 Genomic DNA extraction of *Pediococcus pentosaceus* K1 56
- 3.3.4 PCR amplification of 16S rRNA gene fragment 56
4 COMPUTATIONAL AND SYSTEMATIC ANALYSIS OF THE SIGNAL PEPTIDE OF PEDIOCOCCUS PENTOSACEUS ISOLATED FROM Polygonum minus (Kesum)

4.1 Introduction 61
4.2 Materials and Methods
   4.2.1 Prediction of transmembrane region and protein location 64
   4.2.2 Prediction of signal peptide 66
   4.2.3 In silico analysis of physicochemical properties of Signal peptide 66
4.3 Results
   4.3.1 Prediction of Sub-cellular localization of cell wall binding protein of Pediococcus pentosaceus 68
   4.3.2 Prediction of signal peptide and signal peptide probability 70
   4.3.3 Computational analysis of physicochemical properties of signal peptide 72
4.4 Discussion 73
4.5 Conclusion 78

5 DEVELOPMENT OF LACTOCOCCUS LACTIS SECRETION VECTOR 79

5.1 Introduction 79
5.2 Materials and Methods
   5.2.1 Bacterial strains, plasmids and growth conditions 82
   5.2.2 Preparation of stock culture 83
   5.2.3 Preparation of competent cell 84
   5.2.4 Plasmid DNA extraction 85
   5.2.5 Polymerase chain reaction 86
   5.2.6 Cloning of the DNA fragment 90
   5.2.7 DNA transformation 92
5.2.8 Protein expression analysis

5.3 Results

| 5.3.1 Amplification of the gene coding regions for cell wall binding protein of *Pediococcus pentosaceus*  | 98 |
| 5.3.2 Cloning of the cell wall binding protein gene into TOPO Zero Blunt                                  | 100 |
| 5.3.3 Amplification of signal peptide from cell wall binding protein                                    | 101 |
| 5.3.4 Amplification of GFP fragment                                                                    | 103 |
| 5.3.5 Construction of SP-GFP cassette                                                                  | 104 |
| 5.3.6 Construction of expression/secretion system                                                      | 106 |
| 5.3.7 SDS-PAGE and Western Blot Analysis                                                               | 112 |
| 5.3.8 Quantification of extracellular GFP using ELISA kit                                              | 116 |

5.4 Discussion

5.4 Conclusion

6 GENERAL DISCUSSION

7 CONCLUSION AND RECOMMENDATION

8 REFERENCES

9 APPENDICES

10 BIODATA OF STUDENT