UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION OF LACTOBACILLUS PLANTARIUM ISOLATED FROM MALAYSIAN FERMENTED FOODS (TEMPEH AND TEMPOYAK)

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By

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

April 2002

Dedicated to:

Dearest Husband, Adam bin Zakaria

And

Charming and Lovely Son, Muhammad Amirul Akhtar bin Adam

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : Professor Gulam Rusul Rahmat Ali, Ph.D.

Faculty : Food Science and Biotechnology

Ten and fifteen strains of *Lactobacillus plantarum* were isolated from tempeh and tempoyak, respectively. The identity of these isolates was confirmed with API 50 CHL kit and the polymerase chain reaction (PCR) using a pair of specific primers. All strains were resistant towards nalidixic acid, kanamycin, gentamycin, streptomycin, bacitracin, moxalactam, norfloxacin and vancomycin. 9, 22,19,22 and 22 strains of *L. plantarum* were resistant against penicillin, cefuroxime, tetracycline, cephalothin and ceftazidime, respectively. Six (60%) and 13 (86.7%) strains of *L. plantarum* isolated from tempeh and tempoyak, respectively harboured small plasmid DNA ranging in size from 4.7 to 1.2 MDa, and 10 (40%) of these strains were harboured two plasmids. These strains could be grouped into 13 groups based on the size and number of plasmid DNA. Different random amplified polymorphic DNA (RAPD)

pattern was obtained when the DNA of these strains were amplified with four 10-mer primers that known as GEN1-50-05, GEN1-50-06, GEN1-50-07 and GEN1-50-08. The strains were separated into 7 clusters in a constructed dendrogram at low similarity index (0.15 to 0.42). The whole cell protein profiles of 14 representatives *L. plantarum* strains were obtained using sodium dodecyl sulphate-polyacylamide gel electrophoresis (SDS-PAGE) and analysed with the Gelcompar Version 4.1 (Applied Maths, Kortrijk, Belgium). These strains have almost the same number of protein bands ranging in size from 34.7 to 100 kilodalton (kDa). The whole cell protein profiles demonstrated intra-species differences and enabled to separate three distinct clusters of *L. plantarum* at 84% similarity. A dendrogram of *L. plantarum* strains was obtained by the unweighted average pair group matrix analysis of correlation values. Twelve strains were grouped into three main clusters and two strains were separated. The whole cell proteins of ten strains were resolved into 13 bands and 12 bands were resolved from two strains, whereas the other two strains were exhibited 11 and 14 bands, respectively. Among the characterization techniques used, RAPD has been concluded as the best, rapid and reproducible tool in characterising the *L. plantarum* strains isolated from tempeh and tempoyak.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN MOLEKULAR *LACTOBACILLUS PLANTARUM* YANG DIPENCILKAN DARIPADA MAKANAN MALAYSIA YANG DIFERMENTASIKAN (TEMPEH DAN TEMPOYAK)

Oleh

ZURAINI MAT ISSA @ ZAKARIA

April 2002

Pengerusi: **Profesor Gulam Rusul Rahmat Ali, Ph.D.**

Fakulti: **Sains Makanan dan Bioteknologi**

Sepuluh dan lima belas pencilan *Lactobacillus plantarum* telah dipencilkan daripada tempeh dan tempoyak. Ciri pencilan-pencilan ini telah dikenalpasti dengan menggunakan alat API 50 CHL dan tindakbalas rantaian *polymerase* (PCR) dengan sepasang primer khas. Semua pencilan didapati rentan terhadap *nalidixic acid*, *kanamycin*, *gentamycin*, *streptomycin*, *bacitracin*, *moxalactam*, *norfloxacin* dan *vancomycin*. Sebanyak 9, 22, 19, 22 dan 22 pencilan, masing-masing telah didapati rentan terhadap *penicillin*, *cefuroxime*, *tetracycline*, *cephalothin* dan *ceftazidime*. Enam (60%) dan 13 (86.7%) pencilan *L. plantarum* daripada tempeh dan tempoyak, mempunyai plasmid DNA kecil yang bersaiz di antara 4.7 hingga 1.2 megadalton (MDa), dan 10 (40%) pencilan ini mengandungi dua plasmid. Berdasarkan saiz plasmid, pencilan in boleh dikumpulkan kepada 13 kumpulan. Corak yang berlainan dihasilkan apabila pencirian menggunakan empat 10-mer primer yang dikenali sebagai

GEN1-50-05, GEN1-50-06, GEN1-50-07 dan GEN1-50-08 digunakan dalam amplifikasi DNA polimofik (RAPD). Pencilan-pencilan ini boleh dikelaskan kepada tujuh kelas dengan persamaan indeks yang rendah (0.15 hingga 0.42) bila dendrogram didirikan berdasarkan corak RAPD yang dihasilkan. Corak protin sel untuk 14 pencilan wakil *L. plantarum* diperolehi dengan teknik *sodium dodecyl sulphate-polyacrylamide* gel elektroforesis (SDS-PAGE) dan dianalisa dengan menggunakan GelCompar Versi 4.1 (Applied Maths, Kortrijk, Belgium). Corak protin sel electroforesis menunjukkan perbezaan intra-spesis dan membolehkan pengasingan pencilan-pencilan *L. plantarum* kepada tiga kelas yang berlainan pada persamaan indeks 84%. Dendrogram untuk pencilan-pencilan ini telah didapati dengan menggunakan analisa peyeimbangan pasangan kumpulan matriks dengan nilai korelasi. Dua belas pencilan telah membentuk tiga pengkelasan utama dan dua pencilan yang berasingan. Pencilan-pencilan *L. plantarum* mempunyai hampir sama banyak bilangan jalur protin yang bersaiz di antara 34.7 dan 100 kilodalton (kDa). Protin sel bagi sepuluh pencilan menghasilkan 13 jalur, dua pencilan menghasilkan 12 jalur dan dua pencilan masing-masing menghasilkan 11 dan 14 jalur. Dengan membandingkan kesemua teknik yang digunakan untuk pencirian, RAPD telah disimpulkan sebagai kaedah terbaik, cepat dan keputusan boleh diperolehi semula, dalam mencirikan pencilan-pencilan *L. plantarum* yang dipencilkan daripada tempeh dan tempoyak.

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"The reward of deeds depends upon the intentions and every person will get the reward according to what he has intended"

Prophet Muhammad SAW (pbuh)

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LIST OF ABBREVIATIONS

APS	Ammonium Persulphate
bp	base pair
CBR	Coomassie Brilliant Blue R-250
ccc dsDNA	covalently closed and circular double-stranded DNA
DNA	Deoxyribonucleic acid
kb	kilo base
µl	micro litre
kDa	Kilo dalton
KOH	Potassium hydroxide
LAB	Lactic acid bacteria
MDa	Mega dalton
nt	Nucleotide
PAG	Polyacrylamide gel
PCR	Polymerase chain reaction
pmol	*pico* mol
RAPD	Random amplified polymorphic DNA
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	*N,N,N',N'* tetramythyelethylenediamine
UPGMA	Unweighted pair group method analysis

CHAPTER 1

INTRODUCTION

Lactic acid bacteria (LAB) have been known to be the most widely used microorganisms in the production of fermented foods (Ray, 1996). Their presence in foods could serve as a preservative or a spoilage role. LAB improve foods by having better shelf life due to their ability to inhibit the growth of spoilage and pathogenic microorganisms by lowering the pH (Franz *et al.*, 1998). Besides providing a good aroma and flavour to the food products, they also provide some health benefits especially in preventing food poisoning due to botulinal toxins and enterotoxins, and some intestinal ailments. LAB produce antimicrobial compounds (Stiles, 1996) such as bacteriocin, organic acids, hydrogen peroxide and enzymes that can be beneficial or deleterious.

Lactobacillus plantarum is one of the *Lactobacillus* species of interest in this study. It is normally used as a starter organism in fermented foods such as sausages, vegetables and cereal products. Although it is a part of the adventitious LAB flora growing in fermented vegetables and meat products, but it is considered as spoilage organism in citrus juice (Kennes *et al.*, 1991; Lindgren *et al.*, 1990; Cselovszky *et al.*, 1992), wine (Liu and Pilone, 1998) and some cheeses (Silla Santos, 1996). Orla-Jensen has classified *L. plantarum* as streptobacteria due to its inability to grow at 45°C. Eventhough there are some strains that can grow at 45°C, their ability to grow at 15°C

serves as confirmation for the allocation of *L. plantarum* to the streptobacteria. *L. plantarum* and *L. pentosus* are highly related (Dellaglo *et al.*, 1975). Work done by Collins *et al.* (1991) confirmed their relatedness due to high 16S rRNA similarity between these two species.

The purpose of identification using the API 50 CHL kit is to rapidly and provides good identification of the isolated strains. Phenotypic characteristics such as carbohydrate fermentation patterns have been a benchmark of bacterial classification and identification as it is the principal method available for the conventional microbiology laboratory. Previous studies have shown that the used of original classical methods based on morphological characteristics could cause unresolved or partly resolved problems, or unidentifiable strains (Shaw and Charmaigne, 1984; Stiles and Holzapfel, 1997). The emergence of molecular genetic technique such as polymerase chain reaction (PCR) method has a great influenced in taxonomic classification of LAB. It has been implemented in the study as a further guide to their identification as this method has been proven as the most accurate and rapid method for strains classification and identification (Charteris *et al.*, 1997; Stiles and Holzapfel., 1997) and allows better understanding of the genetic relationships of bacteria (Stiles and Holzapfel, 1997). Specific primers have been developed to enable the specific identification of LAB strains, even below the species level.

The beneficial roles of LAB in food have been acknowledged. However, there are some reports on the involvement of LAB in human infection (Klein *et al.*, 1995; Aguirre and Collins, 1993). These reports have concluded that some LAB are the potential or opportunistic pathogens (Aguirre and Collins, 1993; Harty *et al.*, 1994). Wide spectrum antimicrobials resistance posses by LAB strains could lead to allergies and/or resistance towards other intestinal microorganisms (Holzapfel *et al.*, 1995). The resistance characteristic is might be due to the presence of certain plasmids, or carried by the chromosomes. Therefore, it is important for the food manufacturers to study and use the LAB strains that are resistant to as few antibiotics as possible.

The emergence of new molecular techniques such as PCR-based DNA fingerprinting methods such as randomly amplified polymorphic DNA (RAPD) analysis, DNA-DNA hybridization, whole-cell protein analysis by SDS-PAGE and probing techniques (Pot *et al.*, 1994b), plays a significant role in characterizing the microorganisms whether they are pathogenic or beneficial microorganisms. However, these techniques have their own strengths and limitations. Therefore, in this study, RAPD and SDS-PAGE analyses have been performed in the characterization of the LAB. Both of the methods are reliable tools in distinguishing among the bacterial strains. However, due to time consumption and difficult to standardized all factors and conditions involved in electrophoretic procedures of the SDS-PAGE, RAPD

have been proven to be one of the most rapid and reliable methods in distinguishing among bacterial species and strains.

Objectives

The objectives of this study were as follows:

1. To confirm the identity of the predominant lactic acid bacteria (LAB) present in tempeh and tempoyak by API 50 CHL kit and PCR method.
2. To determine the relationship between the antibiotic and plasmid profiles of *L. plantarum*.
3. To determine the degree of relatedness of *L. plantarum* strains isolated from tempeh and tempoyak by random amplified polymorphic DNA (RAPD) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

CHAPTER 2

LITERATURE REVIEW

Fermented Foods

Numerous fermented foods are consumed around the world. Each nation has its own types of fermented food, representing the staple diet and the raw ingredients available in that particular place. In Malaysia, these foods are produced on a small scale and are normally consumed as they are or used as condiments, flavouring and seasoning agents in a variety of foods. Examples of Malaysian traditional fermented foods are "budu", "cincaluk", "tapai", tempeh, tempoyak and "belacan". Other fermented foods found worldwide are vegetable and fruit pickles, cheeses, sausages, beverages such as coffee, tea and cocoa, and alcoholic drinks such as wine, beer, ciders and sake. Japan, Europe and other Western have produced these foods commercially in a large industrial scale.

Fermented foods are defined as modified products of raw materials of vegetables and animals origin by the most desirable microorganisms (Ray, 1996). Bacteria, yeast and moulds are commonly used in producing a diverse range of products that differ in flavour, texture and stability from the original raw materials. According to Buckenhuskes (1993) lactic acid bacteria (LAB) are the most common bacteria involved in the food fermentation processes such as in dairy and meat products, fruits and vegetable pickles and beverages.