UNIVERSITI PUTRA MALAYSIA

# *CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM PROBIOTIC Lactobacillus STRAINS ISOLATED FROM TRADITIONAL MALAYSIAN FOODS*

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By

**EILAF SULIMAN KHALIL SULIMAN**

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

**April 2018**

# DEDICATION

*This work is dedicated to the soul of my late mother*
(May Allah rest her soul and forgive her)
*My father who always prays for my success*
*My brothers and sisters for their constant encouragement and support*
*My husband and children for being my inspiration*
*My friends and colleagues*

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

**CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM PROBIOTIC *Lactobacillus* STRAINS ISOLATED FROM TRADITIONAL MALAYSIAN FOODS**

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**Chairman : Professor Mohd Yazid B Abd Manap, PhD**
**Faculty : Food Science and Technology**

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) microorganisms, and play an important role in food and animal feed as potential probiotics. LAB are used for the production of several industrially interesting metabolites such as bioactive peptides, antibacterial compounds, aroma compounds, and cells wall component like exopolysaccharides (EPS). EPS are generally related to all forms of polysaccharides that are present outside of the microbial cell wall. Although, EPS produced by LAB have multifunctional, technological, and health benefits, their industrial applications are hindered by their low yield. The present study was conducted to isolate and identify varieties of LAB from some traditional Malaysian foods and to investigate their probiotic characteristics, functional properties, ability to produce EPS, and to study their bioactivities. One hundred and twenty LAB strains were isolated from five different traditional Malaysian foods. The preliminary selection carried out optically by screening the strains' resistance to low pH 2.0 and bile salts 0.3% as well as ability to produce EPS, and a total of 13 isolates were accordingly selected. The 13 isolates were identified using the carbohydrates fermentation profile and 16S rRNA gene sequences. Six of the *Lactobacillus* strains were associated with the species *Lb. fermentum* (DUR18, TAP1, PIC7, BU11, BU14, and TAP16) and four strains belonged to *Lb. plantarum* (DUR2, DUR5, DUR8, and TEMP9). Besides, *Lb. crispatus* (DUR4) *Lb. reuteri* (DUR12) and *Lb. pentosus* (DUR20) were also identified. Two commercial *Lactobacillus* strains namely: *Lb. rhamnosus* (ATCC53103) and *Lb. plantarum* (ATCC8014) were used as reference strains. The isolated strains exhibited high tolerance to acid (<80%) and bile (<65 %) as well as high survival rate in the gastrointestinal tract. The results of the cells wall properties showed that auto-aggregation and cell surface hydrophobicity were ranged between 53-80 and 16-80%, respectively, whereas, the highest co-aggregation value (66%) was reported by *Lb. plantarum* (DUR8) with *Pseudomonas aeruginosa*. The in vitro assay for the functional properties of the isolated strains showed antioxidant activity equivalent to trolox (30-70%), high cholesterol assimilated. Also, the isolated strains exhibited good inhibitory activity against some tested pathogens due to organic acids production. The EPS production by the 13 *Lactobacillus* isolated strains were ranged between 50 and 850 mg/L. Based on that, two strains; *Lb. pentosus* and *Lb. reuteri* which showed the highest EPS yield were screened out for further characterization. The purified EPS were composed of glucose, arabinose, and rhamnose. FTIR analysis indicated a specific spectrum of neutral polysaccharides in the region of 1577 $cm^{-1}$ which attributed to C=O stretching. An extracellular glucosytransferase (GTF) enzyme corresponding to EPS from the selected strains were produced with the molecular weight falls between 120 and 150 kDa. The GTF exhibited high activity under static condition at pH 5 and incubation temperature 28°C. EPS yield from *Lb. reuteri* and *Lb. pentosus* were optimized using response surface methodology (RSM). The maximum EPS yields attained were 1.0 g/L and 1.07 g/L produced by *Lb. reuteri* and *Lb. pentosus*, respectively. Moreover, the optimal values for the tested variables were: 41 and 39.5 °C for incubation temperature, pH 6.4 and 6.5, and the incubation times were 33.7 and 48 h for *Lb. reuteri* and *Lb. pentosus*, respectively. The RSM displayed a significant ($p < 0.05$) response fit for the studied variables with a high coefficient of determination ($R^2$). The predicted and observed values displayed no significant ($p > 0.05$) differences. Both incubation time and the pH significantly ($p < 0.05$) affected the EPS production in both strains. The optimum points were practically validated. The EPS samples revealed potent antioxidant activity to scavenging DPPH (65.8 and 68.0% for *Lb. pentosus* and *Lb. reuteri*, respectively) and antibiofilm activity against some pathogens (above 50%). It can be concluded that *Lactobacillus* strains isolated from traditional Malaysian foods in this study are promising probiotics which have the potential to produce EPS with functional attributes.

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

**PENCIRIAN EKSOPOLISAKARIDA DARIPADA STRAIN LAKTOBASILUS PROBIOTIK TERASING DARIPADA MAKANAN TRADISIONAL MALAYSIA**

Oleh

**EILAF SULIMAN KHALIL SULIMAN**

**April 2018**

**Pengerusi : Profesor Mohd Yazid B Abd Manap, PhD**
**Fakulti : Sains dan Teknologi Makanan**

Secara umumnya, bakteria asid laktik (LAB) telah diakui sebagai mikroorganisma selamat (GRAS) dan ia memainkan peranan penting dalam makanan dan makanan haiwan sebagai probiotik yang berpotensi. LAB telah digunakan untuk penghasilan beberapa metabolit menarik berunsurkan industri seperti peptida bioaktif, kompoun antibakteria, kompoun aroma dan komponen dinding sel seperti eksopolisakarida (EPS). EPS secara umumnya berkaitan dengan semua bentuk polisakarida yang wujud di luar dinding sel mikroba. Walaupun, EPS yang dihasilkan oleh LAB mempunyai pelbagai manfaat multifungsi, teknologikal dan kesihatan, aplikasi perindustrian telah dihalang oleh hasil rendah mereka. Kajian ini telah dijalankan untuk mengasing dan mengenal pasti pelbagai jenis LAB daripada beberapa makanan tradisional Malaysia dan juga bertujuan untuk menyelidiki ciri probiotik mereka, sifat fungsian dan kemampuan untuk menghasilkan EPS dan mengkaji bioaktiviti mereka. Seratus dua puluh strain LAB telah diasingkan daripada lima makanan tradisional Malaysia yang berbeza. Pemilihan awal telah dijalankan secara optik dengan menyaring ketahanan strain pada pH rendah 2.0 dan garam hempedu 0.3% di samping kemampuan untuk menghasilkan EPS, dan sejumlah 13 isolat telah dipilih sewajarnya.13 isolat telah dikenal pasti menggunakan profil fermentasi karbohidrat dan susunan gen rRNA 16S. Enam strain Laktobasilus telah dikaitkan dengan spesis *Lb. fermentum* (DUR18, TAP1, PIC7, BU11, BU14 dan TAP16) dan empat strain tergolong dalam *Lb. plantarum* (DUR2, DUR5, DUR8 dan TEMP9). Selain itu, *Lb. crispatus* (DUR4), *Lb. reuteri* . (DUR12), dan *Lb. pentosus* (DUR20) juga telah dikenal pasti. Dua strain Lb. yang dihasilkan secara komersial, iaitu *Lb. rhmnosus* (ATCC53103) dan *Lb. plantarum* (ATCC8014) telah digunakan sebagai strain rujukan. Strain yang diasingkan itu menunjukkan sifat toleransi yang tinggi terhadap asid (<80%) dan hempedu (<65%) di samping kadar kelangsungan yang tinggi dalam saluran gastrosus. Dapatan mengenai sifat dinding sel sel dinding menunjukkan bahawa autoagregasi dan hidrofobisiti permukaan sel adalah masing-masing antara 53-80 dan 16-80%, manakala, nilai koagregasi tertinggi ( 66%) telah dilaporkan oleh *Lb. plantarum* (DUR8) dengan P*seudomonas aeruginosa*. Cerakinan in vitro bagi sifat fungsian strain terasing menunjukkan aktiviti antioksidan bersamaan dengan troloks (30-70%), kolesterol tinggi berasimilasi. Di samping itu, strain terasing tersebut memperlihatkan aktiviti penentangan yang kuat terhadap beberapa patogen yang diuji akibat penghasilan asid organik. Penghasilan EPS oleh 13 strain *Lactobacillus* terasing berjulat antara 50 dan 850 mg/L. Berdasarkan julat tersebut, dua strain; *Lb. pentosus* dan *Lb. reuteri* yang menunjukkan hasil EPS tertinggi telah disaring bagi pencirian selanjutnya. EPS tulen terdiri daripada glukosa, arabinosa, dan rhamnosa. Analisis FTIR menunjukkan spektrum khusus bagi polisakarida neutral dalam lingkungan 1577 $cm^{-1}$ yang ditentukan pada peluasan C=O. Enzim glukositransferas ekstraselular (GTF) sepadan dengan EPS daripada strain terpilih telah dihasilkan dengan berat molekular turun antara 120 dan 150 kDa. GTF memperlihatkan aktiviti yang tinggi pada pH 5 dan suhu inkubasi 28°C. Hasil EPS daripada *Lb. reuteri* dan *Lb. pentosus* telah dioptimumkan menggunakan metodologi permukaan tindak balas (RSM). Hasil EPS maksimum yang diperoleh ialah 1.0 g/L bagi *Lb. reuteri* dan 1.07 g / L bagi *Lb. pentosus* yang dihasilkan, masing-masing oleh *Lb. reuteri* dan *Lb. Pentosus*. Tambahan pula, nilai optimum bagi pemboleh ubah yang diuji ialah: 41 dan 39.5 °C untuk suhu inkubasi, pH 6.4 dan 6.5, dan masa inkubasi ialah masing-masing 33.7 dan 48 bagi *Lb. reuteri* dan *Lb. pentosus*. RSM menunjukkan tindak balas ($p \leq 0.05$) yang signifikan sesuai bagi pemboleh ubah yang dikaji dengan pekali penentuan yang tinggi ($R^2$). Nilai yang diramal dan diperhatikan memperlihatkan tiada perbezaan yang signifikan ($p > 0.05$). Kedua-dua masa inkubasi dan pH ($p \leq 0.05$) secara signifikan memberi kesan pada penghasilan EPS dalam kedua-dua strain. Titik optimum secara praktikal telah disahkan. Sampel EPS menunjukkan aktiviti antioksidan poten pada menghapus-sisa DPPH (masing-masing 65.8 dan 68.0% untuk *Lb. pentosus* dan *Lb. reuteri*) dan aktiviti antibiofilem terhadap beberapa patogen (melebihi 50%). Kesimpulannya, strain *Lactobacillus* terasing daripada makanan tradisional Malaysia dalam kajian ini merupakan probiotik yang menggalakkan yang mempunyai potensi untuk menghasilkan EPS dengan atribut fungsian.

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This thesis is 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 by Members of Supervisory Committee**

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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## TABLE OF CONTENTS

## LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| µL | Micro liter |
| µM | microMolar |
| Amp | Ampicillin |
| ANOVA | Analysis of Variance |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| BHI | Brain heart infusion |
| BLAST | Basic local alignment search tool |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| CaCl2 | Calcium Chloride |
| CCD | Central composite design |
| CFU | Colony Forming Unit |
| DNA | Deoxyribonucleic acid |
| DSC | Differential Scanning Calorimetry |
| EPS | Exopolysaccharides |
| Ery | Erythromycin |
| FAO | Food and Agriculture Organization |
| FTF | Fructosyltransferase |
| FT-IR | Fourier Transmission Infrared Spectroscopy |
| G | Gram |
| GIT | Gastrointestinal tract |
| GRAS | Generally recognized as safe |
| GTFs | Glycosyltransferase(s) |

| | |
|---|---|
| H | Hour |
| $H_2SO_4$ | Sulphuric acid |
| HCl | Hydrochloride acid |
| HPLC | High Performance Liquid Chromatography |
| $K_2HPO_4$ | Potassium dihydrogen phosphate |
| kb | Kilo Base |
| kDa | Kilo Dalton |
| $KH_2PO_4$ | Potassium phosphate |
| KOH | Potassium hydroxide |
| L | Liter |
| LAB | Lactic Acid Bacteria |
| MIC | Minimum Inhibitory Concentration |
| Log | Logarithm |
| min | Minutes |
| mL | Milliliter |
| MRS | de Man, Rogosa Sharpe |
| NaCl | Sodium Chloride |
| NaHCO3 | Sodium Bicarbonate |
| NaOH | Sodium Hydroxide |
| nm | Nanometre |
| P | Probability |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| rRNA | Ribosomal RNA |

| | |
|---|---|
| RSM | Response Surface Methodology |
| SDS | Sodium Dodecyl Sulphate |
| SD | Standard deviation |
| SE | Standard error of mean |
| Tm | Melting Temperature |
| UV | Ultraviolet |
| UV | Ultra violet |
| w/v | Weight Per Volume |
| WHO | World Health Organization |
| µg | Microgram |

# CHAPTER 1

# GENERAL INTRODUCTION

In the last decade, polysaccharides have drawn increasing attention for their technological properties and biological activities. Polysaccharides can be derived from plants, animals, algae, and microorganisms (fungi and bacteria). Plant and algal polysaccharides are the most predominant sources (Badel *et al.*, 2011). Microbial polysaccharides have many merits compared to other sources of polysaccharides; these advantages include (1) the production of polysaccharides is simple and less susceptible to the effect of environmental conditions – they are of high-quality with a stable supply and a short production cycle (Wang *et al.*, 2010); (2) diversity – the structure of EPS depends on the species of microorganism and the culture conditions (growth factors and growth conditions) (Harutoshi, 2013); (3) microbial EPS have unique physiochemical features that add functional, technological and economic value when used as food additives or functional foods (Li *et al.*, 2014a); (4) the low-cost possibility when the industrial wastes, such as glycerol, whey, molasses, hydrocarbon residue and $CO_2$, are exploited as carbon substrates (Zannini *et al.*, 2016).

Among the microbial polysaccharides, the exopolysaccharides (EPS) produced by lactic acid bacteria (LAB), in particular, the *Lactobacillus* species, are the most widely used groups of bacteria that have obtained the status of Generally Recognized As Safe (GRAS) (Bourdichon *et al.*, 2012). EPS produced from LAB have received considerable attention due to their importance in the pharmaceutical, medical and food industries. The health benefits of EPS have been claimed to include the following properties: antioxidant activity, immune modulation, aiding in tolerance to acid/bile salts, facilitating adherence and colonization within the host, lowering the blood cholesterol, immunostimulatory activity, and antitumor effects (Ruas-Madiedo *et al.*, 2007; Hidalgo-Cantabrana *et al.*, 2012; Li *et al.*, 2014b). The technological functions of EPS in the food industry include their use as viscosifying agents, stabilizers, emulsifiers, gelling agents, and water-binding agents (Torino *et al.*, 2015).

Based on their monomeric composition, lactic acid bacteria EPS can be classified into homopolysaccharides (HoPS) and heteropolysaccharides (HePS). HoPS contain only one type of monosaccharide repeating unit (e.g., glucans and fructans made of glucose and fructose, respectively), whereas HePS consist of two or more types of monosccharides (De Vuyst and Degeest, 1999). As reported by Abid *et al.* (2017) the chemical characterization is crucial for determining their potential application, sugar composition, chain length, and sugar linkage, as the presence of repeated units affect the biotechnological properties of EPS. The optimum technological and functional proprieties of EPS require sufficient production of these molecules to improve their yields and to obtain a particular functionality. As compared to some other bacteria, *Lactobacillus* is not a very efficient producer of EPS (Leroy and De Vuyst, 2016). Recently, EPS from various *Lactobacillus* species have been studied and characterized (Wang *et al.*, 2010; Li *et al.*, 2014a; Wang *et al.*, 2015; Wang *et al.*, 2016), but to date no work has been carried out on EPS produced by *Lactobacillus* isolated from traditional Malaysian.

In order to achieve maximum EPS production, optimization is required, which includes various production factors starting from the selection of an appropriate strain and optimization of the culture conditions to the protocol used for EPS isolation. Furthermore, there is great interest in identifying new EPS that are suitable for special applications, or that have potential industrial relevance, either by applying different culture conditions or by using novel bacterial strains (Aslim *et al.*, 2005).

To ensure that the EPS produced from *Lactobacillus* act as a natural antioxidant or antibiofilm or displays another bioactive attribute, adequate research on the improvement of the yield and characterization of physiochemical and bioactivity properties is necessary. This study assumes that LAB isolated from traditional Malaysian foods have a potential probiotic characteristics and have the ability to produce EPS with functional attributes under optimal conditions.

Based on the above-mentioned hypothesis the specific objectives of the study in this thesis were:

i. To isolate and identify LAB producing EPS from several traditional Malaysian fermented foods and evaluating their characteristics as potential probiotics.
ii. To optimize incubation temperature, pH, and time for the production of EPS from *Lb. pentosus* and *Lb. reuteri.*
iii. To investigate the production of EPS from *Lb. pentosus* and *Lb. reuteri*, and to study the activity of glucosyltransferase (GTF) enzyme.
iv. To determine the antioxidant activity and antibiofilm activity of EPS from *Lb. pentosus* and *Lb. reuteri.*

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