PRODUCTION OF LACTIC ACID BACTERIUM EXOPOLYSACCHARIDES AND THEIR IMPACT ON THE IMMUNE SYSTEM

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การผลิตเอกโซพอลิแซ็กคาไรด์ของแบคทีเรียกรดแล็กติก และผลของสารต่อระบบภูมิคุ้มกัน

นางจินตนา ต๊ะย่วน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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จินตนา ต๊ะย่วน : การผลิตเอกโซพอลิแซ็กคาไรด์ของแบคทีเรียกรดแล็กติกและผลของสารต่อระบบภูมิคุ้มกัน (PRODUCTION OF LACTIC ACID BACTERIUM EXOPOLYSACCHARIDES AND THEIR IMPACT ON THE IMMUNE SYSTEM) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุรีลักษณ์ รอดทอง, 186 หน้า.

เอกโซพอลิแซ็กคาไรด์เป็นพอลิเมอร์น้ำหนักโมเลกุลสูงที่ประกอบด้วยน้ำตาลมอโนแซ็กคาไรด์ทั้งชนิดเดี่ยวและต่างชนิดกันที่จุลินทรีย์ผลิตขึ้นนอกเซลล์ เอกโซพอลิแซ็กคาไรด์บางชนิดเป็นที่สนใจใช้เป็นสารทำให้เกิดความคงตัวของอาหาร จากการทดสอบความสามารถในการผลิตเอกโซพอลิแซ็กคาไรด์ของแบคทีเรียกรดแล็กติกที่แยกได้จากแหล่งที่พบเชื้อตามธรรมชาติจำนวน 566 ไอโซเลท พบว่าร้อยละ 10 และ 19 สร้างโคโลนีที่มีลักษณะเป็นเมือกขนาดเส้นผ่านศูนย์กลาง 0.2 ถึง 0.9 และ 0.2 ถึง 2.1 เซนติเมตร บนอาหารแข็ง พร้อมทั้งผลิตสารได้ในช่วง 0.1 ถึง 0.6 กรัมต่อลิตร และ 0.1 ถึง 6.9 กรัมต่อลิตร ในอาหารเหลวที่มีน้ำตาลกลูโคสและซูโครสร้อยละ 2 เป็นแหล่งคาร์บอน ตามลำดับ เมื่อศึกษาเพื่อการระบุชนิดของแบคทีเรียที่สร้างสารพอลิเมอร์ได้ในปริมาณสูงจำนวน 8 ไอโซเลท ด้วยลักษณะทางสัณฐานวิทยา สรีรวิทยา และความเหมือนของลำดับนิวคลีโอไทด์ของ 16S rRNA gene พบว่าไอโซเลท C56 มีความเหมือนกับ *Lactobacillus salivarius* ร้อยละ 99 และอีก 7 ไอโซเลท ระบุได้เพียงว่าอยู่ในสกุล *Weissella* (PSMS4-4), *Pediococcus* (P14), *Leuconostoc* (PSMS1-5), *Lactobacillus* (FKU23 และ RMS3-1) และ *Streptococcus* (I5 และ G3) สภาวะที่เหมาะสมต่อการผลิตเอกโซพอลิแซ็กคาไรด์ของแบคทีเรียเหล่านี้แตกต่างกันทั้งชนิดและความเข้มข้นของแหล่งคาร์บอน ค่าความเป็นกรด-ด่างเริ่มต้นของอาหารเลี้ยงเชื้อ และอุณหภูมิที่เลี้ยงเชื้อ น้ำตาลทรายขาวจากอ้อยจัดได้ว่าเป็นแหล่งคาร์บอนที่เหมาะสมที่ให้การผลิตจำเพาะของสารพอลิเมอร์แตกต่างกันตามสายพันธุ์ของแบคทีเรียในช่วง 0.03 ถึง 106.94 พิโกกรัมต่อเซลล์ เอกโซพอลิแซ็กคาไรด์ที่ผลิตโดย *Streptococcus* sp. I5 ประกอบด้วยน้ำตาลแมนโนสและกลูโคสร้อยละ 86.91 และ 13.09 ตามลำดับ เป็นสารที่มีผลต่อระบบภูมิคุ้มกัน เมื่อให้สารปริมาณ 100 ไมโครกรัมแก่หนูปลอดเชื้อจำเพาะทางช่องท้อง สามารถกระตุ้นการเพิ่มจำนวนของเซลล์ลิมโฟซัยท์ของม้ามหนูด้วยดัชนีการกระตุ้นเท่ากับ 13.96 และกระตุ้นการหลั่งสารไซโตไคน์ชนิด IL-10 เท่ากับ 218.49 พิโกกรัมต่อมิลลิลิตร เมื่อผลิตสารพอลิเมอร์ในถังหมักที่มีอาหารเหลว MRS ปริมาตร 5 ลิตร ใช้น้ำตาลทรายขาวจากอ้อย 150 กรัมต่อลิตร เป็นแหล่งคาร์บอนแทนกลูโคส ควบคุมค่าความเป็นกรด-ด่างของอาหารที่ 6.0 และอุณหภูมิ 40 องศาเซลเซียส พบเอกโซพอลิแซ็กคาไรด์ที่ผลิตได้ปริมาณสูงสุด 53.45 กรัมต่อลิตร

(อัตราการเปลี่ยนน้ำตาลเป็นพอลิเมอร์ร้อยละ 42.66) เมื่อเลี้ยงเชื้อเป็นเวลา 30 ชั่วโมง ซึ่งเป็นปริมาณสูงกว่าค่าที่มีการรายงานสำหรับเฮเทอโรพอลิแซ็กคาไรด์ที่ผลิตโดยแบคทีเรียกรดแล็กติกถึง 1.7 เท่า จึงเป็นที่น่าสนใจที่จะศึกษาเอกโซพอลิแซ็กคาไรด์ที่ผลิตได้ในเชิงลึกต่อไป เพื่อใช้ประโยชน์ทางการแพทย์และอุตสาหกรรมอาหาร

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CHINTANA TAYUAN : PRODUCTION OF LACTIC ACID BACTERIUM EXOPOLYSACCHARIDES AND THEIR IMPACT ON THE IMMUNE SYSTEM. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 186 PP.

EXOPOLYSACCHARIDES/LACTIC ACID BACTERIA/IMMUNOSTIMULATORY ACTIVITY/EXOPOLYSACCHARIDE PRODUCTION

Exopolysaccharides (EPS) are high molecular mass polymers consisting of monosaccharide residues, and produced by microorganisms. Some of these polysaccharides are of interest to be used as alternative biothickeners in food. A total of 566 strains of lactic acid bacteria isolated from their natural habitats were tested for EPS production using glucose or sucrose as a sole carbon source. Approximately 10 and 19% of lactic acid bacterial strains were able to produce slimy colonies of 0.2-0.9 and 0.2-2.1 cm diameter, and produced EPS ranging from 0.1-0.6 and 0.1-6.9 g equivalent glucose/l when cultivated in MRS medium containing 2% of glucose and sucrose, respectively. Bacterial identification of eight EPS-producing isolates was performed using morphological and physiological characteristics, and 16S rRNA gene sequences. The isolate C56 had 99% similarity to *Lactobacillus salivarius*. Other EPS-producing isolates belonged to the genera *Weissella* (PSMS4-4), *Pediococcus* (P14), *Leuconostoc* (PSMS1-5), *Lactobacillus* (FKU23 and RMS3-1), and *Streptococcus* (I5 and G3). Types and concentrations of carbon sources, the initial pH of the culture medium, and cultivation temperature influenced the production of EPS by each strain. White sugar from sugar cane gave the highest specific EPS production

ranging from 0.03-106.94 pg/cell. Mannose-rich EPS (86.91% mannose and 13.09% glucose) produced by *Streptococcus* sp. I5 stimulated mouse splenocyte proliferation (stimulation index of 13.96) and anti-inflammatory (interleukin-10) cytokine secretion (218.49 pg/ml) after intraperitoneal inoculation of mice with 100 μg of EPS (primary and booster inoculations). *Streptococcus* sp. I5 was used to produce EPS in 5 l MRS broth containing 150 g/l of white sugar from sugar cane as carbon source. Cultivation temperature and pH of the medium were kept constant at 40°C and 6.0, respectively. The maximum yield of EPS (53.45 g/l; % yield = 42.66) was achieved after 30 h of fermentation. This yield was 1.7 times higher than those reported for heteropolysaccharide production in other lactic acid bacteria. The information obtained from this research is of potential use in the food industry and for medical (anti-inflammatory) applications.

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LIST OF ABBREVIATIONS

BMM	Basal minimum medium
bp	Base pair
BSA	Bovine serum albumin
ccpm	Corrected counts per minute
ConA	Concanavalin A
CFU	Colony forming unit
°C	Degree Celsius
Da	Daltons
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTPs	Deoxynucleoside triphosphate
dTTP	Deoxythymidine triphosphate
EPS	Exopolysaccharide
e.g.	for example
et al.	et alia (and others)
Fru	Fructose
g	Gram
Gal	Galactose
Glc	Glucose
GRAS	Generally recognized as safe
h	Hour

LIST OF ABBREVIATIONS (Continued)

HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
l	Liter
LPS	Lipopolysaccharide
LAB	Lactic acid bacteria
M	Molar
mM	Millimolar
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
MRS	Man Rogosa Sharpe
NAc Glc	*N*-Acetylglucosamine
NAc Gal	*N*-Acetylgalactosamine
ng	Nanogram
nm	Nanometer
OD	Optical density
%	Percentage
pg	Picogram
PCR	Polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

Rha	Rhamnose
rpm	Revolutions per minute
sec	Second
SEM	Scanning electron microscopy
SI	Stimulation index
subsp.	Subspecies
TCA	Trichloroacetic acid
µg	Microgram
µl	Microliter
U.S.A.	United States of America
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZPSs	Zwitterionic polysaccharides

CHAPTER I

INTRODUCTION

1.1 Introduction

Polysaccharides are macromolecules consisting of monosaccharide residues joined by glycosidic linkages. Food industries use polysaccharides as thickeners, emulsifiers, gelling agent and stabilizers (Laws and Marshall, 2001). The polysaccharides are mainly derived from plants (starch, pectin, and guar gum) or seaweeds (carrageenan and alginate) (De Vuyst *et al*., 2001). In the last decade, exopolysaccharides (EPS), long-chain polysaccharides, produced by microorganisms have been of interest to be used as alternative biothickeners (De Vuyst and Degeest, 1999). The microbial polysaccharides have rheological properties that match the industrial demands, and could be produced in large amounts and at high purities. The EPS are found to be secreted mainly by bacteria and microalgae into their surroundings during growth (Sutherland, 1977, quote in Laws *et al*., 2001). Some generally recognized as safe (GRAS) bacteria, particularly lactic acid bacteria (LAB), propionibacteria, and bifidobacteria, are known for their EPS production ability (Gorret *et al*., 2001; De Vuyst and Degeest, 1999; Andaloussi *et al*., 1995). EPS are potentially useful as safe additives to improve texture and viscosity of natural fermented milk products and decrease syneresis (Duboc and Mollet, 2001; Ruas-Madiedo *et al*., 2005). Moreover, it has been suggested that some EPS produced by lactic acid bacteria may be useful for health-promoting properties, which include

immunostimulatory actions (Chabot *et al*., 2001; Hosono *et al*., 1997). Ruas-Madiedo *et al.* (2002) reported that EPS from lactic acid bacteria could influence the immune system by enhancing lymphocyte proliferation, macrophage activation and cytokine production.

A number of papers have reported on the capability of several bacterial strains to produce EPS by using sugars, such as glucose, lactose, and sucrose, as a carbon source (Gamar *et al*., 1997; Smitinont *et al*., 1999; Tallon *et al*., 2003). The bacterial strains inhabiting the cheap and abundant agricultural products (grains and vegetables) would be beneficial to both EPS production and application. Also several factors particularly the composition of the medium (carbon and nitrogen sources) as well as chemical and physical factors have been shown to have their impact on EPS production, concentrations and structures (Tallon *et al*., 2003; De Vuyst *et al*., 1998; Gamar-Nourani *et al*., 1998). This research focused on the production of EPS by some selected strains of lactic acid bacteria and the immunostimulatory activity of the EPS.

1.2 Research objectives

The objectives of this research were:

1) To select EPS-producing lactic acid bacteria and produce the EPS that could have potential for medical and food industry applications

2) To optimize EPS production conditions of the selected strains

3) To investigate the immunostimulatory activity of EPS produced by the selected strains

4) To preliminarily characterize EPS produced by the selected strains

1.3 Research hypothesis

High quantities of EPS could be produced by selected strains of lactic acid bacteria with abundant agricultural products or low cost sugar as a carbon source at optimum conditions. Purified EPS may influence immune system. The EPS produced by selected strains may have potential for medical and food industry applications.

1.4 Scope of the study

EPS-producing lactic acid bacterium strains isolated from agricultural products, animal intestinal tracts and Thai fermented foods were selected for EPS production. Then, some selected isolates or strains, that have not been identified were identified by morphological and biochemical characteristics and/or ribosomal gene characterization. EPS were produced by the selected lactic acid bacterium isolates. EPS production conditions (particularly type and concentration of carbon source, the initial pH of the culture medium, and cultivation temperature) were optimized. Then, the immunostimulatory activity of purified EPS was determined by the measurement of cell proliferation and cytokine production. Sugar compositions of EPS were preliminarily characterized.

1.5 Expected results

From this study, the following results are expected. EPS could be produced by lactic acid bacterium isolates inhabiting agricultural products, animal intestinal tracts and Thai fermented foods, and the EPS could have potential for medical and food industry applications. High quantities of EPS could be produced by selected strains of lactic acid bacteria with an abundant agricultural product as a carbon source at optimum conditions. Data of the immunostimulatory activity of EPS produced by the selected strains will be achieved. Information of basic structures of the EPS, that could be suitable for medical and food industry applications, will be obtained.

CHAPTER II

LITERATURE REVIEWS

2.1 Thickening and gelling agents for food industries

Thickening and gelling agents are invaluable for providing high quality foods with consistent properties, shelf stability and good consumer appeal and acceptance. Modern lifestyles and consumer demands are expected to increase the requirement for these products. Polysaccharides, derived from plants (starch, pectin, and guar gum) or seaweeds (carrageenan and alginate), and animal protein (gelatin) have been used to provide the desired textural properties in foods. The polysaccharides are incorporated into foods essentially to alter the balance between free and bound water and to change rheological properties, mouth feel, and texture of the product. Most recently, microbial polysaccharides are also applied to food industry (Imeson, 1992). Examples of industrially important microbial exopolysaccharides are dextran, xanthan, gellan, pullulan, yeast glucan and bacterial alginate (De Vuyst and Degeest, 1999). The gelling properties of the polymers are of importance in dairy products, fabricated foods, icings and frostings, jams and jellies, and pet foods (Crescenzi, 1995).

2.2 Microorganisms and their exopolysaccharides

Microbial exopolysaccharides are extracellular, long-chain, and high molecular mass polysaccharides (branched, containing α- and β-linkages) which are either associated with the cell surface in the form of a capsule or secreted into the extracellular environment in the form of slime. EPS occur widely among bacteria and microalgae and less among fungi (De Vuyst and Degeest, 1999). The capsular structure may protect the cell against unfavorable environmental conditions, macrophages, and cell wall-degrading enzymes (Looijesteijn *et al*., 2001). Moreover, the presence of EPS induces the interaction between substrates and bacterial cells, which is involved in the development of biofilms (Vuong *et al*., 2004). Dextran from *Leuconostoc mesenteriodes*, xanthan from *Xanthomonas campestris*, and EPS of the gellan family from *Sphingomonas paucimobilis* are examples of industrially important microbial EPS (De Vuyst *et al*., 2001). However, a number of papers have reported EPS production by other bacterial strains, such as sugars with manno-pyranosidic configuration from *Bacillus licheniformis* (Arena *et al.*, 2006), insoluble EPS consisting of rhamnose, mannose, galactose, glucose, mannuronic acid, and glucuronic acid from *Rahnella aquatilis* (Matsuyama *et al*., 1999), sphingan from *Novosphingobium rosa* (Matsuyama *et al*., 2003), O-deacylated EPS from *Erwinia persicina* (Kiessling *et al*., 2005) as well as homopoysaccharides and heteropolysaccharides from lactic acid bacterial strains (De Vuyst and Degeest, 1999). Cynobacteria can also be included among the potential sources of EPS, such as *Spirulina platensis* (Mouhim *et al.*, 1993), *Cyanospira capsulata* (Cesáro *et al*., 1990; Garozzo *et al.*, 1998) and *Cyanothece* strains (De Philippis and Vincenzini, 1998). Some strains of mushrooms (such as *Collybia maculate* and *Phellinus linteus*) and

yeasts (such as *Rhodotorula bacarum* and *Aureobasidium pullulans*) have been reported to produce EPS (Chi and Zhao, 2003; Kim *et al.*, 2003; Lim *et al.*, 2005; Yurlova and De Hoog, 1997).

2.3 Lactic acid bacteria as potential microorganisms for exopolysaccharide production

2.3.1 Lactic acid bacteria

Lactic acid bacteria comprise a diverse group of Gram-positive, non-spore-forming bacteria. They occur as cocci or rods and generally lack catalase, although pseudo-catalase can be found in rare cases. They are chemo-organotrophic and grow only in complex media. Fermentable carbohydrates are used as energy source. Hexoses are degraded mainly to lactic acid (homofermentatives) or to lactic acid and additional products such as acetic acid, ethanol, carbon dioxide (CO_2), formic acid or succinic acid (heterofermentatives). At least 21 genera of lactic acid bacteria, *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosicoccus*, *Dolosigranulum*, *Enterococcus*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* have been recorded (Axelsson, 2004).

2.3.2 Exopolysaccharides produced by lactic acid bacteria

Strains of GRAS food grade microorganisms in particular lactic acid

bacteria, propionibacteria and bifidobacteria, are able to produce EPS (Gorret *et al*., 2001; De Vuyst and Degeest, 1999; Andaloussi *et al*., 1995). EPS-producing lactic acid bacteria are commonly found in dairy products, such as Scandinavian ropy fermented milk products, various yogurts, fermented milks, milky, and sugary kefir grains, cheese, fermented meat, and vegetables (De Vuyst and Degeest, 1999). EPS produced by the bacteria could be either homopolysaccharides or heteropolysaccharides in nature (De Vuyst and Degeest, 1999).

2.3.2.1 Homopolysaccharides

Homopolysaccharides consist of repeating units of only one type of monosaccharide. The monosaccharides are joined by either a single linkage type or by a combination of a limited number of linkage types. The homopolysaccharides in current reports consisting of four subgroups: α-D-glucans, β-D-glucans, fructans, and polygalactans (Table 2.1). The production of homopolysaccharides requires the presence of sucrose. The assembly of monosaccharide units takes place outside the bacterial cell (Ruas-Madiedo *et al*., 2002).

2.3.2.2 Heteropolysaccharides

Heteropolysaccharides are constructed from multiple units of an oligosaccharide. The oligosaccharide can contain three and seven residues. The heteropolysaccharides are formed by repeating units that most often contain a combination of D-glucose, D-galactose, and L-rhamnose and in a few cases, *N*-acetylglucosamine, *N*-acetylgalactosamine or glucoronic acid (De vuyst *et al*., 2001) (Table 2.2). Sometimes, non-carbohydrate substituents, such as phosphate, acetyl, and

glycerol are present (Robijn *et al*., 1995; 1996). Precursor-repeating units for heteropolysaccharide synthesis are formed intracellularly and isoprenoid glycosyl carried lipids are involved in the process (Cerning, 1990).

Table 2.1 Homopolysaccharides produced by lactic acid bacteria.

EPS	Bacterial strains	Linkages[a]
α-D-Glucans		
Dextran	*Leuconostoc mesenteroides* subsp. *mesenteroides* *Leuconostoc mesenteroides* subsp. *dextranicum*	α-D-Glc*p*[b] (1-6)
Mutan	*Streptococcus mutans* *Streptococcus sobrinus*	α-D-Glc*p*[b] (1-3)
Alternan	*Leuconostoc mesenteroides*	α-D-Glc*p* (1-3)/(1-6)
β-D-Glucans	*Pediococcus* spp. *Streptococcus* spp.	β-D-Glc*p* (1-3)
Fructans		
Levans	*Streptococcus salivarius*	β-D-Fru*p* (2-6)
Inulin-like	*Streptococcus mutans*	β-D-Fru*p* (2-1)
Polygalactan	*Lactobacillus lactis* subsp. *lactis* H414	β-D-Gal*p*/β-D-Gal*p*[c]

[a] Glc, glucose; Gal, galactose; Fru, fructose.

[b] At least 50% of the respective linkage.

[c] Homopolysaccharide containing a pentameric repeating unit of galactose.

Source: Ruas-Madiedo *et al*. (2002)

Table 2.2 Classification of heteropolysaccharides produced by lactic acid bacteria according to the structure of repeating units and their monosaccharide composition.

Structure of repeating unit	Glc	NAc Glc	Gal	NAc Gal	Rha	Fru
Trisaccharide						
Lactobacillus spp. G-77	3					
Tetrasaccharide						
Streptococcus thermophilus SFi39	2		2			
Streptococcus thermophilus SY89 and SY102			2		2	
Lactobacillus paracasei 34-1			3	1		
Streptococcus thermophilus CNCMI 733	1	2	1			
Streptococcus thermophilus Sfi6	1	2	1			
Streptococcus thermophilus IMDO 01, IMDO 02, IMDO 03, NCFB 859, and 21	1	2	1			
Streptococcus thermophilus SFi20	1	2	1			
Lactococcus lactis subsp. *cremoris* H414			5			
Lactobacillus rhamnosus C83	2		3			
Lactococcus lactis subsp. *cremoris* NIZO B891	3		2			
Lactobacillus delbrueckii subsp. *bulgaricus* 291	3		2			
Lactococcus lactis subsp. *cremoris* SBT 0495	2		2		1	

Table 2.2 (Continued) Classification of heteropolysaccharides produced by lactic acid bacteria according to the structure of repeating units and their monosaccharide composition.

Structure of repeating unit	Glc	NAc Glc	Gal	NAc Gal	Rha	Fru
Lactococcus lactis subsp. *cremoris* NIZO B40	2		2		1	
Lactococcus lactis subsp. *cremoris* AHR 53	2		2		1	
Lactobacillus sakei 0-1	2				3	
Lactobacillus acidophilus LMG 9433	3	1	1			
Hexasaccharide						
Lactobacillus helveticus NCDO 766	4		2			
Lactobacillus helveticus TN-4	3		3			
Lactobacillus helveticus 2091	2		4			
Lactobacillus helveticus Lh59	3		3			
Lactobacillus helveticus K16	4		2			
Streptococcus macedonicus Sc136	3	1	2			
Streptococcus thermophilus SFi12	1		3		2	
Streptococcus thermophilus S3			2		1	
Heptasaccharide						
Lactobacillus helveticus Lb161	5		2			
Streptococcus thermophilus OR901			5		2	
Streptococcus thermophilus Rs and Sts			5		2	
Lactobacillus delbrueckii subsp. *bulgaricus* rr	1		5		1	

Table 2.2 (Continued) Classification of heteropolysaccharides produced by lactic acid bacteria according to the structure of repeating units and their monosaccharide composition.

Structure of repeating unit	Glc	NAc Glc	Gal	NAc Gal	Rha	Fru
Lactococcus lactis subsp. *cremoris* NIZO B39	2		3		2	
Lactobacillus delbrueckii subsp. *bulgaricus* LY03, 24, and 25	1		5		1	
Streptococcus thermophilus EU20	2		3		2	
Lactobacillus helveticus TY1-2	3	1	3			
Streptococcus thermophilus NCFB 2393	1		3	1	2	
Octasaccharide						
Streptococcus thermophilus MR-1C			5		2	1

Source: De vuyst *et al*. (2001)

2.3.3 Biosynthesis of lactic acid bacterial exopolysaccharides

2.3.3.1 Homopolysaccharide biosynthesis

For a small number of homopolysaccharides, including dextrans, mutans, alternans, and levans, the biosynthesis process is extracellular and requires sucrose as specific substrate. A highly specific glycosyltransferase enzyme (e.g. dextransucrase or levansucrase for dextran and levan biosyntheses, respectively) is involved in the polymerization reaction. The energy needed for polymerization, comes from the hydrolysis of sucrose (Figure 2.1) (Monsan *et al*., 2001).

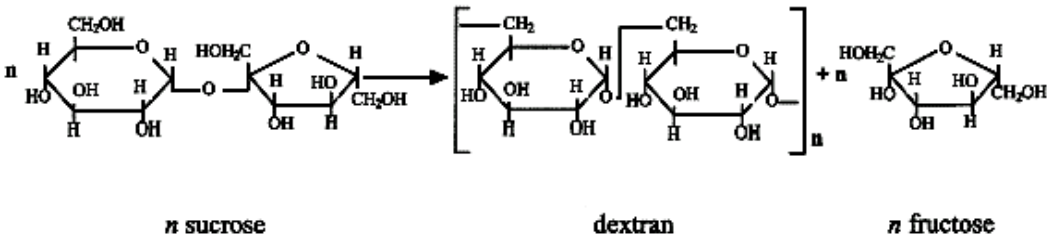

Figure 2.1 Biosynthesis of the homopolysaccharide dextran.

Source: De Vuyst and Degeest (1999)

2.3.3.2 Heteropolysaccharide biosynthesis

The polymerization of repeating unit precursors are formed in cytoplasm. Several enzymes and/or proteins are involved in the biosynthesis and secretion of heterotype EPS which are not necessarily unique to EPS formation (Figure 2.2). The sugar nucleotide, derived from sugar-1-phosphate, plays an essential role in heteropolysaccharide biosynthesis due to their roles in sugar activation, which is necessary for monosaccharide polymerization, as well as sugar interconversions (epimerization, decarboxylation, and dehydrogenation, etc.). Together with the sugar activation and modification enzymes, they play a crucial role in the formation of the building blocks and, thus, the final EPS composition. Glucose or the glucose moiety from lactose hydrolysis seems to be the source of sugar for heteropolysaccharide biosynthesis in lactic acid bacteria. Glucose-1-phosphate derived from glucose-6-phosphate, which is in turn an important metabolic intermediate from sugar break down, is most probably a precursor for polysaccharide formation. Phosphoglucomutase could be a key enzyme linking the lactose degradation pathway to EPS biosynthesis (Figure 2.2). The EPS monomeric composition may not only be dependent on the sugar nucleotide level inside the cell but probably also on the

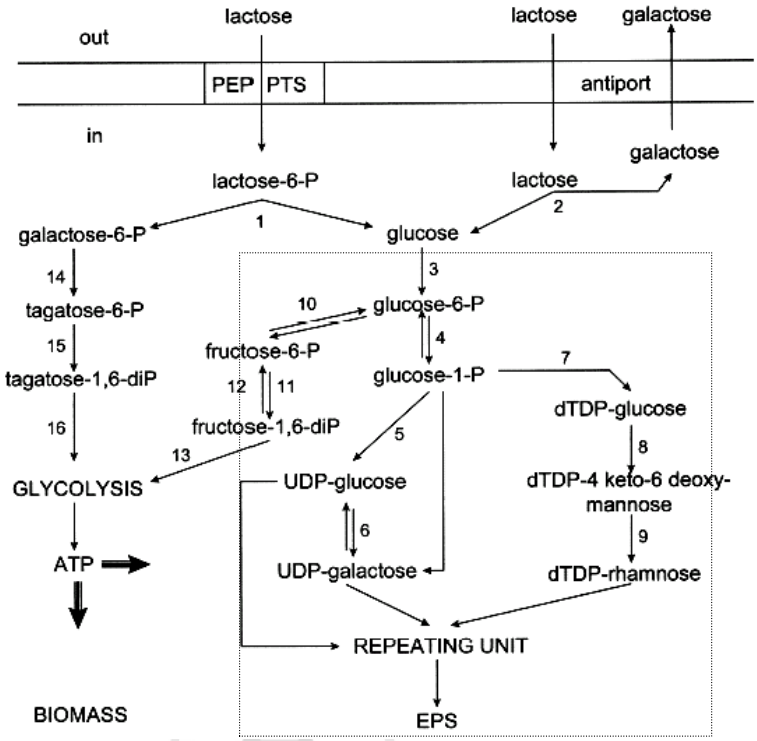

Figure 2.2 Schematic representation of pathways involved in lactose catabolism (left and upper right) and exopolysaccharide biosynthesis (in box) in lactose-fermenting *Lactococcus lactis* (lactose transport via a lactose-specific phosphotransferase primary transport system) and galactose-negative *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (lactose transport via a lactose/galactose antiport secondary transport system) strains. The numbers refer to the enzymes involved: 1, phospho-L-galactosidase; 2, L-galactosidase; 3, glucokinase; 4, phosphoglucomutase; 5, UDP-glucose pyrophosphorylase; 6, UDP-galactose-4-epimerase; 7, dTDP-glucose pyrophosphorylase; 8, dehydratase; 9, epimerase reductase; 10, phosphoglucose isomerase; 11, 6-phosphofructokinase; 12, fructose-1,6-bisphosphatase; 13, fructose-1,6-diphosphate aldolase; 14, galactose-6-phosphate isomerase; 15, tagatose-6-phosphate kinase; 16, tagatose-1,6-diphosphate aldolase.
Source: De Vuyst and Degeest (1999)

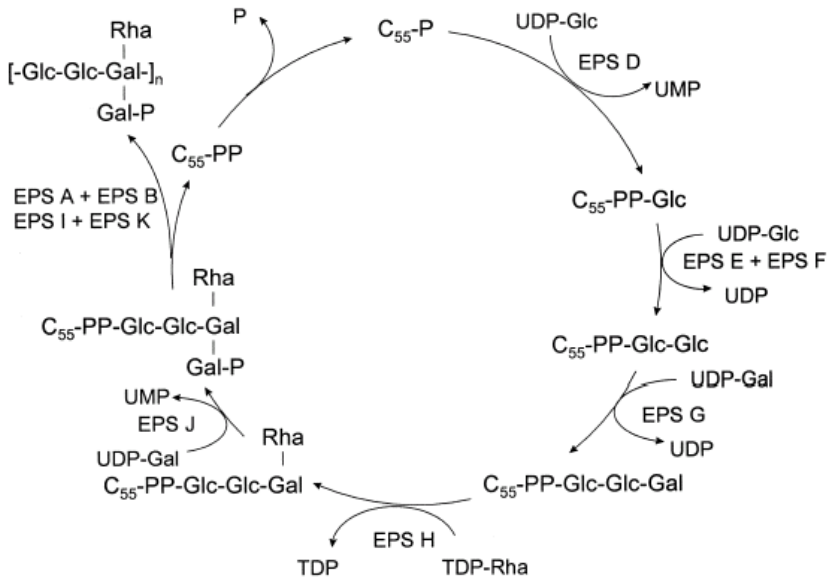

Figure 2.3 Model for EPS biosynthesis in *Lactococcus lactis* NIZO B40. C55-P, lipid carrier; Glc, glucose; Gal, galactose; Rha, rhamnose; UDP-Glc, UDP-Gal and TDP-Rha are nucleotide sugars.
Source: De Vuyst and Degeest (1999)

assembly of the EPS repeating unit. Polymerization of some hundreds to several thousands of the repeating units takes place through sequential addition of sugar residues by specific glycosyl transferases from nucleotide sugars to a growing repeating unit that is coupled to the undecaprenylphosphate carrier yielding the final EPS (Figure 2.3). This isoprenoid glycosyl lipid carrier located in the cell membrane would act as the recipient molecule for the first sugar residue. As a last step of EPS biosynthesis, the synthesized polysaccharide is translocated across the membrane to the exterior of the cell, and is excreted in the environment (slime EPS) or remains attached to the cell (capsular EPS). Both polymerization and transport may affect the amount or the sugar composition of the EPS. The biosynthesis of polysaccharides is

an energy-demanding process. First, one ATP is required for the conversion of each hexose substrate molecule to a hexose phosphate. A further high-energy phosphate bond is needed for the synthesis of each sugar nucleotide, and one ATP is required for the phosphorylation of the isoprenoid C55 lipid carrier. Finally, polymerization and transport need much energy (De Vuyst and Degeest, 1999; De vuyst *et al*., 2001; Law *et al*., 2001; Ruas-Madiedo *et al.*, 2002).

2.3.4 Production of exopolysaccharides for food industry applications

Industrially important microbial EPS are dextran from *Leuconostoc mesenteriodes*, xanthan from *Xanthomonas campestris*, and EPS of the gellan family from *Sphingomonas paucimobilis* (De Vuyst *et al*., 2001). However, EPS synthesized by lactic acid bacteria (LAB) play a major role in the manufacturing of fermented dairy products such as yoghurt, drinking yoghurt, cheese, fermented cream, milk based desserts. The polymers may act both as texturizers and stabilizers (Duboc and Mollet, 2001). The amount and the composition of the EPS produced by lactic acid bacteria are strongly influenced by cultures and fermentation conditions, and are growth associated. The production of EPS depends on temperature and pH of medium as well as the composition of the medium in terms of carbon and nitrogen sources, and mineral and vitamin contents (Gancel and Novel, 1994; De Vuyst *et al.*, 1998; Gamar-Nourani *et al*., 1998; Gorret *et al*., 2001). The carbohydrate composition of EPS is unique to different strains of bacteria, and may vary depending on growth conditions; however, glucose and galactose in particular are frequently detected in the EPS composition of many bacterial species (Cerning, 1990; De Vuyst and Degeest, 1999). Some main factors affecting the EPS production are as follows:

2.3.4.1 Chemical factors

Compositions of the medium (carbon and nitrogen sources) are known to have impact on EPS production (Cerning *et al*., 1994; Degeest and De Vuyst 1999; Tallon *et al*., 2003). A number papers reported on the capability of several bacterial strains to produce EPS by using sugars, such as glucose, lactose, and sucrose, as carbon sources (Gamar *et al*., 1997; Smitinont *et al*., 1999; Tallon *et al*., 2003; Tieking *et al*., 2003). However, some strains can produce EPS in oat-based non-dairy medium (Martensson *et al*., 2000; 2003).

2.3.4.2 Physical factors

The effects of temperature and pH on EPS production are highly variable, and depend on the strain used and the experimental conditions. Cerning *et al*. (1992) observed that the optimal EPS production takes place at temperatures below the optimal growth temperature. While others showed EPS production to be favored at much higher temperatures (De Vuyst *et al*., 1998). The optimum pH for EPS production generally ranges between 5 and 7. The optimal conditions for EPS production and growth of *Streptococcus thermophilus* LY03 were at 42°C and pH 6.2 (De Vuyst *et al*., 1998). Mozzi *et al.* (1996) demonstrated that the regulation of pH during fermentation was more suitable than pH adjustment before the fermentation and EPS production was higher at pH 6, whereas the specific production yield ($Y_{EPS/X}$) was better at pH 4. Gassem *et al.* (1997) also found that optimal pH in EPS production by continuous fermentation at pH 6.5 compared to results obtained at pH 5.2. Van den Berg *et al*. (1995) established that EPS production by *Lactobacillus sake* varied widely with small pH variations.

The production of heteropolysaccharides by lactic acid bacterial strains under optimal cultivation conditions varies from 0.150 to 0.600 g/l, which is higher than non-optimized condition production (0.045 to 0.350 g/l). EPS produced by lactic acid bacteria range from 60 to 150 mg/l for *Lactobacillus bulgaricus* (Cerning *et al.*, 1986; Garcia-Garibay and Marshall, 1991) and from 80 to 600 mg/l for *Lactococcus lactis* subsp. *cremoris* (Cerning *et al*., 1992). EPS production of 1.5, 1.4, and 1.3 g/l were obtained with *Streptococcus thermophilus* LY03, *Lactobacillus sakei* 0-1 and *Lactobacillus rhamnosus* 9595M, respectively (De Vuyst *et al*., 2001). *Lacobacillus sakei* strain 0±1 was isolated from a Belgian salami was able to produce 1.4 g/l of EPS (Van den Berg *et al*., 1995). The concentration of EPS of 2.13 g/l was obtained from *Lactobacillus delbrueckii* subsp. *bulgaricus* RR (Gassem, 1997). EPS production of 0.12-4.10 g/l by *Pediococcus parvulus* 2.6 was reported by Velasco (2006). Vijayendra and Babu (2008) showed that fermentation using EPS medium (pH 6.7), containing sucrose at 5% (w⁄v) and 5% (v⁄v) inoculum, at 25°C resulted in maximum production of HePS (18.38 g/l) by *Leuconostoc* sp. CFR-2181 in 4 h of fermentation. However, these quantities are limited compared to the production of polymers from other bacteria, for example, xanthan (10-25 g/l) produced by *Xanthomonas campestris*, which is one of the major commercial polymers (Becker *et al*., 1998, quote in Ruas-Madiedo *et al*., 2002).

2.3.5 Genes encoding exopolysaccharide synthesis in lactic acid bacteria

In most strains of *Lactococcus lactis*, genes encoding EPS synthesis may be located on a plasmid (Van Kranenburg *et al*., 1997). In contrast, all *eps* gene

clusters of all thermophilic lactic acid bacterium strains, such as *Streptococcus thermophilus*, were located on the chromosome (Broadbent *et al.*, 2003).

Van Kranenburg *et al*. (1997) determined that the essential information needed for the biosynthesis of EPS by *Lactococcus lactis* NIZO B40 was encoded in a single 12-kb gene cluster located on a single 40-kb plasmid (*epsRXABCDEFGHIJKL*), driven by a promoter upstream of *epsR* (Figure 2.4). The predicted gene products of 11 of the 14 genes were homologous in sequence to gene products involved in EPS, capsular polysaccharide, lipopolysaccharide (LPS) or teichoic acid biosynthesis of other bacteria, and putative functions were assigned to these genes.

Partial DNA sequence data of the *eps* gene clusters from the *Lactococcus lactis* NIZO B891 and *Lactococcus lactis* NIZO B35 strains revealed that the genetic organization and sequence of the first five genes are highly conserved. The organization of the other known *eps* genes of *Lactococcus lactis* NIZO B891 appears to be conserved relative to *Lactococcus lactis* NIZO B40 (Van Kranenburg *et al.*, 1999).

The *eps* locus of *Streptococcus thermophilus* Sfi6 comprises 13 genes in the 14.5-kb *eps* gene cluster *epsABCDEFGHIJKLM*. The related *cps* locus of *Streptococcus thermophilus* NCFB 2393, the complete *cps* gene cluster *cpsABCDEFGHIJKL* of about 11.2 kb, have six genes from the *eps* locus *epsABCDEF* of *Streptococcus thermophilus* MR-1C. Recently, a 32.5-kb variable *eps* locus was found in *Streptococcus thermophilus* CNRZ 368; 17 of 25 open reading frames are related to proteins involved in the synthesis of polysaccharides in various

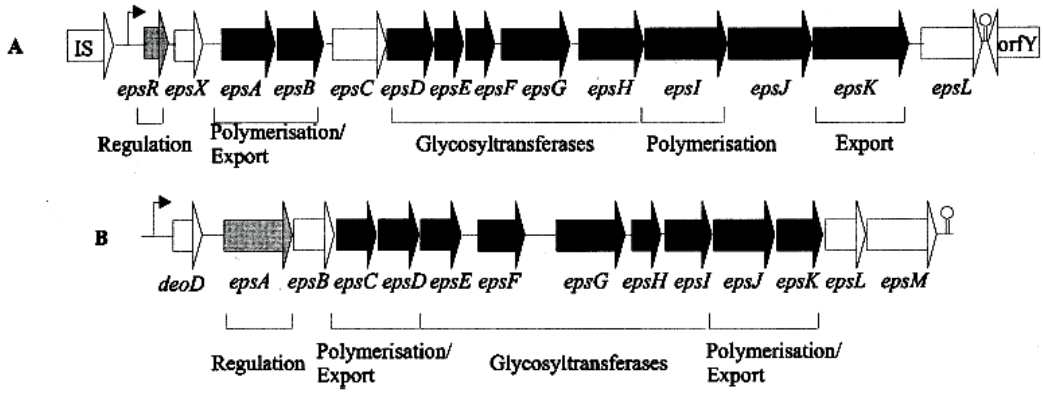

Figure 2.4 Organisation of the *eps* gene clusters involved in the EPS biosynthesis of (A) *Lactococcus lactis* NIZO B40 (plasmid-localized) and (B) *Streptococcus thermophilus* Sfi6 (chromosomally encoded). The (possible) functions of the different gene products are indicated.
Source: De Vuyst and Degeest (1999)

bacteria. Several other genes of the *eps* locus of *Streptococcus thermophilus* CNRZ368 contain frameshifts or stop codons and, therefore, are considered as pseudo-genes.

The genes *epsA*, *epsB*, *epsC*, and *epsF*, that have highly variable divergence with related sequences, are mosaic genes. The two distal regions *epsAB* and *pgm*, and a small central region that contains orf14.9, are constant and present in most *Streptococcus thermophilus* strains examined. The other regions are variable (De Vuyst *et al*., 2001). The functional sequences of these clusters in Gram-positive bacteria, which synthesize polysaccharides at the cell surface, appear to follow a

similar trend of regulation, chain-length determination, biosynthesis of the repeating unit, polymerization and export (Jolly and Stingele, 2001).

2.3.6 Benefits of exopolysaccharides to human health

Lactic acid bacterial EPS have potential for application in food industries. EPS might contribute to human health as prebiotics (Bello *et al*., 2001). Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus, attempt, to improve host health. (Gibson and Roberfroid, 1995). The yoghurt starter *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL 1073R-1, which produces an EPS, has been reported to exert a host-mediated antitumor activity (Kitazawa *et al*., 1998). Moreover, EPS produced by *Lactococcus lactis* subsp. *cremoris* SBT 0495 has been claimed to lower blood cholesterol (Nakajima *et al*., 1992). Kitazawa *et al*. (1993) showed a significant increase of the B cell dependent mitogenic activity induced by the slime material products from *Lactococcus lactis* subsp. *cremoris* KVS 20. Also, Nakajima *et al*. (1995) found that EPS of *Lactococcus lactis* subsp. *cremoris* SBT 0495 administered intraperitoneally enhanced the production of specific antibodies in mice indicating that this EPS may act as adjuvant. Chabot *et al*. (2001) has shown the possibility of enhancing the immune system through EPS from lactic acid bacteria. Extracellular polysaccharides produced by *Lactococcus lactis* subsp. *cremoris*, enhance macrophage activation and cytokine induction (interferon-γ and interleukin-1α) production (Kitazawa *et al*., 1996). For oral administration, the exopolysaccharide produced by *Lactobacillus kefiranofaciens* induced a gut mucosal response and it was able to up and down

regulate it for protective immunity, maintaining intestinal homeostasis, enhancing the IgA production at both the small and large intestine level and influencing the systemic immunity through the cytokines released to the circulating blood (Vinderola *et al*., 2006). Although no evidence of lactic acid bacterial EPS on human immunostimulatory activity has been reported yet, cellular immune responses to the bacterial polysaccharide of *Bacteriodes fragilis* have been investigated. Capsular polysaccharides possess a switterionic charge motif. This motif is critical to modulation of abscess formation. The biological basis for this activity depends on the interaction of these polysaccharides with T cells and subsequent release of cytokines that confer protection against abscess formation (Tzianabos *et al*., 1992; Tzianabos, 2000; Stingele *et al*., 2004). Some evidences of immunostimulatory activity have been reported. However, this knowledge is still importance for functional effect when EPS incorporated into food matrix.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, reagents, and media

Reagents and media used in each step were as follows:

3.1.1 Lactic acid bacterial isolation and culture maintenance

De Man, Rogosa Sharpe (MRS) medium (Appendix A3.1) used for culturing lactic acid bacteria, was obtained from Himedia (Himedia laboratories, India). Other microbiological medium components, proteose peptone, yeast extract, and beef extract, were purchased from Himedia (Himedia laboratories, India). Tri-Ammonium citrate, di-potassium hydrogen orthophosphate, sodium acetate, and manganese sulphate monohydrate were obtained from Carlo Erba (Carlo Erba Reagenti, Italy). Skim milk solution (10%) was used for the maintenance of lactic acid bacteria at -20°C.

3.1.2 Screening of EPS-producing lactic acid bacteria and exopolysaccharide production

Various carbon sources, white sugar from sugar cane, rice flour, molasses and tapioca starch added to MRS medium to replace glucose contained in the MRS broth formulation, were purchased from local supermarket. Analytical grade sucrose was product of Carlo Erba (Carlo Erba Reagenti, Italy).

3.1.3 Exopolysaccharide extraction, purification, and characterization

Chemicals and reagents used for extraction, purification, and characterization of exopolysaccharides were analytical grade. Absolute ethanol, Proteinase K, Pronase E, D-glucose, D-galactose, D-mannose, L-rhamnose, sucrose, *N*-acetylgalactosamine *N*-aetylglucosamine and sodium hydroxide pellets were purchased from Merck (Merck KGaA, Germany). L(+)-Lactic acid, magnesium sulfate, 5-dinitrosalicylic acid, trichloroacetic acid, and trifluoroaetic acid were products of Fluka and Supelco (Sigma-Aldrich Chemical Company, U.S.A.). Quick Start™ Bradford Protein Assay was a product of Bio-Rad (Bio-Rad Laboratories, Inc., U.S.A.). Snakeskin™ pleated dialysis tube was product of Pierce (Thermo Fisher Scientific Inc., U.S.A.).

3.1.4 Immunostimulatory activity test

Roswell Park Memorial Institute 1640 culture (RPMI 1640) medium was product of GIBCO® (Invitrogen Corporation, U.S.A.). Concanvalin A (ConA), phosphate buffer saline (PBS) and 2-mercaptoethanol were purchased from Sigma (Sigma-Aldrich Chemical Company, U.S.A.). [methyl-^{3}H]Thymidine was a product of Amersham (GE Healthcare U.K. ltd., U.K.). Bioplex cytokine kits used for cytokine assay were purchased from Bio-Rad (Bio-Rad Laboratories, Inc., U.S.A.)

3.1.5 Lactic acid bacterial identification

3.1.5.1 Morphological and physiological characterization

Media and reagents used for biochemical characterization of lactic

acid bacteria were API 50 CHL medium and API 50 CH strips (Bio-Mérieux, bioMérieux, Inc., France).

3.1.5.2 16S Ribosomal RNA gene sequencing

Reagents used for genomic DNA extraction were recommended from the Wizard Genomic DNA Purified kit (Promega, Promega Corporation, U.S.A). Isopropyl alcohol (Merck) was used to precipitate genomic DNA; ethanol (Merck) to wash genomic DNA pellet. Low-melting point agarose (Promega) was used to prepare gel for electrophoresis. Reagents used for polymerase chain reaction (PCR) amplification were 10× PCR buffer, dNTPs (dATP, dCTP, dGTP, and dTTP) and *Taq* DNA polymerase (Invitrogen, Invitrogen life technologies, U.S.A.). Oligonucleotide primers were ordered from the Science Pacific Company, Ltd. (Thailand). The Witzard® SV Gel and PCR Clean-up System (Promega) were used for PCR purification. pGEM-T Easy Vector (Promega) was used for cloning 16S rRNA gene fragment. The BigDye Terminator Ready Reaction kit (Perkin Elmer, Applied Biosystems Inc., U.S.A.) was used for nucleotide sequencing.

3.2 Instrumentations

All instruments required for screening of EPS-producing lactic acid bacteria, optimization of some EPS production conditions, production of EPS using optimum conditions, EPS purification, preliminary characterization of the EPS, and identification of selected isolates of EPS-producing bacteria, were located at the Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand. For immunostimulatory activity test, the

experiment was performed at the Department of Immunology and Microbiology, University of Otago, Dunedin, New Zealand. Instruments required for DNA sequencing were located at the Biotechnology and Development Office, Department of Agriculture, Pathumthani, Thailand.

3.3 Selection of exopolysaccharide-producing lactic acid bacteria

3.3.1 Microorganisms

Lactic acid bacterial isolates were obtained from stock cultures of the Microbial Culture Collection Laboratory, Suranaree University of Technology, and isolated from their natural habitats particularly agricultural products and traditional Thai fermented foods.

3.3.2 Isolation and primary screening for exopolysaccharide-producing bacteria using an agar medium

To isolate lactic acid bacteria from their natural habitats, 25 g of each sample were weighed into 225 ml of sterile normal saline and appropriate dilutions were plated onto MRS agar (De Man, 1960) containing 2% (w/v) of glucose or sucrose (Appendix A3.1). The plates were then incubated under anaerobic conditions in anaerobic chamber (Shel LAB, Sheldon Manufacturing, Inc, U.S.A.) with a gas mixture of CO_2:H_2:N_2 (5:5:90%) at 30°C for 48 h. The strains produced slimy colonies of 0.2 cm in diameter on agar medium containing 2% of glucose and sucrose were selected for secondary screening in section 3.3.3. The isolates were maintained in MRS broth and kept at -20°C with the addition of skim milk to 5% (v/v) final concentration. For cell propagation procedure, the stock cultures were taken from

-20°C freezer, thawed at room temperature. Two hundred µl of each culture were used to inoculate 2 ml of MRS broth. After incubation at 30°C for 18 h under anaerobic conditions, the culture was streaked onto MRS agar, and incubated under the same conditions for 48 h. Then, a single colony was subcultured for further study.

3.3.3 Secondary screening for exopolysaccharide-producing bacteria using a liquid medium

The strains that produced slimy colonies on agar plate containing 2% of glucose or sucrose in the section 3.3.2 were selected. EPS production ability was confirmed in liquid MRS medium containing 2% of either glucose or sucrose (Smitinont *et al*., 1999).

3.3.3.1 Cultivation of lactic acid bacteria for exopolysaccharide production

One loopful of selected isolate grown for 48 h at 30°C on MRS agar was inoculated into 5 ml of MRS broth. The inoculated MRS broth was incubated at 30°C for 18 h. The bacterial growth was monitored spectrophotometically at 600 nm (OD_{600}). Then 2% (v/v) of culture (approximately 10^6 CFU/ml) were inoculated into 15 ml test tube containing 10 ml-working volume of the medium, then incubated at 30°C under anaerobic condition for 48 h.

3.3.3.2 Exopolysaccharide isolation and quantification

After incubation (section 3.3.3.1), cultures were centrifuged at 10,000 rpm for 10 min at 4°C. Polysaccharides in the supernatant were precipitated with 3 volumes of chilled 80% (v/v) ethanol, and kept overnight at 4°C according to

Duenas *et al*. (2003). The precipitate was collected by centrifugation at 4,500 rpm for 20 min at 4°C (Labofuge 400R, Heraeus, Germany), then dissolved in distilled water and freeze dried (Heto drywinner, Heto, Denmark). EPS quantity was determined by measuring the dry weight or total carbohydrate content of the precipitates. The total sugar content was determined by the phenol-sulfuric acid method (Dubois *et al*., 1956), using glucose as a standard. The EPS concentration was thus expressed in g equivalent glucose/l.

3.4 Optimization of some exopolysaccharide production conditions

Some optimal conditions for cultivation of the selected isolates were investigated to obtain the efficient EPS production. Composition of MRS medium used (types and concentrations of carbon source) in section 3.3.2 were modified. EPS production conditions (particularly the initial pH of the culture medium and cultivation temperature) were optimized.

3.4.1 Types and concentrations of carbon sources

To obtain the suitable carbon source based on a cheap and abundant raw material, various carbon sources (soluble starch, tapioca starch, rice flour, molasses, white sugar from sugar cane, and analytical grade sucrose) were used to replace glucose in MRS medium (Appendix A3.1). Also concentrations of 20, 30, 35, 40, 45, and 50 g/l of the suitable carbon source selected were applied to achieve the optimal concentration. Bacterial growth was measured by monitoring viable cell counts or cell dry weight. Viable cell counts (CFU/ml) were estimated by plating serial dilutions of bacterial suspension on MRS agar. Plates were incubated anaerobically at 30°C for 48 h in anaerobic chamber (Shel LAB, Sheldon Manufacturing, Inc, U.S.A.). For the

determination of cell dry weight from broth cultures, cells were harvested by centrifugation, cell pellet was washed twice with distilled water. The washed cells were dried at 105°C for 24 h and then weighed after drying to constant weight in desiccators. The EPS production was determined as described in section 3.3.3.2.

3.4.2 Initial pH of the culture medium for exopolysaccharide production

The initial pH of the optimized medium for the EPS production was studied. The medium was adjusted to pH 4.5, 5.0, 6.0, 7.0, and 8.0 using 1 N HCl and 1 N NaOH, then used for culturing the selected EPS-producing isolate(s). Bacterial cell growth was determined by cell dry weight as described in section 3.4.1. EPS concentration was monitored as described in section 3.3.3.2.

3.4.3 Cultivation temperature

The suitable temperatures for EPS production were investigated. The selected EPS-producing isolate was cultivated in the suitable medium resulted from sections 3.4.1-3.4.2 at various incubation temperatures; 30, 35, 37, 40, and 45°C. Bacterial growth and EPS production were monitored as described in section 3.4.2.

3.5 Production of exopolysaccharides using optimum conditions

3.5.1 EPS production in 500 ml Duran bottle

The selected isolate(s) was cultivated using 500 ml Duran bottle containing 350 ml of the suitable medium and optimal conditions obtained from section 3.4 for 48 h. Selected isolate was grown for 48 h at 30°C on MRS agar, then one loopful of the culture was inoculated into 10 ml of MRS broth, and incubated at 30°C under

anaerobic conditions for 18 h. An inoculum containing approximately 10^6 CFU/ml was inoculated into the suitable medium at 2% (v/v) inoculum size. Changes of pH, EPS concentration, carbon source concentration, and bacterial growth were measured at 0, 3, 6, 9, 15, 18, 24, 30, 36, and 48 h of cultivation.

3.5.2 EPS production in controlled fermenter containing 5 l medium

Five liters of optimized MRS medium was sterilized in an autoclave at 121°C for 30 min. The suitable carbon source from section 3.5.1 was separately sterilized using membrane filtration technique. Fermentation was carried out in 6.6 l of the controlled fermenter (Biostat® *B* plus, Sartorius BBI Systems GmbH, Melsungen, Germany). To keep the medium in the fermenter homogeneous, agitation was performed at 100 rpm. The medium was inoculated with optimum concentration of a fresh culture of the strain. The fermentation temperature was kept constant at optimum temperature. The pH was kept constant at optimum pH during fermentation through automatic addition of 10 N NaOH. The temperature, pH, and agitation were computer-controlled and monitored on line using MFCS SCADA Software (Sartorius, Germany). During fermentation experiments, pH, EPS, and bacterial growth were measured at 0, 3, 6, 9, 15, 18, 24, 30, 36, and 48 h as described in section 3.4.1. Also carbon source and lactic acid concentrations were measured at time intervals. Fermentation samples were prepared for analysis using high pressure liquid chromatography (HPLC) by diluting the supernatant of the centrifuged culture samples with deionized water to concentration of 0.5 to 2 mg/ml. A 100 µl was injected into Vertisep™ OA HPLC (Vertical chromatography, Thailand) (300×7.8 mm). For the mobile phase, a 0.005 M H_2SO_4 solution was used at a constant flow

rate of 0.4 ml/min. Carbon source concentrations and products profiles were detected by a Refractive Index Detector (Waters, USA).

3.6 Exopolysaccharide purification

Various purification treatments according to Ruas-Madiedo and de los Reyes-Gavilan (2005) were compared. After exopolysaccharide isolation in section 3.3.3.2, the crude polysaccharide was purified by trichloroacetic acid (TCA) precipitation and protease digestion.

3.6.1 Trichloroacetic acid precipitation

Briefly, 20% trichloroacetic acid (TCA) was added to the polysaccharide solution at a 1:1 volume ratio (Oliveira *et al.*, 1999). Then, the precipitate was removed by centrifugation at 11,000 rpm for 10 min at 4°C. Supernatant containing EPS was dialyzed using Snakeskin™ pleated dialysis tube of molecular weight cut-off 10,000 against sterile distilled water at 4°C for 3 days, with two daily changes of water, then freeze-dried.

3.6.2 Protease digestion

Pronase E (protease type XIV) from *Streptomyces griseus* and Proteinase K from *Tritirachium album* were used for EPS purification according to Gancel and Novel (1994) and Zisu and Shah (2003), respectively. EPS samples were incubated with 0.25 mg/ml of Pronase E, then incubated at 37°C for 1 h. For Proteinase K, 0.2 mg/ml of Proteinase K were added to EPS solution, then incubated at 37°C for 16 h. The reaction was stopped by heating at 90°C for 10 min. The residual proteins were

determined according to Bradford (Bradford, 1976) using BSA (bovine serum albumin) as a standard.

3.7 Immunostimulatory activity test

The impact of EPS on immune system were determined using lymphocyte proliferation and cytokine production assay (Amrouche *et al*., 2006; Chabot, 2001).

3.7.1 Optimization of exopolysaccharide concentrations for immuno-stimulatory activity test

The optimal concentrations of EPS for mouse inoculation and stimulation of mouse splenocytes *in vitro* were studied. Purified EPS from *Lactobacillus reuteri* 100-23 was used as a positive control in this study. The bacterial EPS has been studied and shown to activate dendritic cells. T lymphocytes that interacted with the activated dendritic cells, produce anti-inflammatory cytokines.

3.7.1.1 Experimental design

Six experiments were conducted using 6 different concentrations of EPS; 250, 500, 750, 1,000, 1,500, and 2,500 µg/ml in sterile PBS pH 7.4. In each experiment, 5 specific pathogen-free (SPF) BALB/c female mice in experiment group were inoculated intraperitonially with 100 µl of EPS in sterile PBS (pH 7.4), whereas 5 BALB/c SPF mice in the control group were inoculated with 100 µl sterile PBS pH 7.4. Proliferation of lymphocytes in response to various concentrations of EPS was determined.

3.7.1.2 Stimulation of mouse splenocytes

Mouse splenocytes were aseptically prepared and suspended in RPMI 1640 medium. Briefly, mouse spleens were aseptically excised and placed in 10 ml plastic-capped test tubes containing 3 ml RPMI 1640 culture medium (Appendix A3.2). Under a lamina flow hood, the spleens were chopped into small pieces with sterile scissors. The spleen tissues were forced up and down through a 1 ml syringe. Then splenic cells were transferred into a 50 ml falcon tube containing 5 ml complete RPMI-1640 medium through a cell strainer. The cells were washed using RPMI 1640 complete medium with 5% fetal bovine serum and then centrifuged at 210 rpm for 7 min and resuspended in the same medium. Cells were counted in a Coulter counter (Beckman coulter Inc, U.S.A.) using 20 µl of splenocyte suspension in 10 mL of Isoton (1:500) with 2 drops of Zap solution. Two hundred microliters of spleen suspensions containing approximately 2×10^6 cells/ml were placed in 96-well plates. Splenocytes were stimulated with 100 µl of EPS preparation at different concentrations (10, 25, 50, 75, and 100 µg/ml). One hundred microliters of ConA (5 µg/ml) and RPMI 1640 medium were used as positive and negative controls, respectively. The plates were then incubated under a 5% CO_2 atmosphere and saturated humidity for 72 h. Splenocyte proliferation was measured as described in section 3.7.1.3.

3.7.1.3 Determination of cell proliferation

After stimulation of mouse splenocytes in section 3.7.1.2, cell proliferation was measured by tritiated thymidine incorporation. Fifty microliters of 0.01 µCi [^{3}H]-thymidine were added to each well and incubated under a 5% CO_2

atmosphere and saturated humidity for 16 h at 37°C. The cultures were then harvested on glass filters with an automatic harvester (Harvester 96 Mach III M; TOMTEC, Wallac Ltd., Turku, Finland) and [^{3}H]-thymidine incorporation was determined by Micro Beta Plus liquid scintillation counter (Wallac Ltd., Turku, Finland). The data were expressed as corrected counts per minute (ccpm). The concentration of EPS that induced the highest lymphocyte proliferative response was chosen for further experiments.

3.7.2 Impact of exopolysaccharides produced by lactic acid bacteria on the immune system

3.7.2.1 Experimental design

Eleven experiments were conducted using purified EPS from different strains of lactic acid bacteria. In each experiment, each of 5 female BALB/c SPF mice in experiment group was inoculated intraperitonially with 100 µl of EPS at optimum concentration in PBS (pH 7.4), whereas each of 5 female BALB/c SPF mice in the control group was inoculated with 100 µl of PBS pH 7.4. EPS produced by *Lactobacillus reuteri* 100-23 was used as the positive control.

3.7.2.2 Stimulation of mouse splenocytes

Splenocytes were aseptically prepared and suspended in RPMI 1640 medium and placed in 96-well plates (2×10^6 cells/ml). Splenocytes were stimulated with EPS at optimal concentration. ConA and RPMI 1640 medium were used as positive and negative control, respectively. The plates were then incubated under a 5% CO_2 atmosphere and saturated humidity for 72 h. Cell suspensions (200 µl) were

centrifuged at 10,000 rpm for 10 min, and the supernatants were kept for cytokine determination. The proliferation of cells were measured as 3.7.2.3.

3.7.2.3 Determination of cell proliferation

Cell proliferation was measured by tritiated thymidine incorporation as described in section 3.7.1.3. Results are presented as a stimulation index (SI), where SI=mean corrected counts per minute (ccpm) of test sample/corrected counts per minute of control sample. The control was RPMI medium. Unless otherwise stated, an SI of ≥2 was considered to be an indication of stimulation of cell proliferation.

3.7.2.4 Measurement of cytokine production

The concentrations of cytokines (IL-4, IL-5, IL-10, IL12 (p70), IFN-g and TNF-α) present in the culture supernatants were determined by Bio-Plex cytokine assay kit. The cytokine levels in the cultured supernatants from section 3.7.2.2 were assayed according to the manufacturer's instructions.

3.8 Preliminary characterization of the purified exopolysaccharides

After EPS production under optimal conditions, the purified EPS was hydrolyzed and analyzed by HPLC. Hydrolysis of dried EPS was carried out by incubating samples for 4 h in 2 M tri-fluoroacetic acid at a concentration of 1 mg/ml at 100°C (Choy *et al.*, 1972). At the end of hydrolysis, the samples were dried by freeze drying. The dry samples, which contained the monomer sugars, were dissolved in deionized water and 100 µl of an appropriate dilution were applied to a Vertisep^{TM} OA HPLC

(Vertical chromatography, Thailand). The separation of sugars was carried out in 30 min at a flow rate of 0.4 ml/min using deionized water as mobile phase at 25°C. Separated sugars were detected by a Refractive Index Detector (Waters, Waters Corporation, U.S.A). D-Glucose, D-galactose, D-mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine and L-rhamnose were used as external standards.

3.9 Identification of selected isolates of exopolysaccharide-producing bacteria

Bacterial isolates that produce high amount of EPS were identified using morphological and physiological characteristics, and 16S ribosomal RNA (rRNA) gene sequence.

3.9.1 Morphological and physiological characterization

The selected isolate(s) was grown on MRS agar at 30°C under ananerobic conditions for 24 h. Cell morphology was observed by Gram staining. Some physiological characteristics were determined according to the criteria established in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). The fermentation patterns of each isolates were also determined by the API 50 CH system (Biome´rieux, RCS Lyon, France) according to the manufacturer instructions. Then the APILAB Plus software version 5.0 from bioMérieux and Analytab Products' computer database were used for comparison of carbohydrate assimilation and/or fermentation patterns.

3.9.2 Sequencing of 16S ribosomal RNA gene

Analysis of the nucleotide sequence of 16S ribosomal RNA gene was used for genetic characterization according to Weisburg *et al.* (1991). There were five major steps including extraction of genomic DNA, PCR amplification of 16S rRNA gene, Cloning of PCR product, sequencing of PCR amplicon, and analysis of 16S rRNA gene sequence.

3.9.2.1 Extraction of genomic DNA

Genomic DNA of the selected isolate of EPS-producing bacteria was extracted using Wizard Genomic DNA Purified kit (Promega). The bacterial isolates were cultivated on MRS agar plate, and incubated at 30°C for 24 h. Bacterial cells were washed by scraping into microcentrifuge tube containing 1 ml of 50 mM EDTA (pH 8) and centrifuged at 12,000 rpm for 5 min at 4°C. The cell pellets were resuspended with 240 µl of 50 mM EDTA (pH 8), then 30 µl of 10 mg/ml lysozyme was added. The mixture was incubated at 37°C for 90 min, followed by the addition of 300 µl of nuclei lysis solution (Promega). Samples were then incubated at 80°C for 5 min and left to cool down to room temperature. Subsequently, the mixture was added with 2 µl of RNase Solution (Appendix A2.2), mixed by inversion, and incubated at 37°C for 30 min. One hundred microliters of protein precipitation solution (Promega) was added and mixed using vortex device at high speed for 20 sec. After incubation on ice for 5 min, the mixtures were centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was transferred into a new sterilized microcentrifuge tube. Then, 300 µl of isopropanol was added into supernatant and centrifuged at 12,500 rpm for 10 min at 4°C. The supernatant was discarded. The DNA pellet was washed with 600 µl of 70% ethanol, then dried at 37°C for 1 h. Fifty

microliters of TE buffer (Appendix A2.1) were added, and kept overnight at 4°C to allow DNA to dissolve. The extracted DNA was detected using 1% agarose (Low EEO Agarose, BIO 101, Inc., U.S.A.) gels electrophoresis in TBE buffer (pH 8.3) (Appendix A2.3).

3.9.2.2 Amplification of the 16S ribosomal RNA gene

Polymerase Chain Reaction (PCR) was performed using Thermo electron corporation Px2 Thermal Cycler (Bioscience Technologies Division, U.S.A.) with a primary heating step for 2 min at 95°C, followed by 35 cycles of denaturation for 45 sec at 95°C, annealing for 45 sec at 55°C, and extension for 2 min at 72°C which was followed by a final extension at 72°C for 7 min. Each 25 µl of reaction mixture contained 2 µl of genomic DNA, 13.5 µl of MilliQ water, 2.5 µl of 10X PCR buffer (Invitrogen), 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTPs mixture (Invitrogen), 1.0 µl of each primer (fD1 and rP2) (10.0 pmoles/µl), and 0.5 µl of *Taq* DNA polymerase (5U/µl) (Invitrogen). The primer sequences were shown in Table 3.1. The PCR amplified products were analyzed by electrophoresis in 1.0% agarose gel followed by staining with ethidium bromide (Appendix A2.5) and visualization under ultraviolet light (UV) using gel documentation (Syngene, Synoptics, U.K.). The size of PCR products was compared with 1 kb DNA ladder (Invitrogen). The expected size of amplified DNA fragments was approximately 1,500 bp. The DNA band of the expected size visualized under the UV light was cut from the gel by a clean blade and placed into a new 1.5 ml microcentrifuge tube. The gel matrix that did not contain DNA material was trimmed off to obtain the minimum volume of the gel. Then, the

PCR products were purified by using Witzard® SV Gel and PCR Clean-up System (Promega) according the manufacturer's instruction.

3.9.2.3 Cloning of 16S rRNA gene

DNA fragments obtained from section 3.9.2.2 were ligated into pGEM-T Easy Vector (Promega). Each 10 µl of reaction mixture contained the following components: 5 µl of 2X Rapid Ligation Buffer, T4 DNA Ligase, 1 µl of pGEM-T Easy Vector (50 ng), 3 µl of PCR product, and 1 µl of T4 DNA Ligase (3 Weiss units/µl). The reactions were mixed by pipetting, and incubated 1 h at room temperature. The ligation mixes were transformed into competent *Escherichia coli* JM109 cells using the heat-shock method. Two microliters of each ligation reaction were added to a sterile 1.5 ml microcentrifuge tube on ice, then transferred 50 µl of cells into each tube. The tubes were gently flicked to mix, and placed on ice for 20 min. The cells were heat-shocked for 45-50 sec in a water bath at exactly 42°C, and immediately returned to ice for 2 min. Subsequently, 950 µl of SOC medium (Appendix A2.8) was added to the tubes, then reactions were incubated for 1.5 h at 37°C with shaking (150 rpm). One hundred microliters of each transformation culture were then spread onto duplicate Luria-Bertani (LB)/ampicillin/isopropyl-β-D-thiogalactopyranoside (IPTG)/X-Gal plates and incubated at 37°C for 16 h. Transformants were selected for the presence of the pGEM-T Easy containing insert by blue/white screening system. White transformant colony of each strain was chosen and cultured overnight in 5 ml of Luria-Bertani broth (Appendix A2.7) containing kanamycin (50 µg/ml) at 37°C with shaking (150 rpm) for 24 h. Plasmids were

extracted from the 5 ml overnight cultures of *Escherichia coli* following the Pure Link^{TM} Quick Plasmid Miniprep kit protocol (Invitrogen).

3.9.2.4 Sequencing of 16S rRNA gene

Sequencing of 16S rRNA gene was performed using M13/pUC universal forward and reverse primers and also walking forward and reverse primers (Table 3.1) and Terminator Ready Reaction kit version 2.0 (Perkin Elmer, U.S.A.) in combination with an automated sequencing system. The gene was amplified using thermal cycler. An estimated amount of 100 ng of DNA was used for each reaction together with 5 pmol of each primer, 4 μl of ready reaction mix and deionized water to attain a 10 μl final volume. The same primers were used as previous PCR amplification. Cycle-sequencing PCR and DNA precipitation with ethanol and sodium acetate were done following the manufacturer's protocol (Applied Bio-Systems, U.S.A.). The precipited DNA was dried, and dissolved in deionized water. Then, sequencing was performed using ABI377 Autometed DNA sequencer (Perkin Elmer, U.S.A.).

3.9.2.5 Analysis of 16S rRNA gene sequence

Nucleotide sequence data obtained from DNA sequencing software of ABI377 Automated DNA Sequencer was interpreted and converted to single letter code in text file format by the Chromas 1.56 program. The sequence was also corrected by manual inspection of the chromatogram. For the identification of closest relatives, newly determined sequences were compared to those available in GenBank

databases [http://www.ncbi.nlm.nih.gov/] using standard nucleotide-nucleotide BLAST program [blastn] to ascertain their closest relatives.

Table 3.1 Oligonucleotide primers used for PCR amplification and sequencing of 16S rRNA gene.

Primer	Primer sequence (5' to 3')	Target region[a]	Reference
fD1	AGAGTTTGATCCTGGCTCAG	8-27	Weisburg *et al.* (1991)
rP2	ACGGCTACCTTGTTACGACTT	1491-1511	Weisburg *et al.* (1991)
Walking forward	TAACTACGTGCCAGCAGCC	515-533	Udomsil (2008)
Walking reverse	CGACAACCATGCACCACCTG	1008-1027	Udomsil (2008)
M13/pUC Forward	GTTTTCCCAGTCACGAC		Messing (1983)
M13/pUC Reverse	CAGGAAACAGCTATGAC		Messing (1983)

[a] *Escherichia coli* numbering

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Selection of exopolysaccharide-producing lactic acid bacteria

Five hundred and sixty six isolates of lactic acid bacteria obtained from stock cultures of the Microbial Culture Collection Laboratory, Suranaree University of Technology, and isolated from their natural habitats (agricultural products and traditional Thai fermented foods) were tested for their EPS production capability using two different types of carbon sources, glucose and sucrose. Approximately 10 and 19% of lactic acid bacterial isolates produced slimy colonies of 0.2-0.9 and 0.2-2.1 cm in diameter on MRS agar containing 2% of glucose and sucrose respectively (Figure 4.1). The isolates produced slimy colonies when culturing on MRS agar containing 2% of glucose were isolated from Thai fermented foods: Pla-som (9.9%) and Sai-krork-prieo (0.5%). Additionally, when culturing on MRS agar containing 2% sucrose, slime-producing isolates were isolated from Thai fermented foods; Pla-som (12.4%), Sai-krork-prieo (1.1%), and Nham (0.2%), agricultural products; rice grain (1.6%), rice husk (1.2%), mung bean (0.5%), silage (0.2%), and topioca waste (0.5%), and intestinal tract; chicken intestine (0.9%) and pig intestine (0.4%) (Figure 4.2). A total of 166 selected bacterial isolates were confirmed their EPS production ability using a liquid medium, MRS medium containing 2% of sugar. EPS concentration was estimated by the phenol/sulfuric acid method, and expressed in g equivalent glucose/l. The EPS production of 0.1-0.6 g equivalent glucose/l was

obtained when glucose was used as carbon source (Figure 4.3). When the selected isolates were cultivated in MRS broth containing sucrose, the amount of EPS produced was varied from 0.1-6.9 g equivalent glucose/l. Twenty isolates of lactic acid bacteria were selected for further study based on their EPS production ability, with specific EPS production ranging from 0.26-44.5 pg/cell, and diversity of bacterial habitats (Table 4.1). In a previous study, Van Geel-Schutten *et al.* (1998) indicated that type of carbon source added to the screening media plays a major role in the detection of the EPS phenotype. The study demonstrated that the sucrose medium was the best media for detecting the EPS phenotype of EPS-producing *Lactobacillus* when compared to the medium containing either glucose, fructose, maltose, raffinose, galactose, or lactose. This observation was similar to EPS-producing lactic acid bacteria isolated from traditional Thai fermented foods reported by Smitinont *et al.* (1999). Our result also showed that MRS containing sucrose improved the detection of EPS-producing strains. A number of papers reported on habitats of EPS-producing lactic acid bacteria such as dairy products (fermented milk, yoghurt and kefir grains) (De Vuyst and Degeest, 1999), cereal fermentations and intestinal tract (Tieking and Ganzle, 2005; Tieking *et al.*, 2003), non-dairy fermented foods (Ludbrook *et al*., 1997), Thai fermented foods (Smitinont *et al.*, 1999) and Nigerian fermented foods (Sanni *et al.*, 2002). However, the ecological function of the EPS produced by LAB is not clearly defined, but they probably have a protective function against unfavorable environmental conditions, macrophages, and bacterial cell wall-degrading enzymes (Looijesteijn *et al.*, 2001). In this study, most of EPS-producing isolates were selected from traditional Thai fermented foods especially

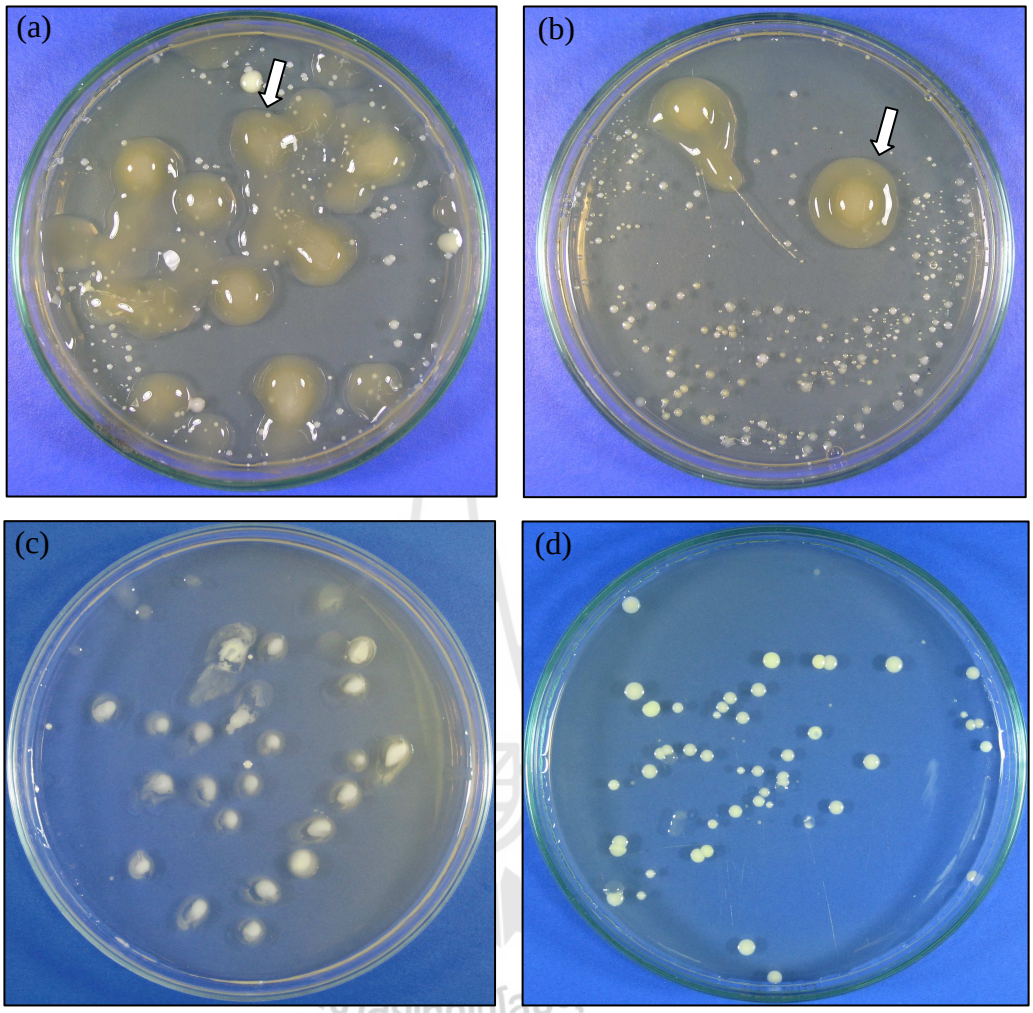

Figure 4.1 Slimy colonies (arrow) of lactic acid bacterial isolates on MRS agar containing 2% of sucrose (a and b) compared to the medium containing 2% of glucose (c and d).

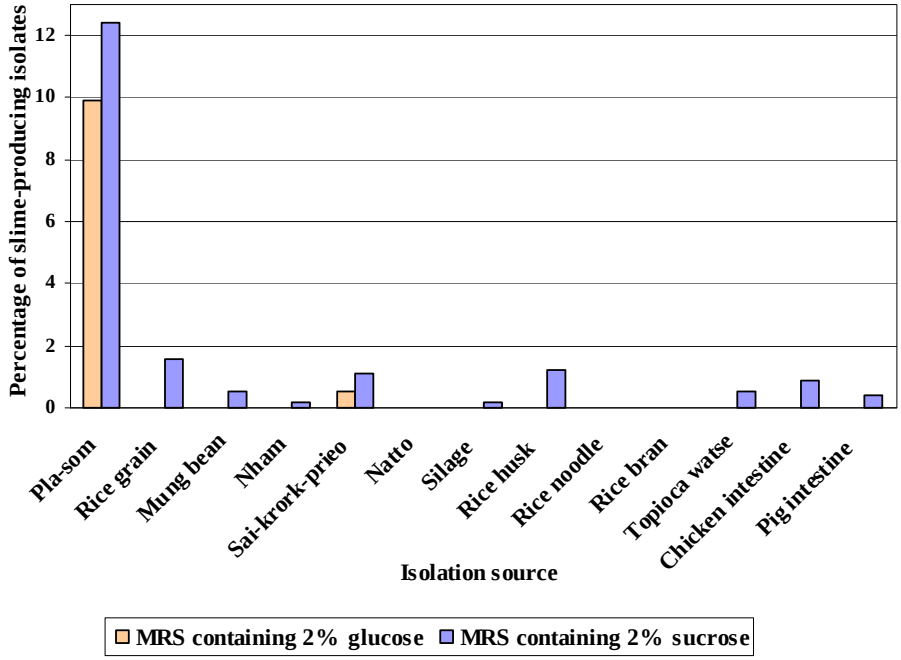

Figure 4.2 Percentages of lactic acid bacterial isolates that produced slimy colonies on MRS agar containing 2% of glucose or sucrose.

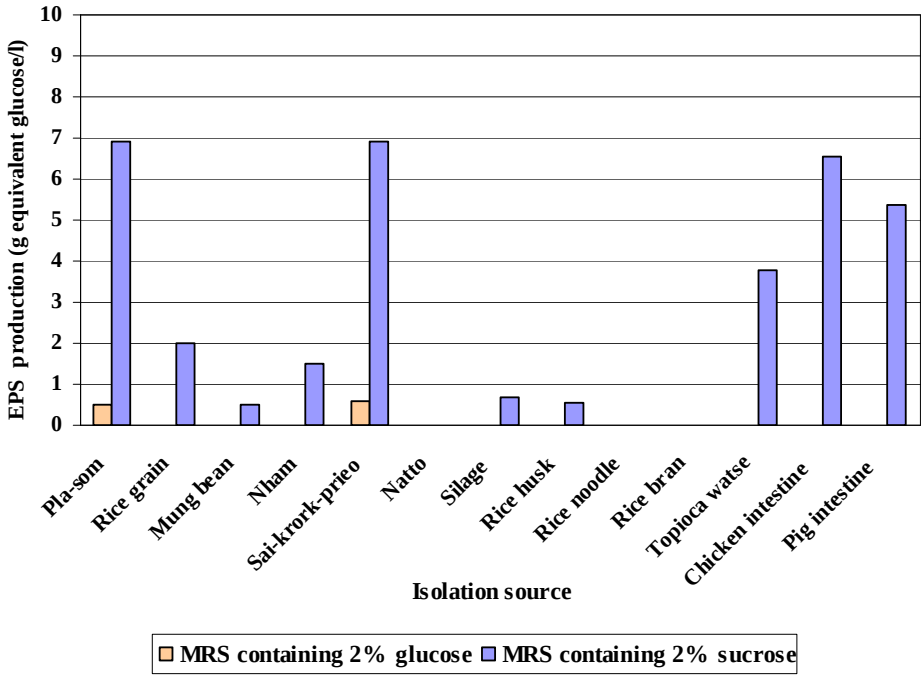

Figure 4.3 Comparison of EPS production (g equivalent glucose/l) by selected isolates using MRS broth containing 2% of glucose or sucrose.

Table 4.1 Specific EPS production of selected isolates when culturing at 30°C in MRS broth containing 2% of analytical grade sucrose for 48 h under anaerobic conditions.

Isolate code	Source of bacteria[a]	Specific EPS production (pg/cell)
C25	Chicken intestine	11.9
C27	Chicken intestine	3.30
C56	Chicken intestine	1.51
C58	Chicken intestine	1.03
FKU5	Pla-som	0.26
FKU23	Pla-som	44.5
FLB1	Pla-som	42.3
G3	Tapioca waste	2.06
I5	Tapioca waste	15.7
NHMS3	Nham	0.35
P14	Pig intestine	1.39
PSMS1-1	Pla-som	22.8
PSMS1-5	Pla-som	35.9
PSMS2-4	Pla-som	13.1
PSMS3-6	Pla-som	7.08
PSMS4-4	Pla-som	8.68
PSMS5-1	Pla-som	34.2
RMS3-1	Rice grain	1.50
SSMS1	Sai-krork-prieo	6.13
SSMS6	Sai-krork-prieo	20.2

[a] Pla-som, Thai fermented fish; Sai-krork-prieo, Thai fermented sausage; Nham, Thai fermented pork

Pla-som containing 2-5% of salt. High osmotic pressure in its natural habitat may induce the formation of polysaccharides.

4.2 Optimization of some exopolysaccharide production conditions

To obtain the maximum production of exopolysaccharide from inexpensive medium, some components of MRS medium and production conditions including types and concentrations of carbon source, initial pH of the culture medium, and incubation temperature for culturing the selected EPS-producing isolates were investigated.

4.2.1 Types and concentrations of carbon sources

Five different carbon sources: soluble starch, tapioca starch, rice flour, molasses, and white sugar from sugar cane were used to identify the suitable type of carbon source in the MRS medium for EPS production. Glucose, an original carbon source of MRS medium, was replaced by the selected carbon sources. Twenty selected isolates obtained from a selecting step, were grown at 30°C for 48 h in MRS medium supplemented with 2% of various carbon sources. The EPS production using MRS containing 2% of analytical grade sucrose was performed as a positive control. The exopolysaccharide production was shown in term of specific EPS production (pg/cell). The highest EPS production of 106.94 pg/cell was observed in the isolate PSMS1-5 when using white sugar from sugar cane as a carbon source (Table 4.2). Jung *et al.* (2008) reported that the specific EPS content of 1.869 EPS/cell (specific EPS production of 1.820 pg/cell) was achieved by *Lactobacillus amylovorus* DU-21 when 15 g/l of glucose was employed as the carbon source. *Lactobacillus casei* CG11 was grown at 25°C for 48 h in BMM supplemented with sucrose at concentrations of

2, 5, 10, and 20 g/l resulted in specific EPS production of 4.86, 5.26, 4.50, 8.93 pg/cell, respectively (Cerning *et al.*, 1994).

Table 4.2 Specific EPS production (pg/cell) of 20 selected isolates after culturing at 30°C for 48 h under anaerobic conditions in MRS broth containing carbon source at the concentration of 20 g/l.

Isolate code	Specific EPS production (pg/cell)					
	Analytical grade sucrose	White sugar from sugar cane	Molasses	Soluble starch	Rice flour	Tapioca starch
C25	11.93	28.36	0.22	0.00	0.00	0.00
C27	3.30	0.04	2.93	6.51	2.86	1.19
C56	1.51	1.40	1.00	0.00	5.48	5.95
C58	1.03	3.69	0.57	0.00	2.01	3.84
FKU5	0.26	0.18	0.00	8.88	0.82	0.00
FKU23	44.53	47.00	0.69	4.03	1.28	0.00
FLB1	42.25	6.88	12.08	0.00	0.00	0.00
G3	2.06	2.65	0.06	0.00	0.00	0.00
I5	15.73	43.19	8.09	0.00	0.00	0.00
NHMS3	0.36	0.14	0.76	3.59	0.00	0.00
P14	1.39	1.27	0.09	0.00	1.28	0.00
PSMS1-1	22.82	13.01	0.01	9.95	0.06	1.54
PSMS1-5	35.91	106.94	1.02	2.36	0.43	0.00
PSMS2-4	13.10	8.96	0.42	2.17	1.50	0.00
PSMS3-6	7.08	5.90	2.37	0.00	0.07	0.00
PSMS4-4	8.68	75.92	8.72	0.00	2.11	0.00
PSMS5-1	34.20	4.73	0.76	0.00	0.00	0.00
RMS3-1	1.49	0.03	0.79	4.70	0.00	0.00
SSMS1	6.13	8.96	1.56	0.00	0.00	9.33
SSMS6	20.25	4.47	0.07	3.98	0.76	0.00

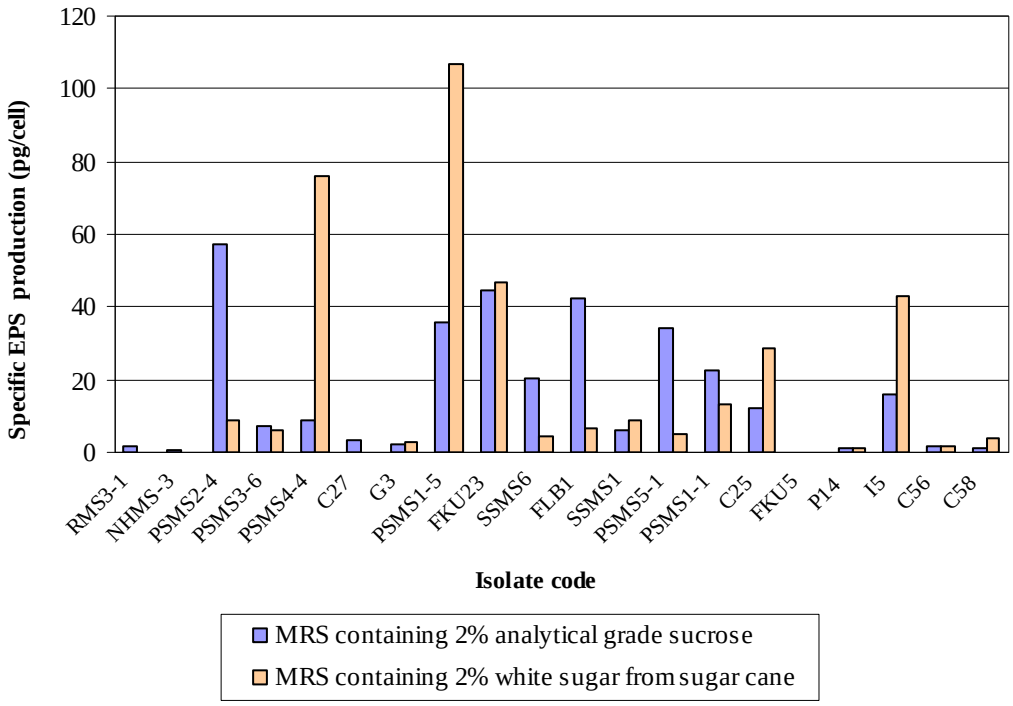

Figure 4.4 Specific EPS production (pg/cell) of 20 selected isolates after culturing at 30°C for 48 h in MRS broth containing white sugar from sugar cane (20 g/l) compared to analytical grade sucrose.

Among the carbon sources tested for selected lactic acid bacterial isolates, white sugar from sugar cane gave the highest specific EPS production ranging from 0.025-106.94 pg/cell followed by molasses (0-12.08 pg/cell), soluble starch (0-9.95 pg/cell), tapioca starch (0-9.33 pg/cell), and rice flour (0-5.48 pg/cell) compared to analytical grade sucrose (0.26-44.5 pg/cell) (Table 4.2). Results from this study revealed that white sugar from sugar cane gave higher EPS specific production (0.025-106.94 pg/cell) than analytical grade sucrose (0.26-44.5 pg/cell) (Figure 4.4). Moreover, from the economic point of view, white sugar from sugar cane is a potential carbon source for EPS production by selected lactic acid bacterial isolates which was about 40 times cheaper than analytical grade sucrose. Prasertsan *et al*. (2008) found that substitution of the analytical grade sucrose by white sugar from

sugar cane resulted in the reduction of the raw material cost by 98%. Therefore, white sugar from sugar cane was chosen to be used as the carbon source in MRS medium for further study.

For study on optimum concentration of white sugar from sugar cane, five isolates of lactic acid bacteria: FKU23, I5, PSMS1-5, PSMS4-4, and RMS3-1, were selected based on their high EPS production ability when culturing in MRS medium containing 2% of white sugar from sugar cane as a carbon source (47.00, 43.19, 106.94, 75.92, and 0.03 pg/cell respectively), and diversity of bacterial habitats (pla-som, tapioca waste, and rice grains). Various concentrations of white sugar from sugar cane: 20, 30, 35, 40, 45, and 50 g/l, were investigated to obtain the optimal concentration. After cultivating the isolates FKU23, I5, PSMS1-5, PSMS4-4, and RMS3-1 for 48 h at 30°C under anaerobic conditions, the isolates could produce the maximum exopolysaccharide of 1.52, 6.28, 13.16, 28.02, and 0.03 g EPS/ g dry weight when using white sugar from sugar cane at concentrations of 45, 35, 45, 50, and 30 g/l, respectively (Figure 4.5). White sugar from sugar cane concentrations of 45, 35, 45, 50, and 30 g/l were suitable for EPS production by the isolates FKU23, I5, PSMS1-5, PSMS4-4, and RMS3-1, respectively, and were selected for further experiment. The sugar concentration had a mark effect on EPS yield. The increase in sucrose concentration from 20 to 50 g/l resulted in increased EPS production. EPS formation is generally favored by the excess of nutrient carbohydrates. Cerning *et al.* (1994) showed that the presence of excess sugar in the medium had a stimulating effect on EPS production of lactic acid bacteria. Yuksekdag and Aslim (2008) studied EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in the medium containing different concentrations of glucose, and found

that EPS production and growth were stimulated by the high glucose concentration (30 g/l). Korakli *et al.* (2003) reported the EPS production by *Lactobacillus sanfranciscensis* LTH2590 increased by increasing the sucrose concentration in the medium. Influence of concentrations of white sugar from sugar cane on growth was also observed. The bacterial growth slightly increased when the sugar concentration was increased from 20 to 50 g/l for the isolates PSMS1-5, FKU23, and RMS3-1. Most mucoid microorganisms produce EPS under all growth conditions, but production is maximal under particular growth conditions on a defined medium (Gancel and Novel, 1994). All parameters that increase or decrease growth rate influence the extracellular concentration of EPS precursors and, therefore, EPS synthesis (Zevenhuizen, 1986). A decrease in growth of bacteria was observed in isolates PSMS4-4 and I5 grown in medium containing high concentration of carbon source (3% white sugar from sugar cane). Less favorable fermentation conditions for growth of these selected strains may decrease total viable counts which resulted in more EPS being produced for protecting the microbial cell itself. Gancel and Novel (1994) found that an industrial strain of *Streptococcus salivarius* subsp. *thermophilus* grown in a semisynthetic medium, produced EPS during the stationary phase of growth. Concentration of sugars that decreased growth rate, increased polymer synthesis.

4.2.2 Initial pH of the culture medium for exopolysaccharide production

Optimization of environment for growth is important to achieve the maximal EPS production. To elucidate the influence of initial pH of the culture medium on growth and exopolysaccharide production, the initial pH of optimum medium were varied at pH 4.5, 5.0, 6.0, 7.0, and 8.0. Results showed that the highest

EPS production by isolate PSMS4-4 was obtained when the cultivating medium was adjusted to pH 5.0 and 7.0. The optimum initial pH for EPS production of isolate RMS3-1 was 5.0 and 8.0. When the influence of initial pH of the culture medium was investigated for the isolates I5, PSMS1-5 and FKU23, the pH of medium at 6.0, 5.0, and 7.0 respectively were found to be suitable (Figure 4.6). The optimum pH for EPS production generally ranges between 5.0 and 7.0. Van den Berg *et al*. (1995) established that EPS production by *Lactobacillus sake* varied with pH variations. De Vuyst *et al*. (1998) reported optimal EPS production and growth for *Streptococcus thermophilus* LY03 in milk medium, were at pH 6.2 and 42°C. Mozzi *et al.* (1996) demonstrated that maximum polymer synthesis (488 mg/l) and cell viability (2.4×10^{10} CFU/ml) occurred when *Lactobacillus casei* was cultured at a constant pH of 6.0 and 30°C for 24 h. Gassem *et al.* (1997) also found that the optimal pH for EPS production by continuous fermentation was at pH 6.5 compared to results obtained at pH 5.2. For bacterial cell dry weight, the increase in pH from 4.5 to 8.0 had tendency to increase biomass production. At high pH value, the bacteria may protected itself by production of slime layer attached to the cell surface resulting in increase of cell dry weight.

4.2.3 Cultivation temperature

The optimal temperature for EPS production was determined by cultivating the selected isolates in the optimized medium at optimum pH for 48 h. The incubation temperatures were varied at 30, 35, 40, 45, and 50°C based on the range of its growth temperatures. Cultivation temperatures affected bacterial growth and EPS production depending on strains (Figure 4.7). Cerning *et al*. (1992) observed that the

optimal EPS production takes place at temperatures below the optimal growth temperature. While others have shown EPS production to be favored at much higher temperatures (De Vuyst *et al*., 1998). Garcia-Garibay and Marshall (1991) found that Specific polymer production (equivalent milligrams of dextran per CFU) by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in skim milk was greater at a temperature (48°C) than at the optimum temperatures for growth (37 to 42°C). The EPS production increased with increasing temperatures, was also found by Grobben *et al.* (1995), who examined EPS production by the same strain in chemically defined medium with glucose or lactose as the substrate carbohydrate. In *Lactobacillus delbruckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains, the EPS production rate was lower at 30, 37 and 42°C than at 45 °C (Aslim *et al*., 2005).

Optimum conditions for the production of exopolysaccharides by selected lactic acid bacterial isolates were concluded in Table 4.3. EPS production rate depended on both bacterial isolates and growth conditions. The results showed that all strains tested provided high amount of EPS (0.08-29.16 g EPS/g cell dry weight). Mozzi *et al.* (1996) found that *Lactobacillus casei* CRL 87 produced the EPS with optimum specific EPS production of 3.9×10^{-5} g EPS/g cell dry weight when culturing at a constant pH of 6.0 and 30°C for 24 h. Shene and Bravo (2007) found that the highest specific EPS production of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the medium containing lactose from deproteinized whey as the carbon source, was 188 mg/g biomass/h.

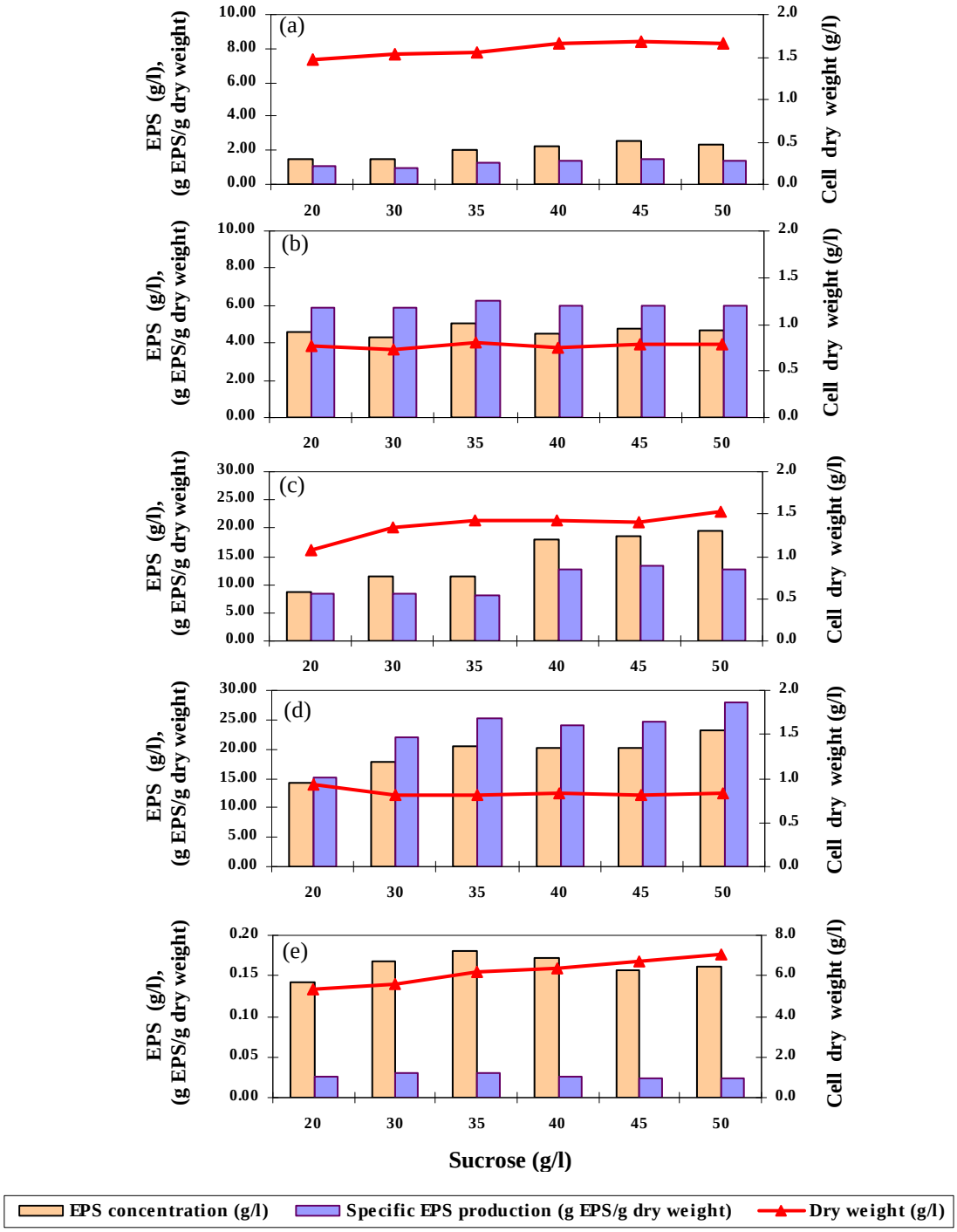

Figure 4.5 Effects of various concentrations of white sugar from sugar cane on growth, EPS production and specific EPS production of selected isolates after cultivating at 30°C for 48 h; FKU23 (a); I5 (b); PSMS 1-5 (c); PSMS4-4 (d) and RMS3-1(e).

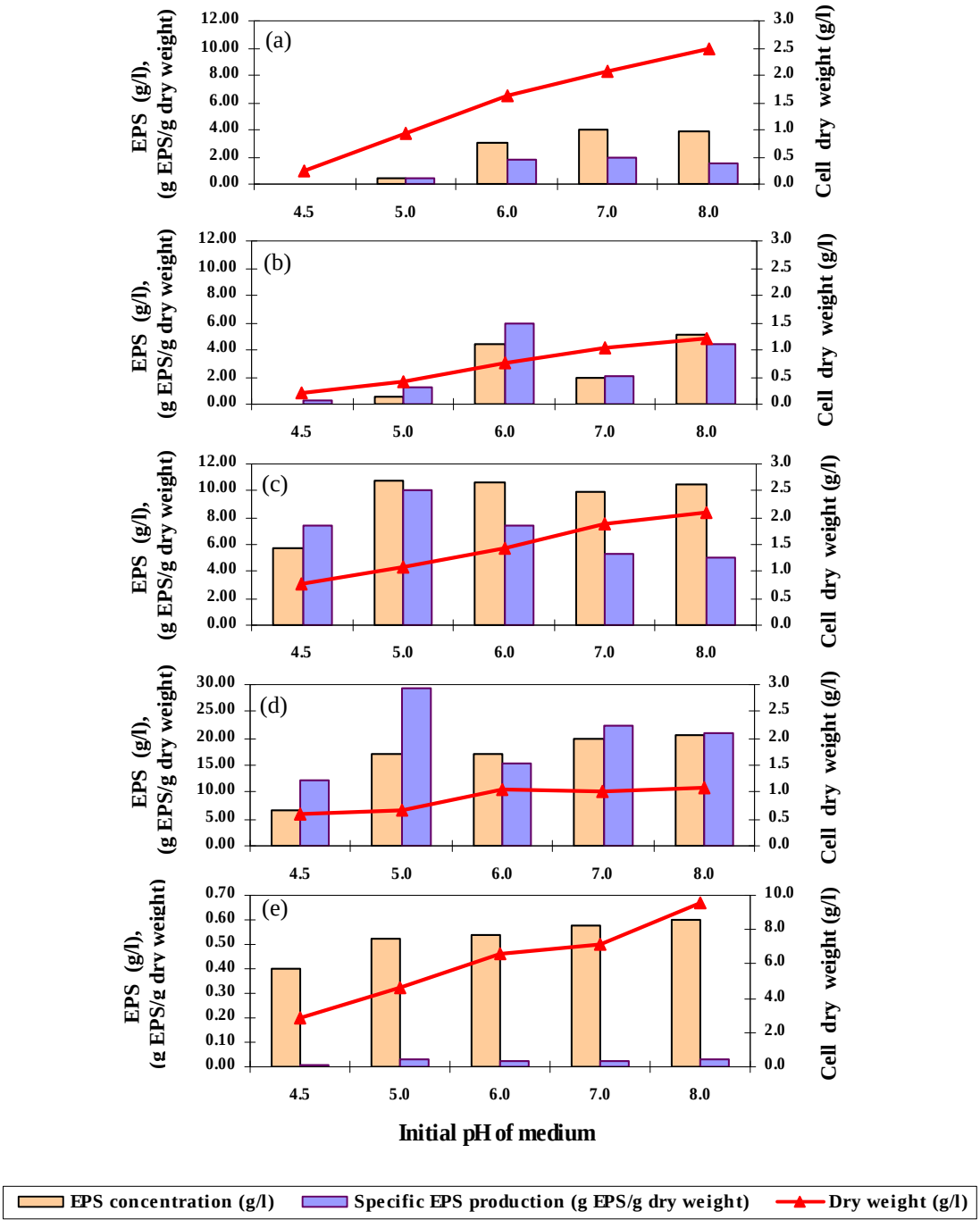

Figure 4.6 Effects of initial pH of the culture medium on growth, EPS production and specific EPS production of selected isolates when cultivating at 30°C for 48 h in MRS broth containing white sugar from sugar cane at selected concentration; FKU23 (a); I5 (b); PSMS1-5 (c); PSMS4-4 (d) and RMS3-1 (e).

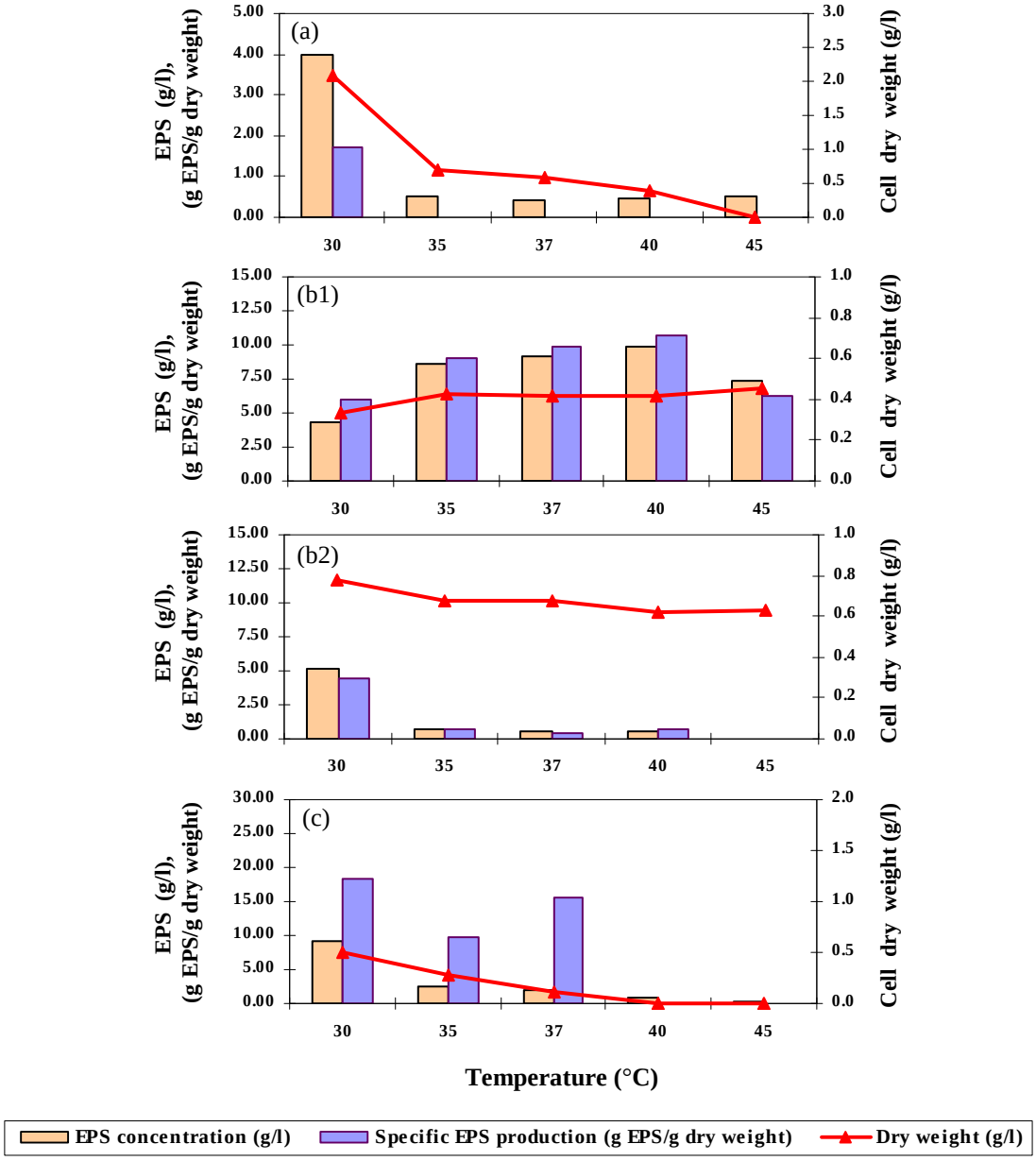

Figure 4.7 Effects of cultivation temperatures on growth, EPS production and specific EPS production of selected isolates when cultivating in MRS broth containing white sugar from sugar cane with optimum initial pH at various temperatures for 48 h. Selected isolates FKU23 (a); I5, pH 6 (b1); I5, pH 8 (b2); PSMS1-5, pH 5 (c); PSMS4-4, pH 5 (d1); PSMS 4-4, pH 7 (d2); RMS3-1, pH 7 (e1) and RMS3-1, pH 8 (e2).

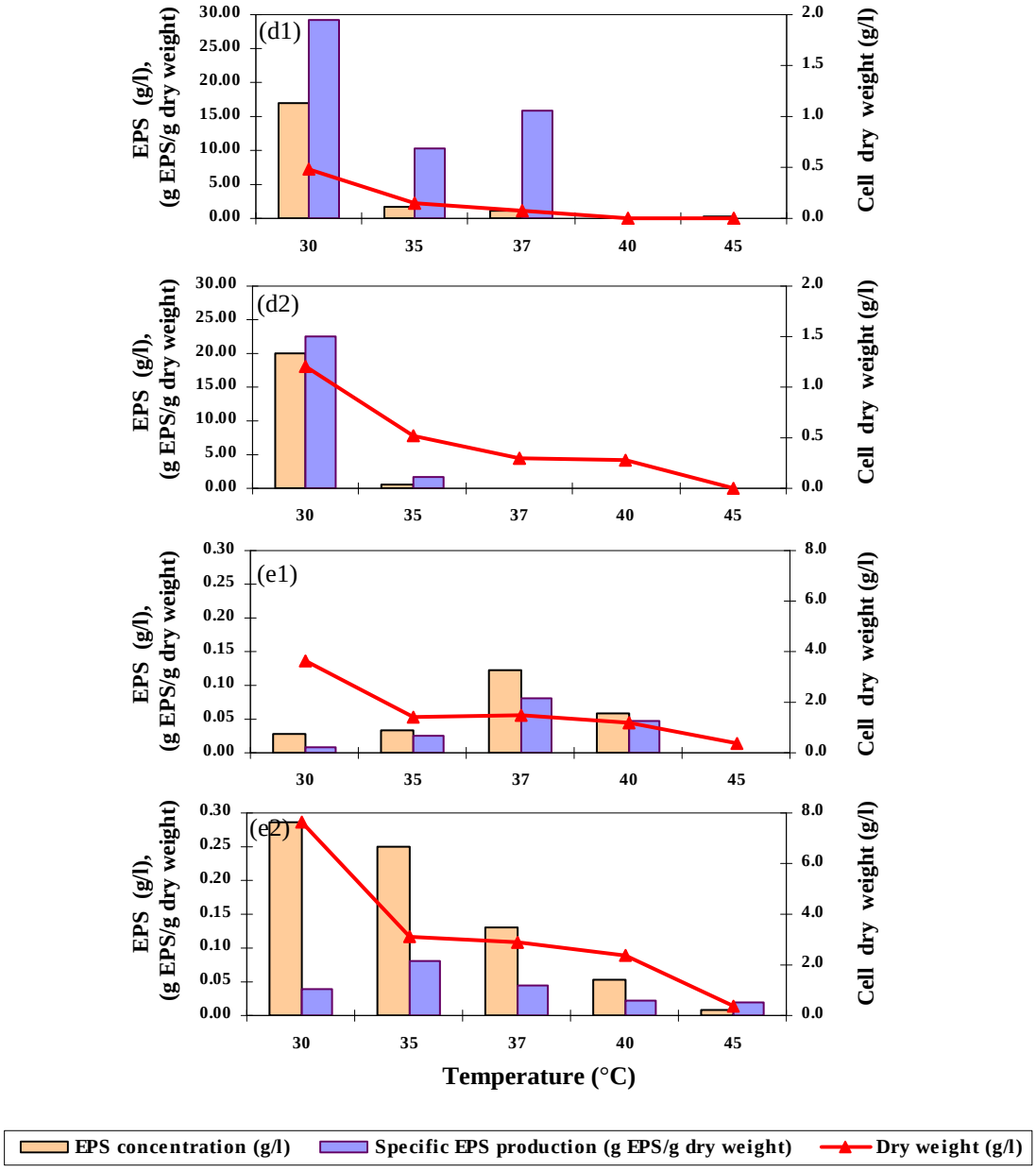

Figure 4.7 (Continued). Effects of cultivation temperatures on growth, EPS production and specific EPS production of selected isolates when cultivating in MRS broth containing white sugar from sugar cane with optimum initial pH at various temperatures for 48 h. Selected isolates FKU23 (a); I5, pH 6 (b1); I5, pH 8 (b2); PSMS1-5, pH 5 (c); PSMS4-4, pH 5 (d1); PSMS4-4, pH 7 (d2); RMS3-1, pH 7 (e1) and RMS3-1, pH 8 (e2).

Table 4.3 Optimum conditions for the production of exopolysaccharides by selected lactic acid bacterial isolates.

Isolate code	EPS production conditions		
	Carbon source[a] (%)	Initial pH of the culture medium	Cultivation temperature (°C)
FKU23	4.5	7	30
I5	3.5	6	40
I5	3.5	8	30
PSMS1-5	4.5	5	30
PSMS1-5	4.5	5	37
PSMS4-4	5	7	30
PSMS4-4	5	5	30
RMS3-1	3	5	37
RMS3-1	3	8	35

[a] Carbon source in MRS base medium for EPS production.

4.3 Production of exopolysaccharides using optimum conditions

4.3.1 Exopolysaccharide production in 500 ml Duran bottle

Time course of EPS production of selected isolates under optimum conditions were investigated to obtain the suitable fermentation time for the maximum EPS production. Selected isolates were cultured in the optimized medium under optimum conditions. The fermentation profiles, in terms of pH, EPS production, cell growth, and the consumption of carbon sources during growth, were exhibited (Figure 4.8). All parameters were detected at intervals when the isolates FKU23, I5, PSMS1-5, PSMS4-4, and RMS3-1 were cultured for 48 h. It was found that EPS production commenced at 3 h of incubation and still increased until 9 h of incubation times before becoming slight increase. The consumption rate of a selected carbon source during the fermentation period varied depending on EPS-producing

strains. The highest specific EPS productions were obtained after 12, 12, 30, 18, and 36 h of fermentation for the isolate FKU23, I5, PSMS1-5, PSMS4-4, and RMS3-1 respectively. After 3 h of incubation, the decrease in pH was found for all isolates tested. Also, the final pH in the MRS medium was below 4.0 after a fermentation period of 48 h. For bacterial growth, the stationary phase of growth was obtained after 6, 9, and 12 h of incubation for the isolate I5, isolates FKU23, PSMS1-5, and RMS 3-1, isolate PSMS4-4 respectively. Moreover, the growth of most strains studied was growth-associated production. However, both growth-associated and non-growth-associated production kinetics were observed for *Lactobacillus* strains (Manca de Nadra *et al.*, 1985; Kojic *et al.*, 1992). Degeest *et al.* (1995) also mentioned that EPS production from thermophilic LAB strains is growth-associated production.

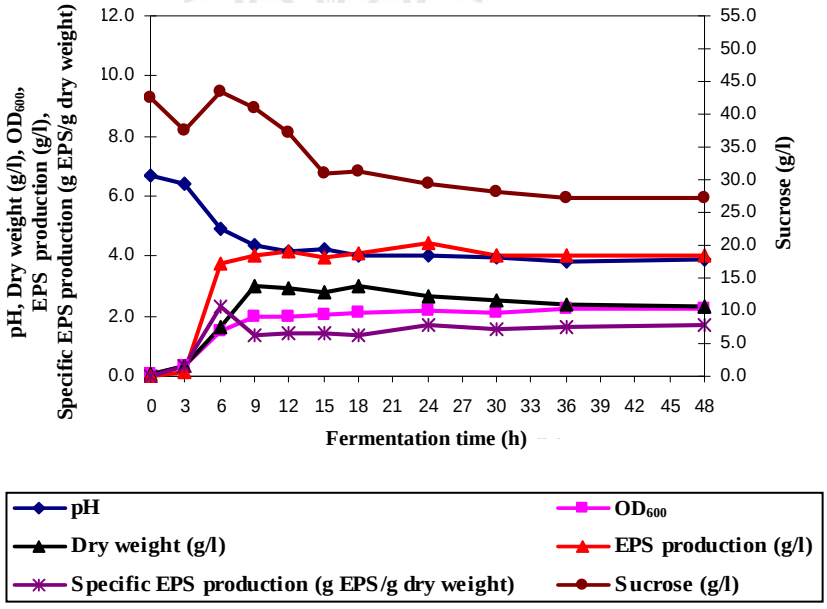

Figure 4.8 (A) Growth and EPS production by isolate FKU23 in MRS medium containing 45 g/l of white sugar from sugar cane at initial pH of 7 and 30°C for 48 h.

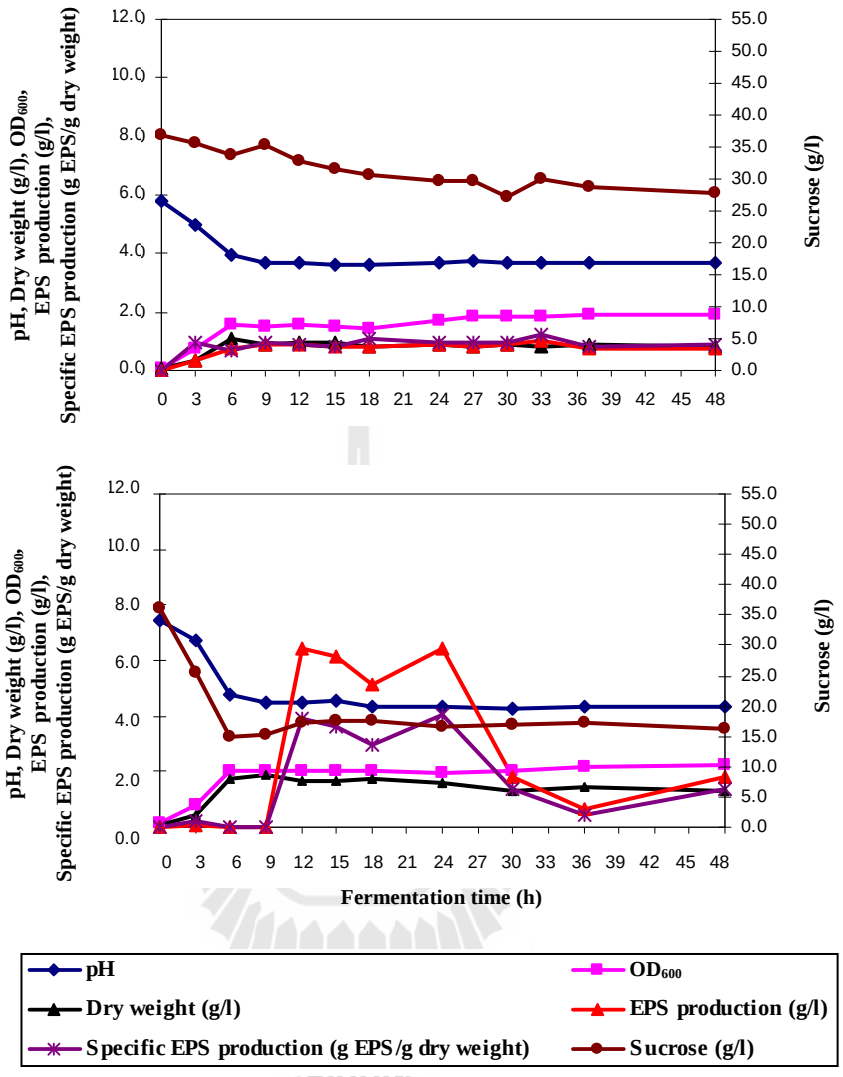

Figure 4.8 (B) Growth and EPS production by isolate I5 in MRS medium containing 35 g/l of white sugar from sugar cane at initial pH of 6 and 40°C (a) and 8 and 30°C (b) for 48 h.

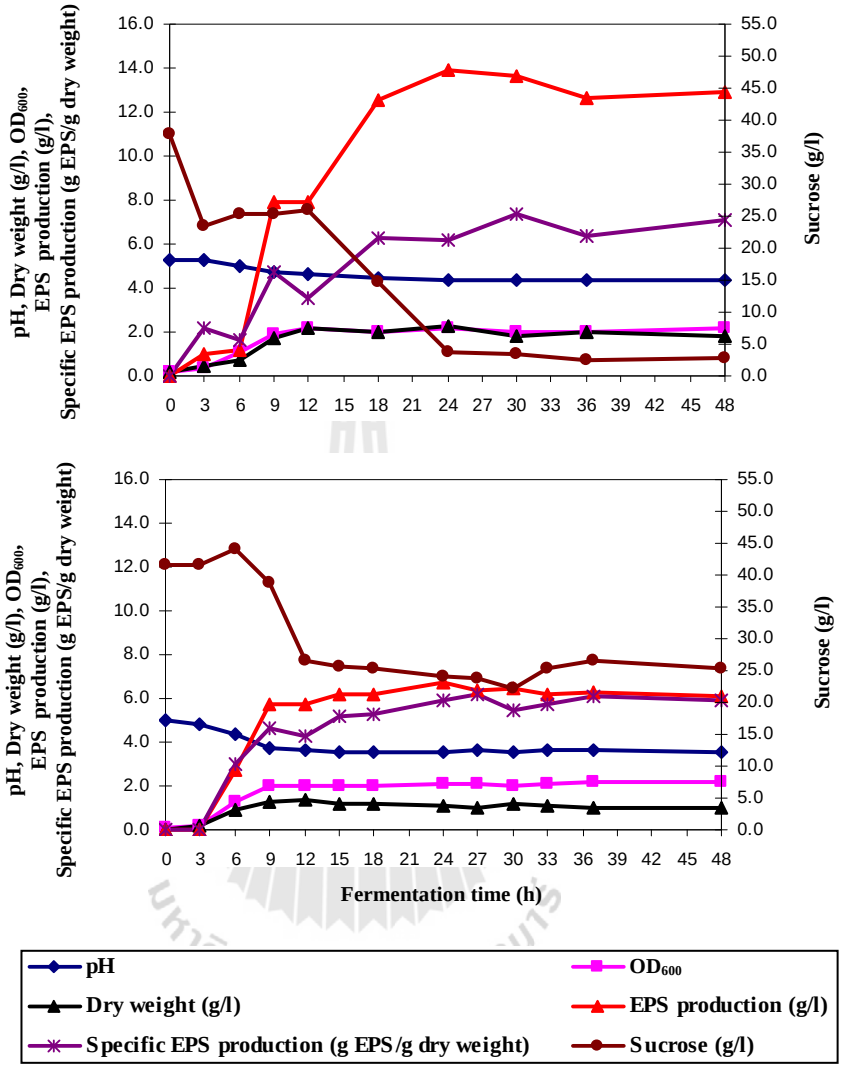

Figure 4.8 (C) Growth and EPS production by isolate PSMS1-5 in MRS medium containing 45 g/l of white sugar from sugar cane at initial pH of 5 and 30°C (a) and 37°C (b) for 48 h.

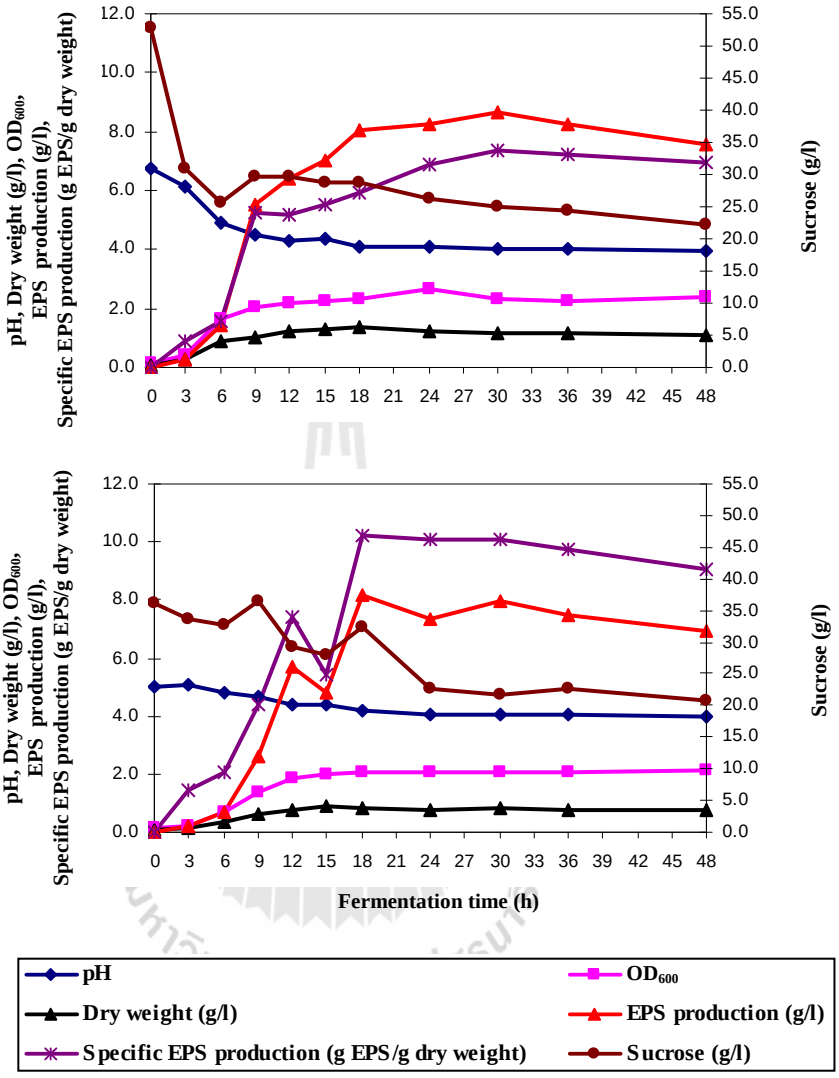

Figure 4.8 (D) Growth and EPS production by isolate PSMS4-4 in MRS medium containing 50 g/l of white sugar from sugar cane at initial pH of 5 (a) and 7 (b) at 30°C for 48 h.

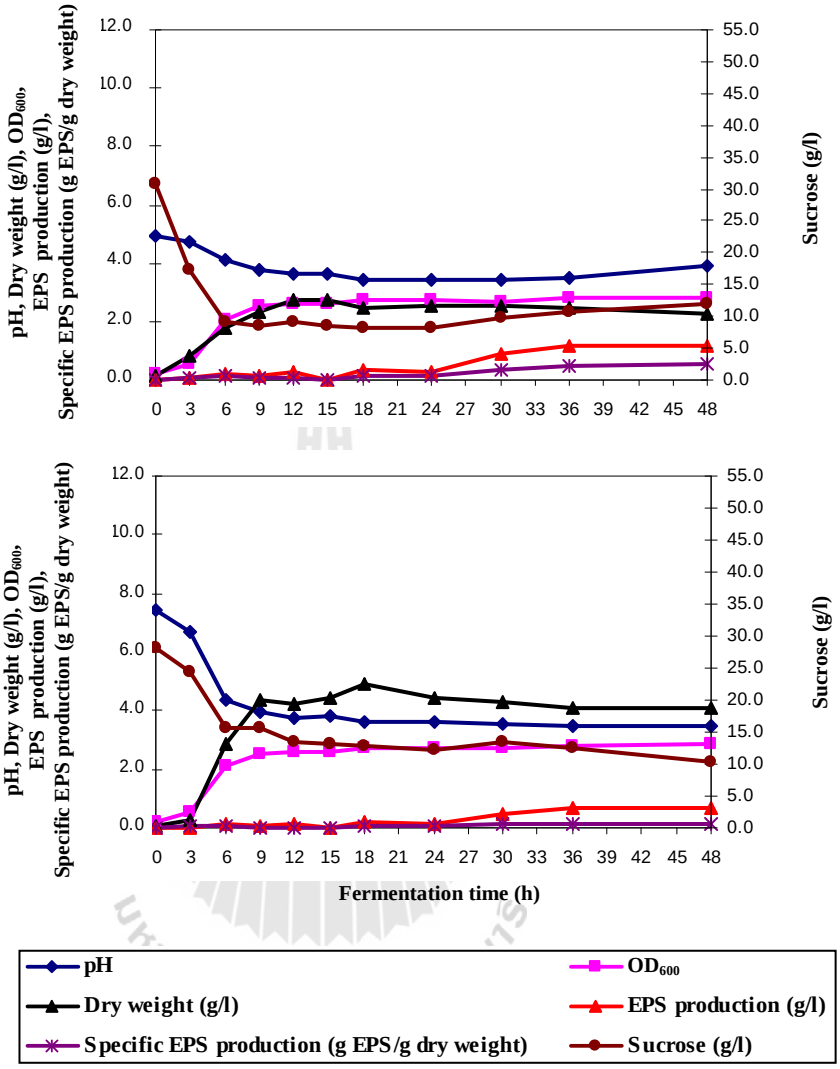

Figure 4.8 (E) Growth and EPS production by isolate RMS3-1 in MRS medium containing 30 g/l of white sugar from sugar cane at initial pH of 5 and 37°C (a) and 8 and 35°C (b) for 48 h.

EPS production in terms of % EPS Yield (Yp/s) ranging from 4.38-79.96% was obtained (Table 4.4). The maximum specific EPS production by *Lactobacillus casei* CRL 87 at a pH of 4.0 after 24 h of fermentation was 4.3% EPS yield. Dueñas *et al.* (2003) found that *Pediococcus damnosus* produced 0.6% EPS yield when culturing in SMD medium containing 30 g/l glucose, without pH control for 19 days at 25°C. *Lactobacillus delbrueckii* subsp. *bulgaricus* RR was reported to produce EPS with yield ranged from 0.013 to 0.046 g EPS/g lactose consumed or 1.3-4.6% EPS yield (Gassem *et al*., 1997).

Table 4.4 EPS yields and percentages of sucrose consumed when culturing in 500 ml Duran bottle under optimum conditions.

Isolate code	EPS production conditions			Fermentation time[b] (h)	EPS production (g/l)	Specific EPS production (g EPS/ g dry weight)	% EPS yield (Yp/s)[c]	% Consumed sucrose
	Carbon source[a] (%)	Initial pH of culture medium	Cultivation temperature (°C)					
FKU23	4.5	7	30	12	4.15	1.42	79.96	12.22
I5	3.5	6	40	18	0.84	1.07	16.99	17.08
I5	3.5	8	30	12	6.47	3.94	34.17	52.49
PSMS1-5	4.5	5	30	30	13.67	7.34	39.75	91.29
PSMS1-5	4.5	5	37	24	6.71	5.94	38.44	41.98
PSMS4-4	5	7	30	30	8.65	7.35	31.09	52.78
PSMS4-4	5	5	30	18	8.16	10.25	67.45	33.59
RMS3-1	3	5	37	36	1.18	0.48	5.87	64.93
RMS3-1	3	8	35	36	0.69	0.17	4.38	55.46

[a] Carbon source in MRS base medium for EPS production

[b] Fermentation time achieving maximum specific EPS production

[c] % EPS yield (Yp/s) = g EPS $\times$ 100/g consumed sucrose.

The isolate I5 was selected for large scale production, based on high % EPS yield, % sucrose consumed, short fermentation time and its impact on the immune system results (section 4.5). Approximately seventeen percent of sucrose consumed produced a 16.99% EPS yield. The maximum specific EPS production was obtained after 18 h of fermentation time. The EPS produced by isolate I5 stimulated the proliferation of lymphocytes as well as the production of IL-10 anti-inflammatory cytokine. EPS production conducted in 500 ml Duran bottle without shaking resulting in only seventeen percent of sucrose being consumed, therefore about 83% of residual sugar still remained in cultured medium. Agitation is important for adequate mixing, mass transfer and heat transfer. It not only assists mass transfer between the different phases present in the culture, but also maintains homogeneous chemical and physical conditions in the culture by continuous mixing (Kongkiattikajorn *et al.*, 2007). In order to improve production of the polymer, agitation was further applied for 5 l fermentation.

In additionally, the amount of inoculum used could affect the growth and EPS production. The production of the polymer by isolate I5 using various inoculum sizes (1, 2, 5, 7, and 10%) of culture (approximately 10^6 CFU/ml) was studied. The decrease in % EPS yield was observed at 5% up to 10% inoculum sizes (Figure 4.9). One percent and 2% inoculum sizes gave similar % EPS yields during 18 h of fermentation. Thus, one percent inoculum size was chosen for the production of EPS in fermenter.

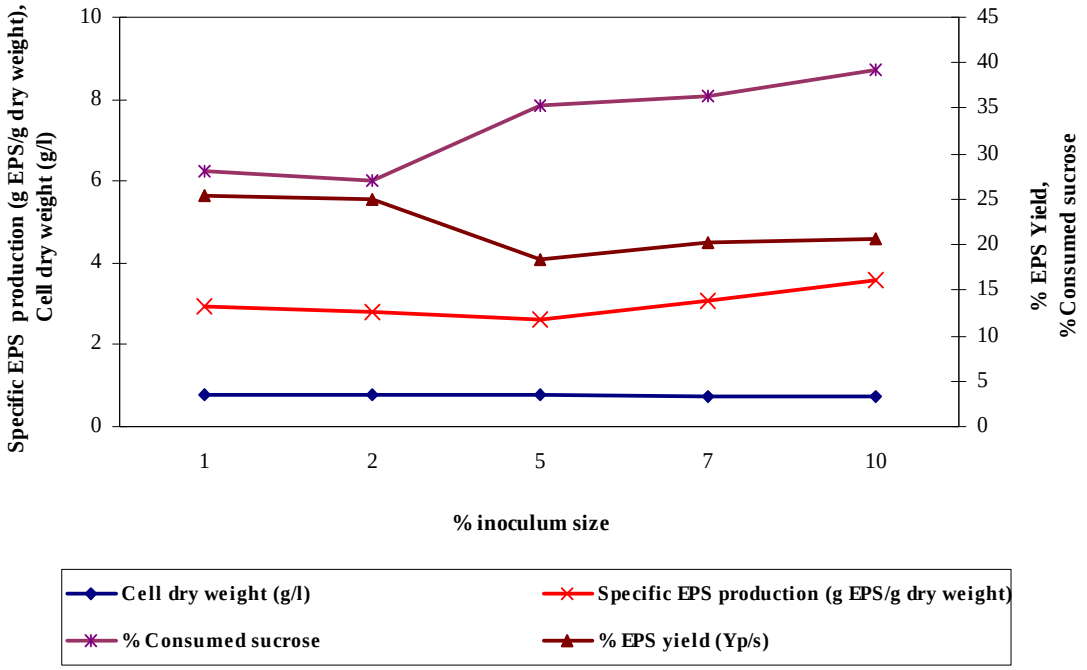

Figure 4.9 Effect of inoculum size on growth (cell dry weight) and EPS production of isolate I5 cultivated in MRS medium containing 35 g/l of white sugar from sugar cane at 40°C for 18 h.

4.3.2 EPS production in controlled fermenter

EPS production was performed in a 6.6 l controlled fermenter containing 5 l of MRS medium containing 35 g/l of white sugar from sugar cane. To keep the medium in the fermenter homogeneous and protect oxygen dissolved into the medium, agitation was performed at 100 rpm. The fermenter was inoculated with 1.0% (v/v) of a late log phase culture. Temperature and pH of the cultivation medium were kept constant at 40°C and 6.0 respectively. EPS production started at 3 h after inoculation and continuously increased until 24 h, which reached the maximum EPS yield of 12.29% (Figure 4.10). EPS production was accompanied by growth. When the bacterial growth reached stationary phase at 24 h of fermentation, specific EPS production reached the highest level of 1.19 g EPS/g dry weight (3.96 g/l).

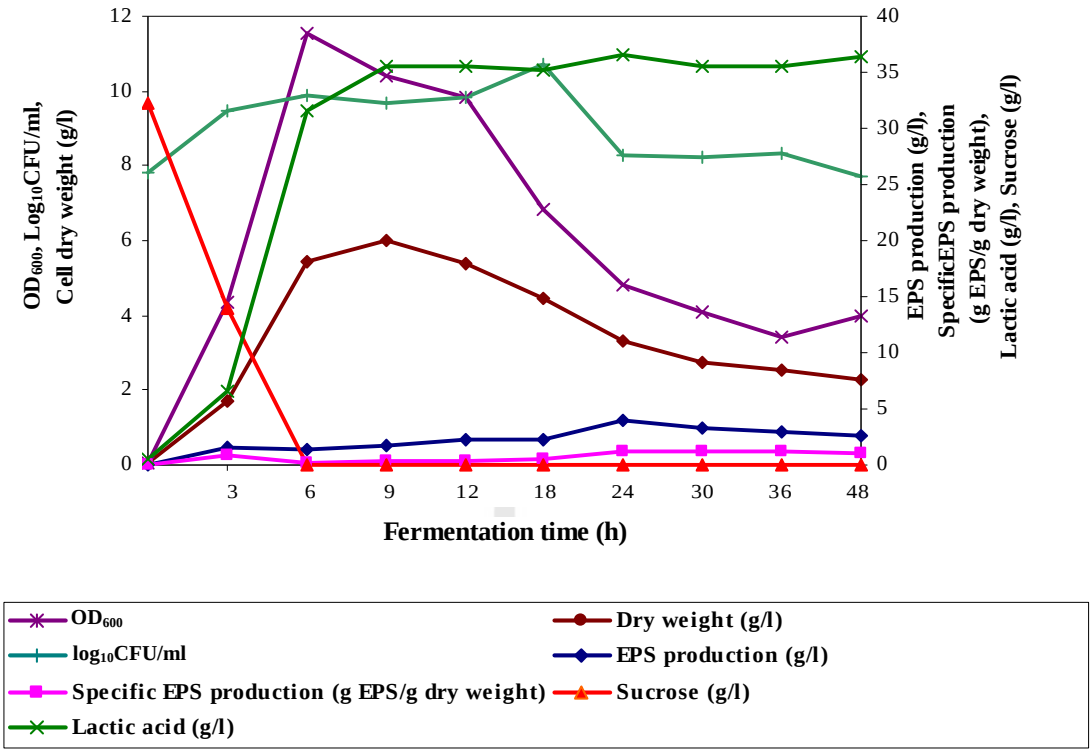

Figure 4.10 Growth and EPS production by isolate I5 at 40°C in a fermenter containing 5 l MRS medium with 35 g/l of white sugar from sugar cane and pH 6.

Bacterial growth was related to the increase in lactic acid. The maximum lactic acid of 36.49 g/l was achieved within 24 h, followed by constant values until the end of the experiment of 48 h. For substrate consumption, the complete consumption of sucrose was found within 6 h of fermentation. The result showed that available substrate limited bacterial growth.

To overcome substrate limitation, the optimum concentration of white sugar was investigated using 500 ml Duran bottle. EPS production was further improved after optimization of concentration of carbon source. The increase in concentration of

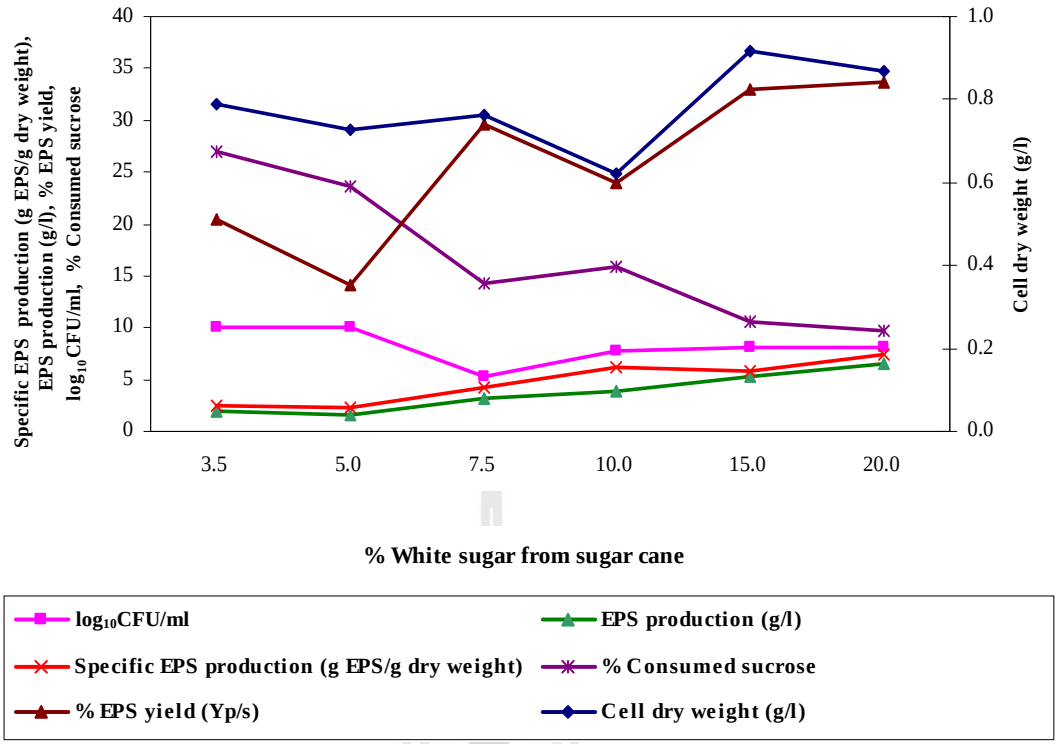

Figure 4.11 Effect of white sugar from sugar cane concentration on growth and EPS production by isolate I5 in MRS medium at 40°C for 18 h.

white sugar from sugar cane from 35 to 150 g/l provided the increase in EPS yield from 20.47% to 33.01% (Figure 4.11). Whereas the increase in concentration of white sugar from sugar cane to 200 g/l provided the increase in EPS yield to 33.74%. Therefore the concentration of 150 g/l was chosen for further study in the fermenter.

The optimum carbon source concentration was used for EPS production. The fermentation temperature and pH were kept constant at 40°C and pH 6.0. The maximum EPS yield of 42.66% was achieved at 30 h of fermentation. The maximum EPS production of 53.45 g/l (Specific EPS production of 26.08 g EPS/g dry weight) was obtained. Sucrose was completely consumed within 78 h (Figure 4.12).

A number of papers reported that EPS production using various sugar by lactic acid bacteria ranged from 60 to 150 mg/l for *Lactobacillus bulgaricus* (Cerning *et al.*, 1986; Garcia-Garibay and Marshall, 1991) and from 80 to 600 mg/l for *Lactococcus lactis* subsp *cremoris* (Cerning *et al.*, 1992). EPS production of 0.12-4.10 g/l by *Pediococcus parvulus* 2.6 was reported by Velasco (2006). *Lactobacillus sakei* strain 0±1 was isolated from a Belgian salami was able to produce 1.4 g/l of EPS (Van den Berg *et al*., 1995). The concentration of EPS of 2.13 g/l was obtained from *Lactobacillus delbrueckii* subsp. *bulgaricus* RR (Gassem, 1997). The EPS production (2.767 g/l) obtained using *Lactobacillus rhamnosus* RW-9595M in whey permeate medium (Macedo *et al*., 2002). However, to make EPS production profitable for the food industry, the productivity should be enhanced to at least 10-15 g/l (Van den Berg *et al*., 1995). Vijayendra and Babu (2008) showed that EPS production using medium containing sucrose at 5% (w⁄v) and 5% (v⁄v) inoculum, at pH 6.7 and 25°C for 4 h resulted in maximum production of heteropolysaccharide (18.38 g/l). Our study show that the isolate I5 has its potential for EPS production was obtained and could be apply for industry. The maximum EPS production of 53.45 g/l and specific EPS production of 26.08 g EPS/g dry weight were approximately 1.7 times higher than those reported for heteropolysaccharide production in other lactic acid bacteria (30.10 g/l and 13.87 g EPS/g dry weight, respectively) (Vijayendra and Babu, 2008).

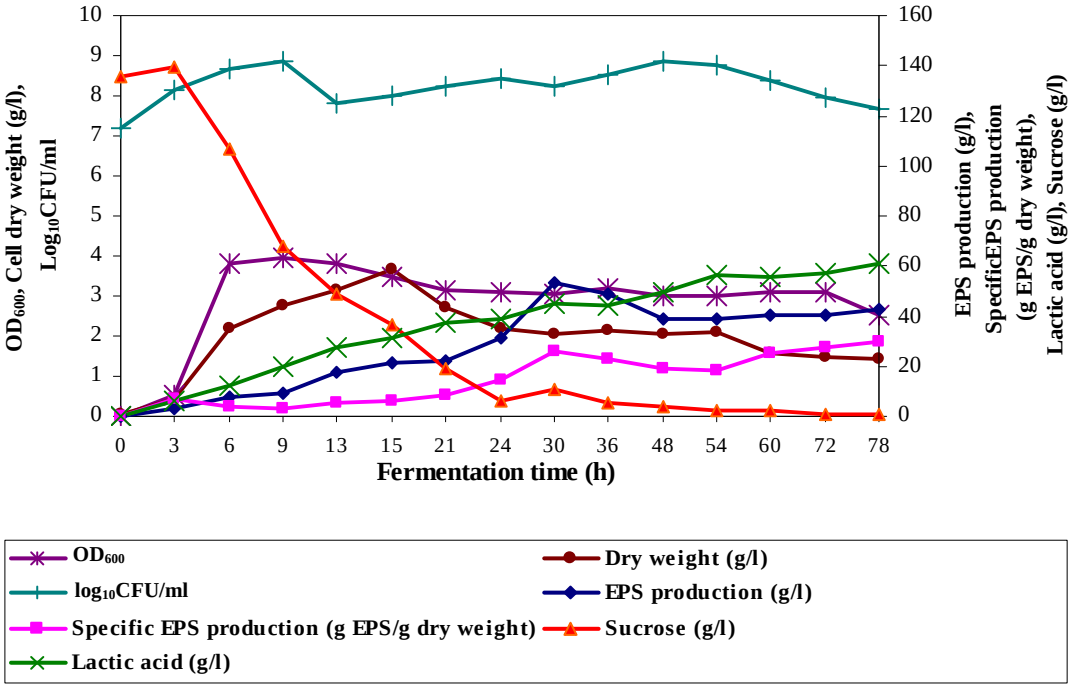

Figure 4.12 Growth and EPS production by isolate I5 in 5 l MRS medium containing 150 g/l of white sugar from sugar cane at pH 6 and 40°C in 6.6 l fermenter.

4.4 Exopolysaccharide purification

Since EPS produced by selected strains using different optimum conditions, there was an opportunity to obtain diverse EPS structures. The EPS were needed to be purified for further characterization and study on their impact on immune system. Crude EPS were precipitated from supernatant of cultured medium using 3 volume of 80% chilled ethanol. After lyophilization, protein content of the polymers was measured. Results showed that crude EPS contained 6.99-8.06% (w/w) protein

Table 4.5 Protein contents in crude EPS and purified EPS (after purification treatment) produced by the selected lactic acid bacterial isolates when culturing at optimum conditions.

EPS according to isolate code	EPS production conditions			% Protein contents		% Protein removal
	Carbon source[a] (%)	Initial pH of culture medium	Cultivation temperature (°C)	Crude EPS	Purified EPS	
C56	2% glucose	6.2	37	8.06±0.34	0.97±0.0	87.99
FKU23	4.5% white sugar from sugar cane	7.0	30	7.06±0.02	0.32±0.02	95.41
G3	2% white sugar from sugar cane	6.2	30	8.06±0.02	0.74±0.02	90.76
I5	3.5% white sugar from sugar cane	6.0	40	7.72±0.23	0.36±0.01	95.39
P14	2% analytical grade sucrose	6.2	30	7.72±0.00	0.58±0.02	92.45
PSMS1-5	4.5% white sugar from sugar cane	5.0	37	6.99±0.02	0.42±0.02	93.98
PSMS4-4	5% white sugar from sugar cane	7.0	30	7.73±0.80	0.26±0.16	96.65
RMS3-1	3% white sugar from sugar cane	8.0	35	7.39±0.05	0.74±0.0	89.98

[a] Carbon source in MRS base medium for EPS production.

Data are mean±SD for duplicate data.

Protein content was calculated as % weight by weight.

contents (Table 4.5). Smitinont *et al.* (1999) reported that two different strains of *Pediococcus pentosaceus* produced EPS comprised 90.3% w/w and 85.2% w/w

sugars while protein contents were found to be 1.0% w/w and 4.0% w/w. Macura and Townsley (1984) showed that *Lactococcus lactis* subsp. *cremoris* (formally *Streptococcus cremoris)* synthesizes slime material containing 47% protein and 29% carbohydrate and suggested a glycoprotein nature of the slime. Although there were many methods recommended for EPS purification by removal of protein, the most common procedure was TCA precipitation and centrifugation. Also a combination of TCA precipitation and protease digestion has been used for purification of the polymers (Ruas-Madiedo and de los Reyes-Gavilán, 2005). In this study, comparisons of various purification treatments for EPS purification were studied. Three approaches: precipitation with variable amounts of TCA, digestion with proteases, and the combination of protease digestion and TCA precipitation were used to reduce protein in the crude EPS. The protein content was determined according to the procedure of Bradford (1976) using bovine serum albumin standard. Different purification steps were compared as follows;

a) Trichloroacetic acid precipitation

Single step of trichloroacetic acid precipitation

Crude EPS sample was added with 20% TCA to final concentration of 0.05 mg/ml, then the mixture was incubated on ice for 1 h followed by centrifugation at 11,000 rpm for 10 min at 4°C. The EPS solution was dialyzed using Snakeskin™ pleated dialysis tube of molecular weight cut-off 10,000 against distilled water for 3 days, at 4°C, with two daily changes of water, then freeze-dried before determination of protein contents in the solution. The result showed that only 7.84% protein contents were removed indicated that the protein precipitation did not have notable effect for protein elimination.

Three repeated trichloroacetic acid precipitation

Protein in crude EPS was removed using 20% TCA as described above and repeated 3 times before dialysis and freeze-drying. After treatment, the protein content of 33.01 ug/mg EPS still remained in crude EPS. Without any protease digestion treatment, approximately 8 to 58% protein contents were removed from the crude EPS indicated that trichloroacetic acid precipitation alone was not efficient treatment for EPS purification (Table 4.6).

b) Protease digestion

Concentration of crude EPS sample was adjusted to 5 mg/ml using phosphate buffer pH 7.4, then Proteinase K (0.2 mg/ml) were added and incubated at 37°C for 16 h according to Zisu and Shah (2003). The enzyme reaction was stopped by heating at 90°C for 10 min and then centrifuged at 11,000 rpm for 10 min at 4°C. Protein content of the sample with protease treatment was 55.02%. Less than 30% protein content was removed by the single step protease digestion and also protein precipitation.

c) Combination of protease digestion and TCA precipitation

EPS was treated with Proteinase K as described in step b) followed by freeze-drying. Then the polymer was treated with 20% TCA as described in step a). The combination of protease digestion and TCA precipitation enabled to eliminate protein approximately 80% (Table 4.6).

d) Combination of 2 steps of protease digestion and TCA precipitation

EPS sample from step c) was added to a final concentration of 0.25 mg/ml Pronase E followed by freeze-drying according to Gancel and Novel (1994). The combination of protease digestion using both Proteinase K and Pronase E, and TCA

precipitation was the most efficient purification treatment of crude EPS. The method resulted in approximately 90% protein removal.

Then, the combination of protease digestion using both Proteinase K and Pronase E, and TCA precipitation was selected and applied for purification of EPS samples. After treatment, negligible amount of protein was detected, about 90% of protein content in EPS were removed. Less than 1% protein content still contained in EPS samples (Table 4.5).

When crude polysaccharide was applied to Sephacryl S-400 gel permeation chromatography, protein in EPS was observed within two of three polysaccharide fractions (Figure 4.13). This result could be used to explain that there were some proteins associated with polysaccharide. Some polysaccharides from plant have been reported to have protein associated with polysaccharide (Yang *et al*., 2008).

Table 4.6 Purification of exopolysaccharide produced by *Lactobacillus reuteri* 100-23.

Treatments of crude EPS	**Protein content (µg) in 1000 µg EPS**	**% Protein removal**
Crude EPS	78.48±0.00	0
TCA precipitation	72.33±1.83	7.84±2.33
3x TCA precipitation	33.01±1.14	57.94±1.46
Protease digestion	55.02±0.23	29.90±0.29
Combination of protease digestion and TCA precipitation	15.09±1.83	79.88±2.33
Combination of 2 steps of protease digestion and TCA precipitation	7.10±0.69	88.04±0.87

Data are mean±SD for duplicate data.

Protein content was calculated as % weight by weight.

However, the protein was removed after purification using both Proteinase K and Pronase E, and TCA precipitation (Figure 4.14).

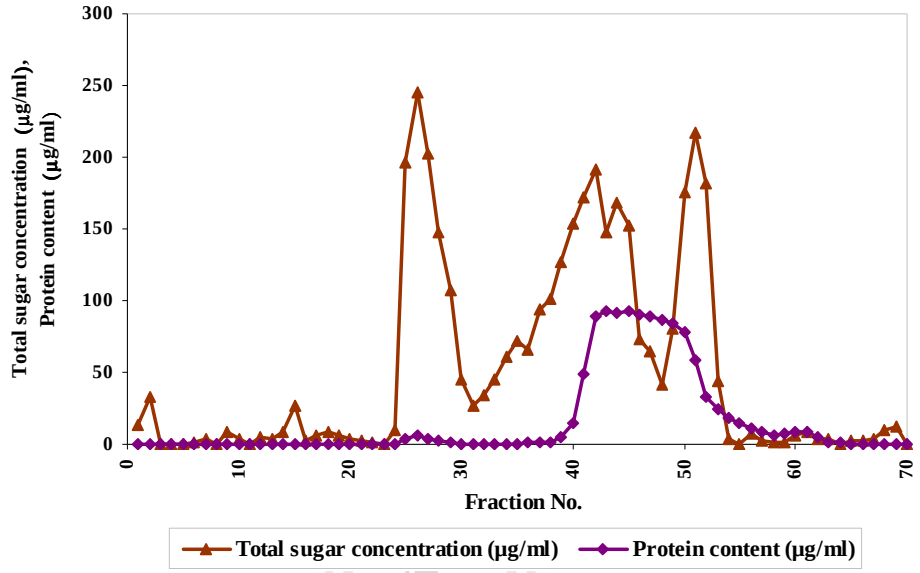

Figure 4.13 Gel filtration chromatogram of the crude polysaccharides produced by isolate I5 on Sephacryl S-400 column.

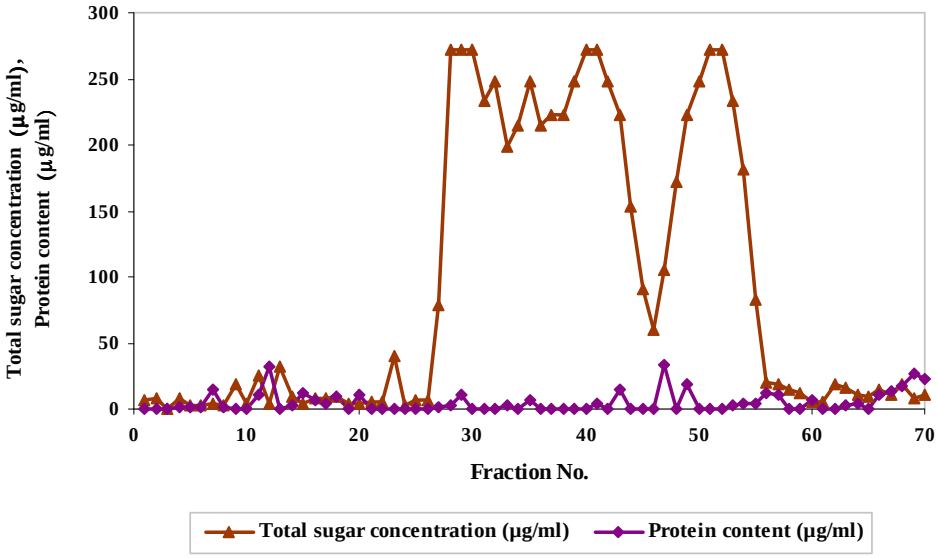

Figure 4.14 Gel filtration chromatogram of the purified polysaccharides produced by isolate I5 on Sephacryl S-400 column.

4.5 Immunostimulatory activity test

4.5.1 Optimization of exopolysaccharide concentration for immunostimulatory activity test

The optimal concentrations of EPS for mouse inoculation, and stimulation of mouse splenocytes *in vitro* were studied. Purified EPS from *Lactobacillus reuteri* 100-23, a positive control EPS, was used in this study. The optimum concentration of EPS for mouse inoculation was investigated by using various concentrations. One hundred microliters of each concentration (250, 500, 750, 1000, 1500, and 2,500 µg/ml) of the EPS in phosphate buffer saline pH 7.4 were injected intraperitonially. Each mouse received a booster dose of each concentration after 3 weeks of inoculation. After 5 weeks of inoculation, splenocytes were aseptically prepared and stimulated with 100 µl of EPS at different concentrations of 10, 25, 50, 75, and 100 µg/ml. Proliferation of lymphocytes in response to various concentrations of EPS was compared. The data demonstrated that optimal concentration of EPS for splenocyte stimulation *in vitro* was 75 µg/ml (Figure 4.15). Also one hundred microliters of 1,000 µg/ml used for mouse inoculation exhibited the highest lymphocyte response (Figure 4.16). Thus 100 µg of EPS was appropriate to use in the further mice inoculation. For this test, a *P* value of < 0.05 was considered significant.

4.5.2 Impact of exopolysaccharide produced by lactic acid bacteria on the immune system

4.5.2.1 Lymphocyte proliferation

Lymphocyte proliferation response to mitogens is widely used to assess immune function. ConA, the T-cell mitogen, was used as a positive control in

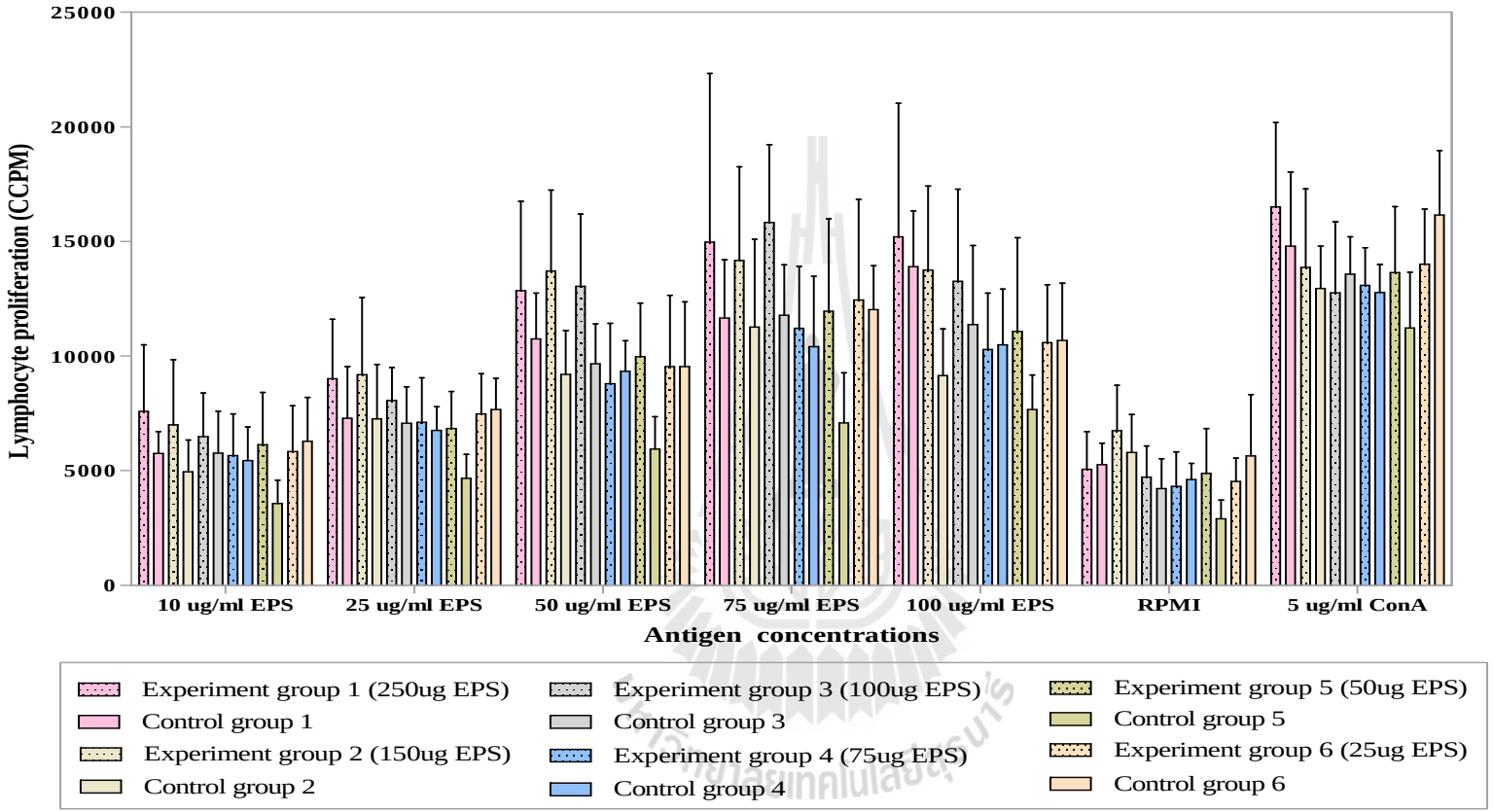

Figure 4.15 Optimum EPS concentration for splenocyte stimulation *in vitro*. Mice in experiment groups were inoculated with EPS at various concentrations (25-250 µg). Mice in control groups were inoculated with PBS. Splenocytes were exposed to 100 µl of EPS preparation at different concentrations (10-100 µg/ml), RPMI, and ConA (5 µg/ml). Values are expressed as mean and standard deviation.

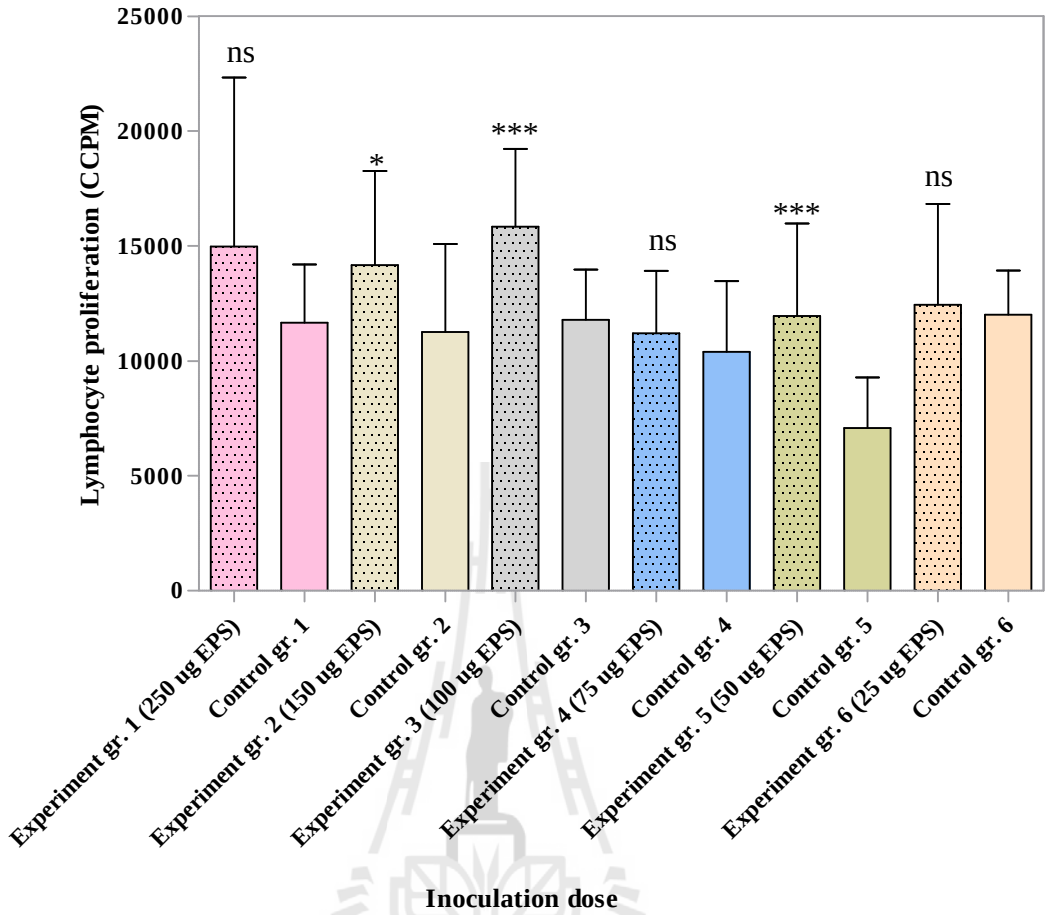

Figure 4.16 Optimum concentrations of EPS for mouse inoculation. The proliferation of splenocytes exposed to 100 µl of EPS (75 µg/ml) were compared. Error bars represent standard deviation of the mean; The differences of mean were calculated by the Mann Whitney U test, ***, Highly significant difference at $p < 0.001$; *, Significant difference at $P < 0.05$; ^{ns}, No significant difference, compared with control group.

this study. Eleven EPS samples derived from different bacterial isolates were tested for their ability to stimulate lymphocyte proliferative responses by detecting the proliferation of spleen cells of experiment mice inoculated with the EPS. EPS produced by the following isolates: C56 when cultivating in MRS containing 2%

Table 4.7 Lymphocyte proliferation responses to EPS and ConA.

EPS according to isolate code	EPS production conditions			EPS-stimulated conditions[b]		ConA-stimulated conditions[b]	
	Carbon source[a] (%)	Initial pH of culture medium	Cultivation temperature (°C)	Experiment group	Control group	Experiment group	Control group
C56	2% glucose	6.2	37	3.96	3.08	11.70	8.66
FKU23	4.5% white sugar from sugar cane	7.0	30	2.31	2.09	1.70	1.65
G3	2% white sugar from sugar cane	6.2	30	9.48	5.93	10.98	9.45
I5	2% glucose	6.2	37	3.04	1.91	7.58	6.22
I5	3.5% white sugar from sugar cane	6.0	40	13.96	12.19	6.06	7.91
I5	3.5% white sugar without yeast extract	6.0	40	7.22	19.61	2.77	21.93
P14	2% analytical grade sucrose	6.2	30	2.32	1.87	2.15	3.00
PSMS1-5	4.5% white sugar from sugar cane	5.0	37	1.66	1.62	3.73	3.82
PSMS4-4	5% white sugar from sugar cane	7.0	30	1.34	1.29	2.29	1.88
PSMS4-4	2% glucose	6.2	37	3.27	2.49	7.34	8.56
RMS3-1	3% white sugar from sugar cane	8.0	35	5.84	3.72	4.43	3.10
L. reuteri 100-23	2% analyical grade sucrose	6.2	37	3.6	3.2	4.21	3.46

[a] Carbon source in MRS base medium for EPS production.

[b] The stimulation index (SI) was expressed as fold stimulation (ratio of mean ccpm of culture with antigen/mean ccpm of culture without antigen).

of glucose at pH 6.2 and 37°C, FKU23 when culturing in MRS medium containing 4.5% of white sugar from sugar cane at pH 7 and 30°C, G3 when culturing in MRS medium containing 2.0% of white sugar from sugar cane at pH 6.2 and 30°C, I5 when culturing in MRS medium containing 3.5% of white sugar from sugar cane at pH 6 and 40°C, P14 when cultivating in MRS medium containing 2% of analytical grade sucrose at pH 6.2 and 30°C, PSMS4-4 when cultivating in MRS medium containing 2% of glucose at pH 6.2 and 37°C, RMS3-1 when cultivating in MRS containing 3% of white sugar from sugar cane at pH 8 and 35°C and I5 when cultivating in MRS medium containing 2% of glucose at pH 6.2 and 37°C, stimulated the proliferation of lymphocyte with the stimulation index (SI)>2 and higher than the control group as shown in Table 4.7. Also, EPS produced by the positive strains, *Lactobacillus reuteri* 100-23 when cultivating in MRS medium containing 2% of analytical grade sucrose at pH 6.2 and 37°C, stimulated the proliferation of lymphocyte. The highest proliferative response was obtained with EPS produced by isolate I5 when culturing in MRS medium containing 3.5% of white sugar from sugar cane at pH 6 and 40°C (SI=13.96, control=12.19). When monomer compositions of the polymer were considered, it was observed that mannose-rich EPS (63-91.44% mannose) from isolates C56, G3, I5, and RMS3-1 when cultivating under optimum conditions and isolate PSMS4-4 when cultivating in MRS medium containing 2% of glucose at pH 6.2 and 37°C exhibited lymphocyte proliferation stimulation. Whereas the EPS contained exclusively glucose as the main components could not stimulated cell proliferation as found in EPS from isolates PSMS4-4 when cultivating in MRS medium containing 5% of white sugar from sugar cane at pH 7.0 and 30°C and PSMS1-5 when cultivating in MRS medium containing 4.5% of white sugar from

sugar cane at pH 5.0 and 37°C. From these results demonstrated that mannose component affected lymphocyte proliferation. Contradictory result found in EPS contained 96.65% glucose and 3.35% mannose produced by isolate FKU23 and also EPS contained 96.58% glucose, 1.27% mannose, and 2.13% unknown sugar produced by isolate P14 could affect lymphocyte proliferation. Jiang *et al.* (2007) found that EPS composed of mannose, glucose and galactose with a molecular weight of 23 kDa could markedly facilitate lymphocyte proliferation, and might be a strong immunomodulator. A mannan from the cell wall of *Candida utilis*, and mannan-type polysaccharides from plant, bacterial, and fungal sources have been described to have immunomodulatory effects. As an example, acemannan, a polydispersed *β*-(1,4)-linked mannan, is an immunostimulant which causes macrophage activation (Ramamoorthy *et al*., 1996). Leung *et al.* (2004) found that the mannose-rich polysaccharide fractions prepared from *Aloe vera* L. *var. chinensis* (Haw.) Berg. stimulated peritoneal macrophages, splenic T and B cell proliferation. The potency of aloe polysaccharide fraction increases as mannose content and molecular weight of the polysaccharide fraction increase. The mechanism of activation of immunity by *β*-glucan may provide clues to the immune activation by mannose-rich polysaccharide. Dectin-1 is now thought to be the major receptor on leukocytes that mediates the biological effects of *β*-glucans as immune cell activators. Dectin-1 shows glucan-independent binding to a subset of T lymphocytes, and is involved in triggering their proliferation (Brown *et al*., 2003; Palma *et al*., 2006). Kalka-Moll *et al.* (2002) emphasized that bacterial polysaccharides with a zwitterionic charge spatial motif, such as capsular polysaccharides, elicit potent $CD4^+$ T-cell responses both *in vivo* and *in vitro*. Tzianabos *et al.* (2003) demonstrated that bacterial polysaccharides have the

ability to modulate the cellular immune system. They are structurally diverse, but all share a zwitterionic charge motif that allows them to directly interact with T cells and antigen-presenting cells to initiate an immunomodulatory T cell response. Zwitterionic polysaccharides bind to the surface of antigen presenting cells, and the high density of charges facilitates electrostatic interacions with T cells. Stingele *et al.* (2004) demonstrated that zwitterionic polysaccharides interact directly with T cells with rapid association/dissociation kinetics. The proliferative response of T cells depends on free amino (positively charged) and carboxyl or phosphate groups (negatively charged) that are part of the repeating unit structure. Chemical neutralization of either of the charged group results in loss of the ability of the polysaccharide to activate T cells (Tzianabos *et al.*, 2003; Tzianabos, 2000). One study suggested that phosphate groups in EPS produced by lactic acid bacteria could play important role in lymphocyte proliferation (Kitazawa *et al*., 1998).

4.5.2.2 Cytokine production

On activation by antigen, T cells synthesize and secrete a variety of cytokines that serve as growth, differentiation and activation factors for other immunocompetent cells. There are two main subsets of T lymphocytes, distinguished by the presence of cell surface molecules known as CD4 and CD8. T lymphocytes expressing CD4 are also known as helper T cells, and these are regarded as being the most prolific cytokine producers. This subset can be further subdivided into Th1 and Th2, and the cytokines they produce are known as Th1-type cytokines and Th2-type cytokines. Th1 cells produced interleukin-2 (IL-2), interferon gamma (IFN-g) and

Table 4.8 Effect of EPS produced by different lactic acid bacterial isolates on cytokine production.

EPS according to isolate code	Content of cytokine (pg/ml±SD)[a]											
	IL-4		IL-5		IL-10		IFN-g		TNF-a		IL-12 (p70)	
	Experimen group	Control group	Experiment group	Control group	Experiment group	Control group	Experiment group	Control group	Experiment group	Control group	Experiment group	Control group
C56, 2% glucose, pH 6.2, 37°C	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.48±1.08	7.63±12.72	-1.31±7.39	0.67±2.75	0.01±0.02	0.00±0.00
FKU23, 4.5% white sugar, pH 7.0, 30°C	0.38±0.85	0.00±0.00	0.00±0.00	0.00±0.00	16.80±4.05*	1.76±7.09	0.06 ±0.83	-0.60±0.70	2.36±4.38	-0.01±3.88	2.78±1.59	0.97±4.76
G3, 2% white sugar, pH 6.2, 30°C	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	24.74±18.88*	-18.74±5.13	-0.41±0.38	-0.41±0.38	-0.78±1.74	-1.56±2.13	-1.06±0.96	-0.68±1.55
I5, 2% glucose, pH 6.2, 37°C	0.02±0.03	0.00±0.00	7.80±39.40	0.00±0.00	1.14±1.56	1.14±1.56	-8.35±1.08	2.21±4.42	-3.02±3.51	3.59±5.83	-0.05±0.00	0.01±0.03
I5, 3.5% white sugar, pH 6.0, 40°C	0.00±0.00	0.02±0.05	0.00±0.00	0.00±0.00	218.49 ±92.02*	23.62 ±16.31	6.80±15.20	3.53±4.84	-5.51±5.51	4.37±3.01	0.02±0.03	0.02±0.03
I5, 3.5% white sugar without yeast extract, pH 6.0, 40°C	-1.97±0.64	0.00±0.00	-317.89 ±184.03	0.00±0.00	237.93 ±192.75	83.46 ±116.19	-19.60±0.00	3.92±8.77	-9.24±5.19	4.16±3.33	-1.06±1.55	0.00±0.00

[a] Background levels of cytokine production in cultures not stimulated with EPS were subtracted from the mean EPS-induced responses to produce secretion values. Negative values indicate that the unstimulated secretion levels were higher than the stimulated secretion levels. The data are expressed as means ± SD.

* Cytokine production in the experiment group was significantly higher than that in the control group ($P < 0.05$).

Table 4.8 (Continued) Effect of EPS produced by different lactic acid bacterial isolates on cytokine production.

EPS according to isolate code	Content of cytokine (pg/ml±SD) [a]											
	IL-4		IL-5		IL-10		IFN-g		TNF-a		IL-12 (p70)	
	Experimen group	Control group	Experiment group	Control group	Experiment group	Control group	Experiment group	Control group	Experiment group	Control group	Experiment group	Control group
P14, 2% analytical grade sucrose, pH 6.2, 30°C	0.35±0.77	0.00±0.00	0.19±0.43	0.00±0.00	9.65±2.90*	0.32±3.38	-0.01±0.48	0.42±0.76	3.89±0.00	-0.75±1.68	2.07±0.79	0.86±1.21
PSMS1-5, 4.5% white sugar, pH 5.0, 37°C	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	6.59±1.43*	-0.50±2.02	-0.41±0.38*	0.55±0.57	2.33±2.13*	-1.92±2.70	1.06±0.96*	-1.25±1.52
PSMS4-4, 5% white sugar, pH 7.0, 30°C	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	5.27±1.97	3.92±5.54	0.41±0.38	1.31±1.85	-2.33±2.13	6.78±4.08	-1.76±0.00	0.88±1.24
PSMS4-4, 2% glucose, pH 6.2, 37°C	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	6.06±13.54	0.00±0.00	1.77±3.95	0.00±0.00	-3.51±3.24	0.67±2.75	0.00±0.00	0.00±0.00
RMS3-1, 3% white sugar, pH 8.0, 35°C	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	19.76±18.57	0.00±0.00	1.77±3.95	1.77±3.95	2.75±2.75	-4.06±2.68	0.00±0.00	0.00±0.00
***L. reuteri* 100-23, 2% analyical grade sucrose, pH 6.2, 37°C**	0.17±0.38	0.00±0.00	0.00±0.00	0.00±0.00	11.87±5.03	9.15±2.90	0.26±0.58	0.00±0.38	3.11±1.74	2.33±2.13	2.95±0.79*	0.88±1.23

[a] Background levels of cytokine production in cultures not stimulated with EPS were subtracted from the mean EPS-induced responses to produce secretion values. Negative values indicate that the unstimulated secretion levels were higher than the stimulated secretion levels. The data are expressed as means ± SD.

* Cytokine production in the experiment group was significantly higher than that in the control group ($P < 0.05$).

tumor necrosis factor and are vital for cell-mediated immunity. On the other hand, Th2 cells predominantly produce interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-10 (IL-10) and are associated with humoral immunity and allergic response (Street and Mosmann, 1991). In this study, EPS of lactic acid bacterium origin stimulated cytokine production of mouse splenocytes (Table 4.8). The cytokines measured in the cultured supernatants showed an increase in the concentrations of IL-10, compared to control mice. Anti-inflammatory cytokine IL-10 production by stimulated splenocytes was obtained in response to EPS produced by the isolates FKU23, G3, P14, and PSMS1-5 when culturing at optimum conditions, and isolate I5 when cultivated in MRS medium containing 3.5% of white sugar from sugar cane at pH 6 and 40°C. Although EPS produced by isolate I5 when cultivated in MRS medium containing 3.5% of white sugar from sugar cane (without yeast extract) at pH 6 and 40°C, and *Lactobacillus reuteri* 100-23 stimulated the production of cytokines in experiment mice with no significant amount compared with control mice, IL-10 was produced in response to stimulation with the produced EPS. EPS produced by isolate P14 when cultivating in MRS medium containing of 2% of analytical grade sucrose at pH 6.2 and 30°C and isolate I5 when cultivated in MRS medium containing 3.5% of white sugar from sugar cane at pH 6 and 40°C had an immunostimulating activity since the EPS stimulated lymphoctytes proliferation and also augmented the IL-10 production. Results from this study showed that EPS produced by selected lactic acid bacterial isolates could stimulate the immune system and thus may increase the capacity of host against inflammations. This knowledge could be important with respect to a functional effect on human health if the EPS has incorporated into a food matrix.

4.6 Preliminary characterization of the exopolysaccharides

The study of the structure of EPS should be considered to understand their physicochemical and biological properties and exploit EPS-producing LAB in industrial or medical applications. Purified EPS were hydrolyzed and analyzed by high performance liquid chromatography (HPLC). Partial acid hydrolysis of the EPS yielded a complex mixture of oligosaccharides however a chromatographic run time of 35 min provided clear separation of all monosaccharide (Appendix C). D-glucose, D-mannose, D-fructose, D-galactose, L-rhamnose, *N*-acetylglucosamine and *N*-acetylgalactosamine were used as standard sugars. The results showed that varieties of monomer compositions of EPS were depending on EPS-producing isolates (Table 4.9). EPS-producing lactic acid bacterial strains including genera *Streptococcus*, *Lactobacillus*, *Leuconostoc*, and *Lactococcus*, have been reported to produce a wide variety of structurally different polymers, in a size that range from 4×10^4 to 6×10^6 Da (De Vuyst and Degeest, 1999). EPS produced by the isolate PSMS4-4 contained only glucose when cultivated in MRS medium with 5% white sugar from sugar cane as carbon source. Monosaccharides detected in EPS produced by isolates PSMS1-5 and FKU23 when cultivated in MRS containing 4.5% white sugar from sugar cane, were glucose and mannose (approximately 97.0 and 3.0%, respectively). The monomer compositions of 96.5% glucose, 1.30% mannose, and 2.13% unknown sugar was found in EPS produced by isolate P14 when cultivated in analytical grade sucrose as carbon source. Mannose-rich exopolysaccharide (63% mannose and 37% glucose) found for EPS produced by isolates RMS3-1 and G3 when cultivated in MRS medium containing white sugar from sugar cane. The EPS produced by C56 and PSMS4-4 composed of 77% mannose when cultivated in MRS medium containing

Table 4.9 Sugar composition of EPS produced by selected isolates under optimum conditions.

EPS according to isolate code	EPS production conditions			Composition of sugar[b] (%)		
	Carbon source[a] (%)	Initial pH of culture medium	Cultivation temperature (°C)	Glucose	Mannose	Unknown sugar
C56	2% glucose	6.2	37	22.85	77.15	-
FKU23	4.5% white sugar from sugar cane	7.0	30	96.65	3.35	-
G3	2% white sugar from sugar cane	6.2	30	36.96	63.04	-
I5	2% glucose	6.2	37	8.56	91.44	-
I5	3.5% white sugar from sugar cane	6.0	40	13.09	86.91	-
P14	2% analytical grade sucrose	6.2	30	96.58[c]	1.27[c]	2.13[c]
PSMS1-5	4.5% white sugar from sugar cane	5.0	37	96.92	3.08	-
PSMS4-4	2% glucose	6.2	37	22.81	77.19	-
PSMS4-4	5% white sugar from sugar cane	7.0	30	100.00	-	-
RMS3-1	3% white sugar from sugar cane	8.0	35	36.11	63.89	-

[a] Carbon source in MRS base medium for EPS production.

[b] Data are presented as percent molar composition.

[c] The relative proportion of the peak areas was calculated to estimate the monomer composition.

-, Negative detection.

2% glucose. Different contents of mannose (86 and 91% mannose) were found in EPS produced by isolate I5 when cultivated in MRS medium containing white sugar from sugar cane (3.5%) and glucose (2%), respectively. The composition of the EPS produced by lactic acid bacteria was strongly influenced by cultures and fermentation conditions. Kojic *et al*. (1992) and Cerning *et al*. (1994) reported that the sugar composition of EPS was influenced by carbon sources. Grobben *et al.* (2000) also found that sugar composition of the EPS was influenced by the type of carbon source. The type of substrate limitation had a remarkable influence on the molecular mass of EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and NIZO B891 (Looijesteijn *et al.*, 2000). However, Torino *et al*. (2005) found that the monomeric composition of the polymers produced by *Lactobacillus helveticus* ATCC 15807 is independent from the carbohydrate used. From our study, the isolate PSMS 4-4 produced homopolysaccharide containing only glucose when cultivated in the medium containing white sugar from sugar cane whereas heteropolysaccharide-EPS was found when the isolate was cultivated in the medium containing glucose as a carbon source. Also the isolate I5 produced polysaccharide composing of different components when different types of carbon source (white sugar from sugar cane compared to glucose) were used. This reveals that the organism is able to produce more than one type of polymer. It has been reported previously that the EPS produced by *Lactobaillus casei* CG11 in the presence of glucose was different from that formed in the presence of lactose (Cerning *et al.*, 1994). Not only types of carbon source, the polymer composition may vary depending on microbial growth conditions (Cerning, 1990; De Vuyst and Degeest, 1999). Results from this study exhibited that different fermentation conditions (initial pH of the culture medium and cultivation temperature)

affect the components of EPS. However, different microbial strains could produce the same type of EPS. For example, composition analysis of EPS produced by two strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* isolated at different growth phases and produced under different fermentation conditions (varying carbon source or pH) revealed that the component sugars were the same (Petry *et al.*, 2000). Based on microbial exopolysaccharide types, EPS are either homopolysaccharides or heteropolysaccharides in nature (De Vuyst and Degeest, 1999). The homopolysaccharides were found to consist of glucans, fructans and polygalactan. The production of these homopolysaccharides requires the presence of sucrose (Ruas-Madiedo *et al*., 2002). Most of homopolysaccharides were reported to be synthesized by extracellular glycansucrases using sucrose as the glycosyl (fructose or glucose) donor. From this study, PSMS4-4 produced homopolysaccharide contained only one type of monosaccharide (100% glucose) when using sucrose as carbon source. Maina *et al.* (2008) found that *W. confusa* E392 produced α-D-glucans named dextran which could be a suitable alternative to widely used in the production of linear dextran. *Leuconostoc*, *Lactobacillus*, and *Streptococcus* are known to synthesize dextran (Padmanabhan and Kim, 2002). Most EPS from LAB recorded are heteropolysaccharides. Their structures consist of a repeating unit containing three to seven sugar residues. The repeating units most often contain a combination of D-glucose, D-galactose, and L-rhamnose. In a few cases, L-fucose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-galactosamine, D-ribose, D-glucuronic acid, and D-nononic acid are present. Each hexose could adopt the pyranose or furanose ring configuration, and be linked with the α or β anomeric configuration to other residues at several possible positions. Furthermore, substituents, such as acetate, phosphate, glycerol or

pyruvate, could be present. Mannose was also found as a monomeric component of EPS produced by lactic acid bacteria, such as *Lactococcus lactis* subsp. *cremoris*, and *Lactobacillus casei* subsp. *casei* (Cerning, 1992). Aslim *et al.* (2006) reported that mannose dominated (99-100%) on the EPS produced by *Lactobacillu delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains in skim milk and MRS/M17 medium. Pavlova and Grigorova (1999) reported that the interaction between the polysaccharide synthesized by *Rhodotorula acheniorum* MC polymer (92.8% mannose and 7.2% glucose) and xanthan showed synergistic effect. The viscosity of a mannan–xanthan (20:80) mixture was 40% higher as compared to the xanthan alone. Proportions of glucose and mannose in EPS found in our study were different from EPS recorded. These biopolymers could offer the opportunity for new applications.

4.7 Identification of selected isolates of exopolysaccharide-producing bacteria

4.7.1 Morphological, cultural and physiological characterization

Twenty lactic acid bacterial isolates were selected according to their EPS-production abilities from section 4.2.1 for their morphological and physiological characterization. These selected isolates were Gram-positive, non-spore forming and catalase negative (Table 4.10). Sixteen, three and one isolates found to be rods, cocci and ovoid, respectively. Cultural and morphological charcteristics of some selected isolates were shown in Figure 4.17, 4.18 and 4.19. For biochemical characterization results compared to API50CH/CHL database, nine isolates of hetero-fermentative rods; C25, FLB1, PSMS1-1, PSMS2-4, PSMS3-6, PSMS4-4, PSMS5-1, SSMS1, and SSMS6 were identified as *Weissella confusa* with preciseness at 99.8, 99.6, 99.8, 99.9,

Table 4.10 Cell morphology and some physiological characteristics of selected isolates of EPS-producing lactic aid bacteria.

Isolate code	Source of bacterial isolation	Characteristics		
		Cell shape	Cell size (µm)	CO_2 production
C25	Chicken intestine	Rods, short chains	0.43-0.53 x 0.63-1.31	+
C27	Chicken intestine	Rods, short chains	0.40-0.52 x 0.62-0.89	+
C56	Chicken intestine	Rods, short chains	0.30-0.45 x 1.12-1.31	-
C58	Chicken intestine	Rods, short chains	059-0.76 x 1.16-1.70	-
FKU5	Pla-som	Rods, short chains	0.42-0.63 x 0.83-1.15	+
FKU23	Pla-som	Rods, short chains	0.43-0.63 x 0.76-1.41	+
FLB1	Pla-som	Rods, short chains	0.45-0.52 x 0.55-1.38	+
G3	Tapioca waste	Cocci, short chains	0.50-0.59 x 0.59-0.80	-
I5	Tapioca waste	Cocci, short chains	0.43-0.52 x 0.52-0.78	-
NHMS3	Nham	Rods, short chains	0.43-0.55 x 0.81-1.50	-
P14	Pig intestine	Cocci, short chains	0.78-0.88 x 0.98-1.26	-
PSMS1-1	Pla-som	Rods, short chains	0.35-0.40 x 0.79-1.16	+
PSMS1-5	Pla-som	Ovoids, short chains	0.31-0.42 x 0.56-1.17	+
PSMS2-4	Pla-som	Rods with a thicken at one end, short chains	0.41-0.76 x 0.90-1.70	+
PSMS3-6	Pla-som	Rods with a thicken at one end, short chains	0.40-0.59 x 0.83-1.36	+
PSMS4-4	Pla-som	Rods, short chains	0.66-0.74 x 1.19-1.25	+
PSMS5-1	Pla-som	Rods, short chains	0.53-0.66 x 0.90-1.07	+
RMS3-1	Rice grain	Rods, short chains	0.54-0.69 x 0.89-1.53	+
SSMS1	Sai-krork-prieo	Rods, short chains	0.38-0.50 x 0.74-1.20	+
SSMS 6	Sai-krork-prieo	Rods, short chains	0.49-0.66 x 0.73-1.26	+

Symbols: +, positive; -, negative.

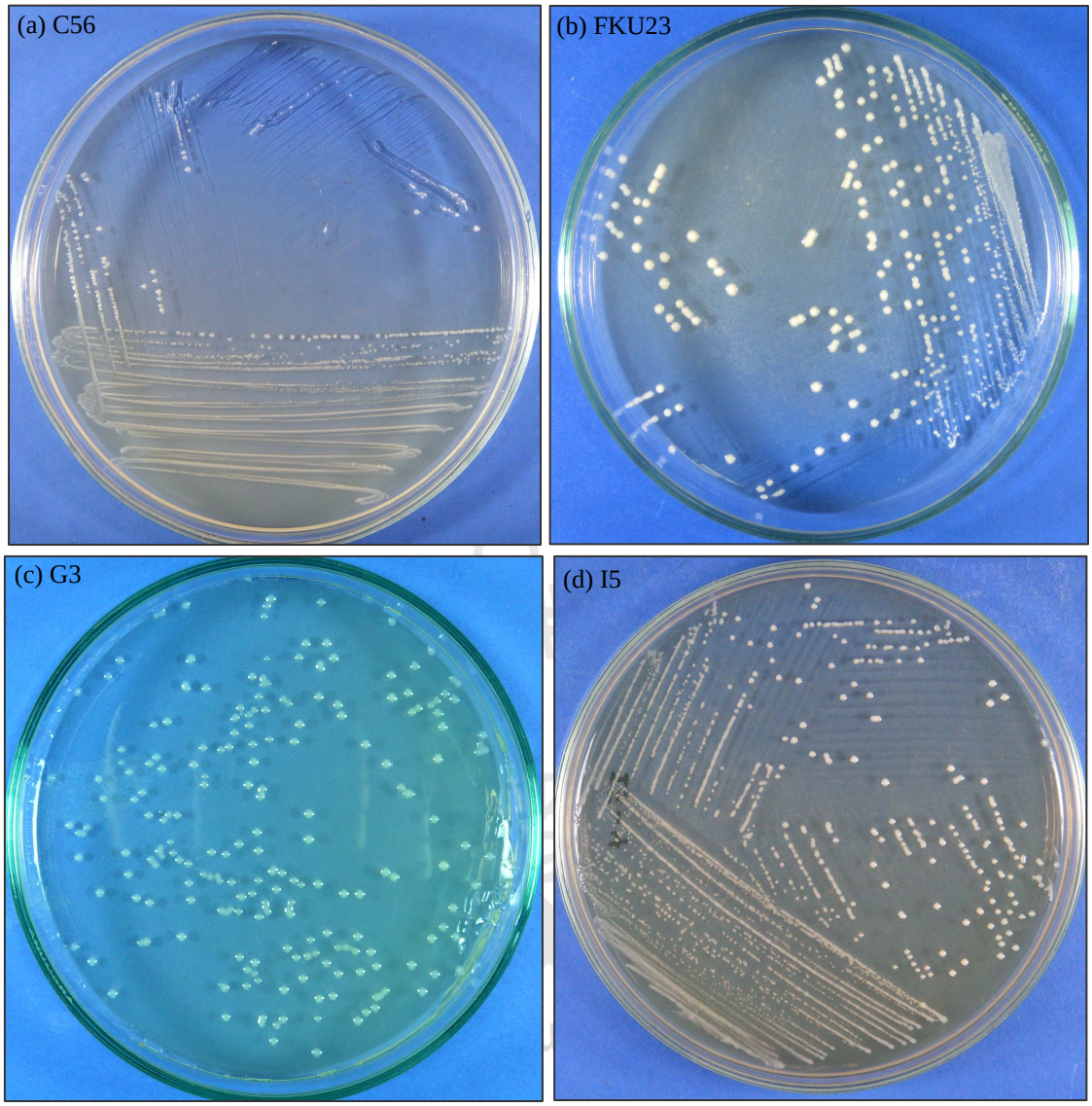

Figure 4.17 Colony morphology of lactic acid bacterial isolates (a) C56, (b) FKU23, (c) G3, and (d) I5 on MRS agar at 30°C for 48 h.

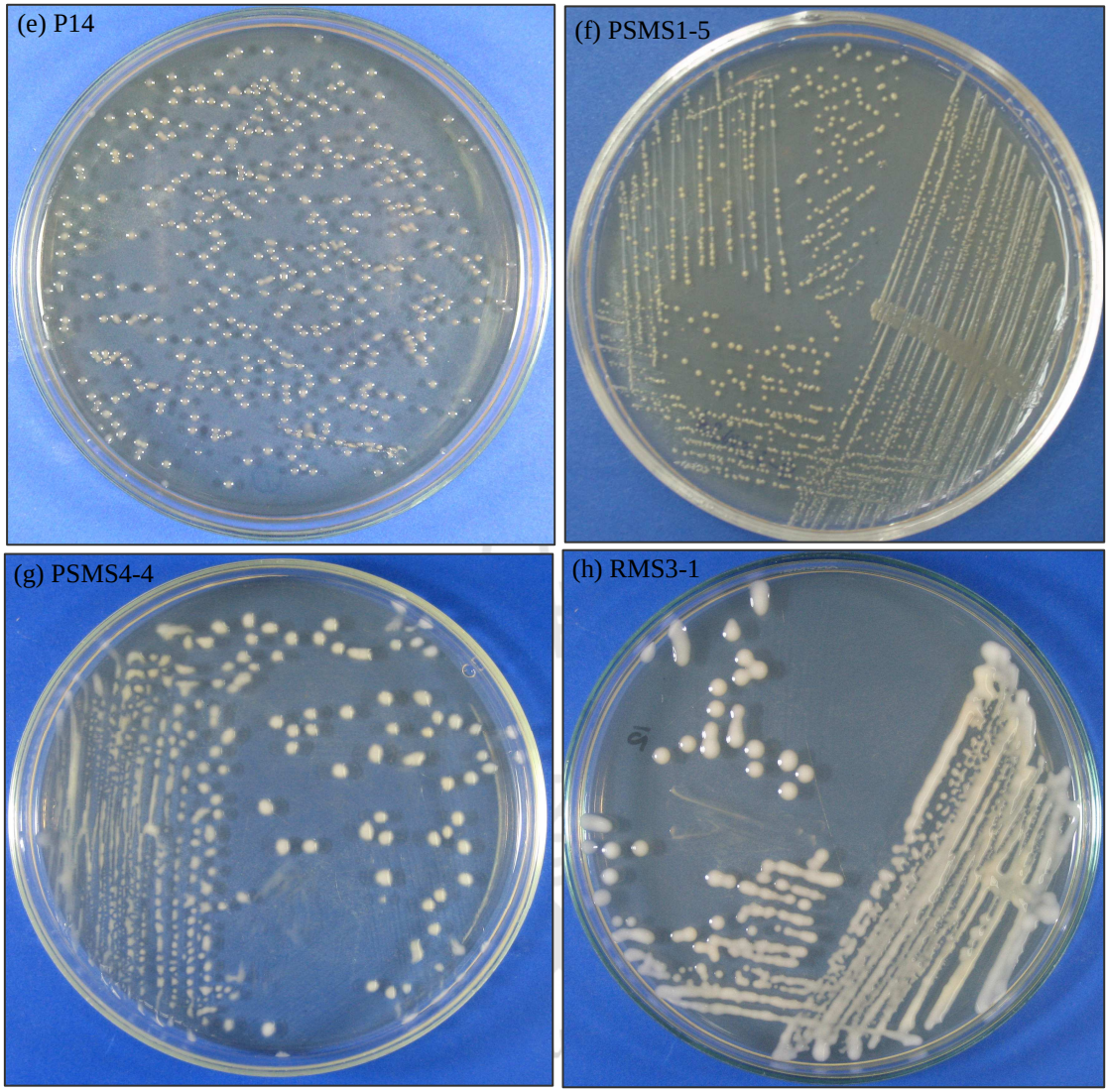

Figure 4.18 Colony morphology of lactic acid bacterial isolates (e) P14, (f) PSMS1-5, (g) PSMS4-4, and (h) RMS3-1 on MRS agar at 30°C for 48 h.

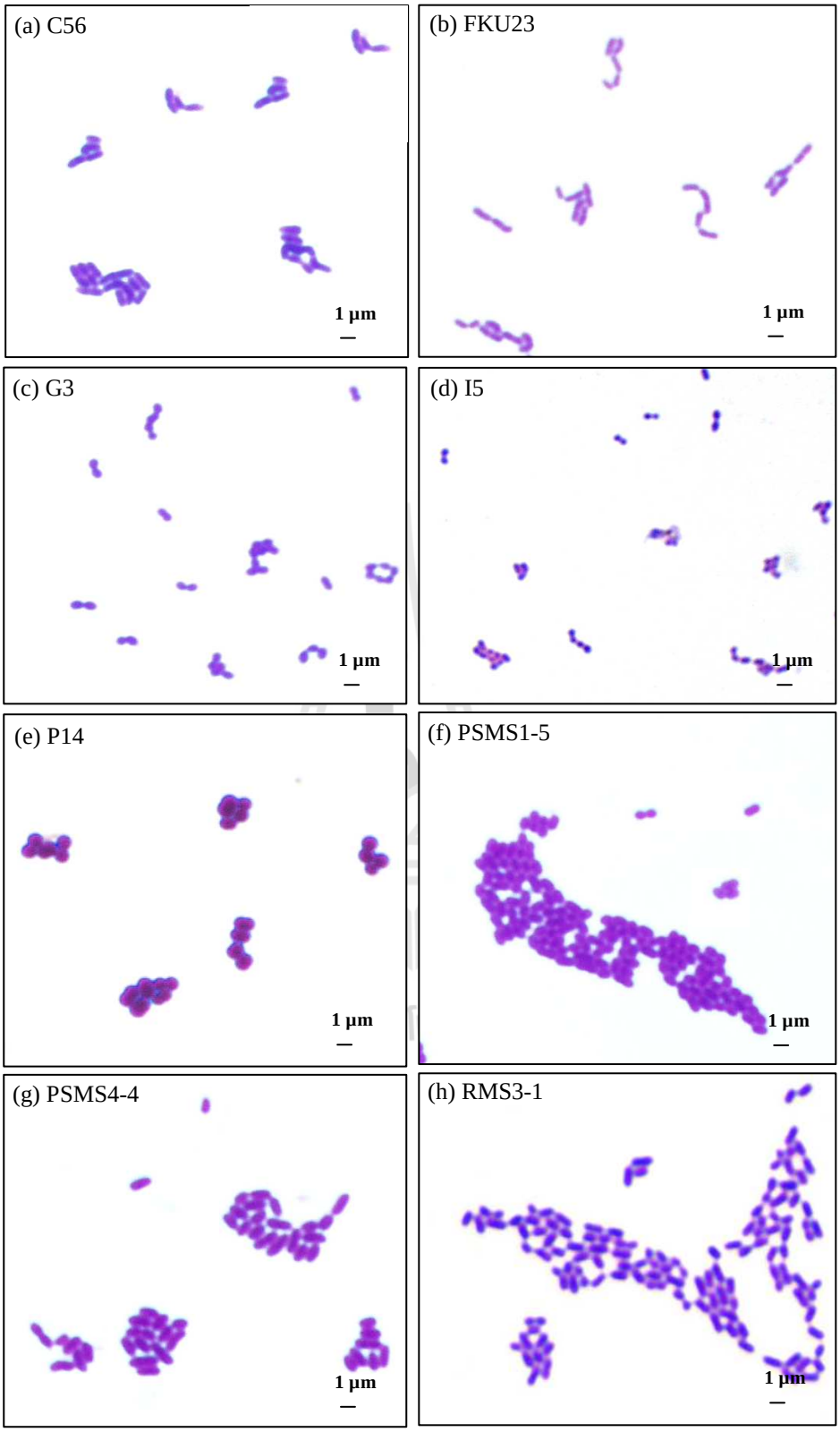

Figure 4.19 Cell morphology from Gram stain of lactic acid bacterial isolates (a) C56, (b) FKU23, (c) G3, (d) I5, (e) P14, (f) PSMS1-5, (g) PSMS4-4, and (h) RMS3-1.

Table 4.11 Identification results of selected lactic acid bacterial isolates according to biochemical characteristics.

Isolate code	Identification (% identity) According to API 50CH/CHL system (Biome´rieux, France)
C25	*Weissella confusa* (99.8%) *Lactobacillus delbrueckii* subsp. *delbrueckii* (58.9%)
C27	*Leuconostoc mesenteriodes* subsp. *mesenteriodes/dextranicum* 2 (99.9%)
C56	*Lactobacillus salivarius* (99.7%)
C58	*Lactobacillus salivarius* (99.9%)
FLB1	*Weissella confusa* (99.6%) *Lactobacillus acidophilus* 3 (78.2%) *Lactobacillus delbrueckii* subsp. *delbrueckii* (58.9%)
FKU5	*Leuconostoc mesenteriodes* subsp. *mesenteriodes/dextranicum* 2 (99.9%)
FKU23	*Leuconostoc mesenteriodes* subsp. *mesenteriodes/dextranicum* 2 (98.4%)
G3	*Lactobacillus crispatus* (99.9%)
I5	*Lactobacillus acidophilus* 2 (99.6%)
NHMS3	*Lactobacillus pentosus* (99.9%)
P14	*Lactococcus lactis* subsp. *lactis* 1 (98.6%) *Pediococcus pentosaceus* 2 (95.8%) *Pediococcus pentosaceus* 1 (91.2%)
PSMS1-1	*Weissella confusa* (99.8%) *Lactobacillus brevis* 1 (94.5%) *Weissella confusa* (65.1%)
PSMS1-5	*Leuconostoc mesenteriodes subsp. mesenteriodes/dextranicum* 2 (99.1%) *Lactobacillus brevis* 1 (99.5%) *Leuconostoc citreum* (93.7%)
PSMS2-4	*Weissella confusa* (99.9%) *Lactobacillus acidophilus* 3 (83.8%) *Lactobacillus delbrueckii subsp. delbrueckii* (15.9%)
PSMS3-6	*Weissella confusa* (99.7%) *Lactobacillus acidophilus* 3 (92.5%)
PSMS4-4	*Weissella confusa* (99.2%) *Lactobacillus acidophilus* 3 (83.6%) *Lactobacillus brevis* 1 (63.5%)
PSMS5-1	*Weissella confusa* (99.2%) *Lactobacillus brevis* 1 (94.5%) *Lactobacillus acidophilus* 3 (73.1%)

Table 4.11 (Continued) Identification results of selected lactic acid bacterial isolates according to biochemical characteristics.

Isolate code	Identification (% identity) according to API 50CH/CHL system (Biome´rieux, France)
SSMS1	*Weissella confusa* (99.8%) *Lactobacillus delbrueckii* subsp. *delbrueckii* (98.6%)
SSMS6	*Weissella confusa* (94.4%) *Lactobacillus delbrueckii* subsp. *delbrueckii* (74.1%)
RMS3-1	*Lactobacillus brevis1* (99.9%)

99.7, 99.2, 99.2, 99.8, and 94.4% homology, respectively (Table 4.11). Three isolates C27, FKU5, and FKU23, were heterofermentative, and could be identified as belonging to the genus *Leuconostoc*. Their biochemical profiles were closed to *Leuconostoc mesenteriodes* at 99.9, 99.9, and 98.4% homology, respectively. Isolate PSMS1-5, the heterofermentative ovoid, was identified as *Leuconostoc mesenteriodes*, *Lactobacillus brevis*, *Leuconosnoc citreum* (99.1, 99.5, and 93.7% respectively). Two isolates C56 and C58 were similar with *Lactobacillus salivarius* (99.7 and 99.9% respectively). Isolate NHMS3 was identified as *Lactobacillus pentosus* (99.9%). Isolate RMS3-1 was identified as *Lactobacillus brevis* (99.9%).

Amongst the twenty EPS-producing isolates, a Gram-positive coccus isolate; P14, was identified as *Lactococcus lactis* and *Pediococcus pentosaceus* (98.6 and 95.8% respectively). The isolates I5 and G3 gave 99.6 and 99.9% homology with *Lactobacillus acidophilus* and *Lactobacillus crispatus* from API50CH/CHL database, respectively. Boyd *et al.* (2005) mentioned that API50CH/CHL database could lead to occasionally misidentification or uninterpretable results for *Lactobacillus* species. Eight potential EPS-producing isolates; C56, FKU23, I5, P14, PSMS1-5, PSMS4-4,

and RMS3-1, were chosen for further characterization using 16S ribosomal RNA sequencing technique.

4.7.2 Sequencing of 16S ribosomal RNA gene

Eight potential EPS-producing isolates were identified using 16S ribosomal RNA sequencing technique (Weisburg *et al.*, 1991). The gene was amplified from genomic DNA using fD1/rP2 primers. The expected size of amplified DNA fragments obtained were approximately 1,500 bp (Figure 4.20).

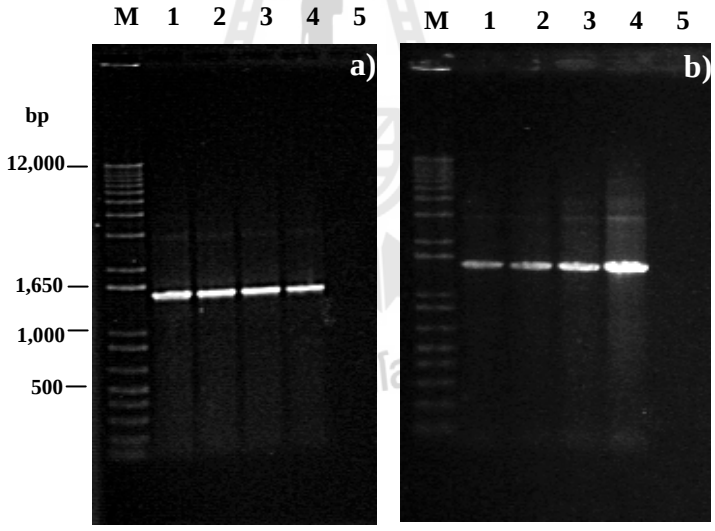

Figure 4.20 Gel electrophoresis of PCR products obtained from the amplification of lactic acid bacterial 16S rRNA gene using primers fD1and rP2. Lanes: M, Molecular weight marker (1 kb plus DNA ladder, Invitrogen); 1a, bacterial isolates C56; 2a, G3; 3a, I5; 4a, P14; 5a, negative control; 1b, P14; 2b, PSMS1-5; 3b, PSMS4-4; 4b, RMS3-1, and 5b, negative control.

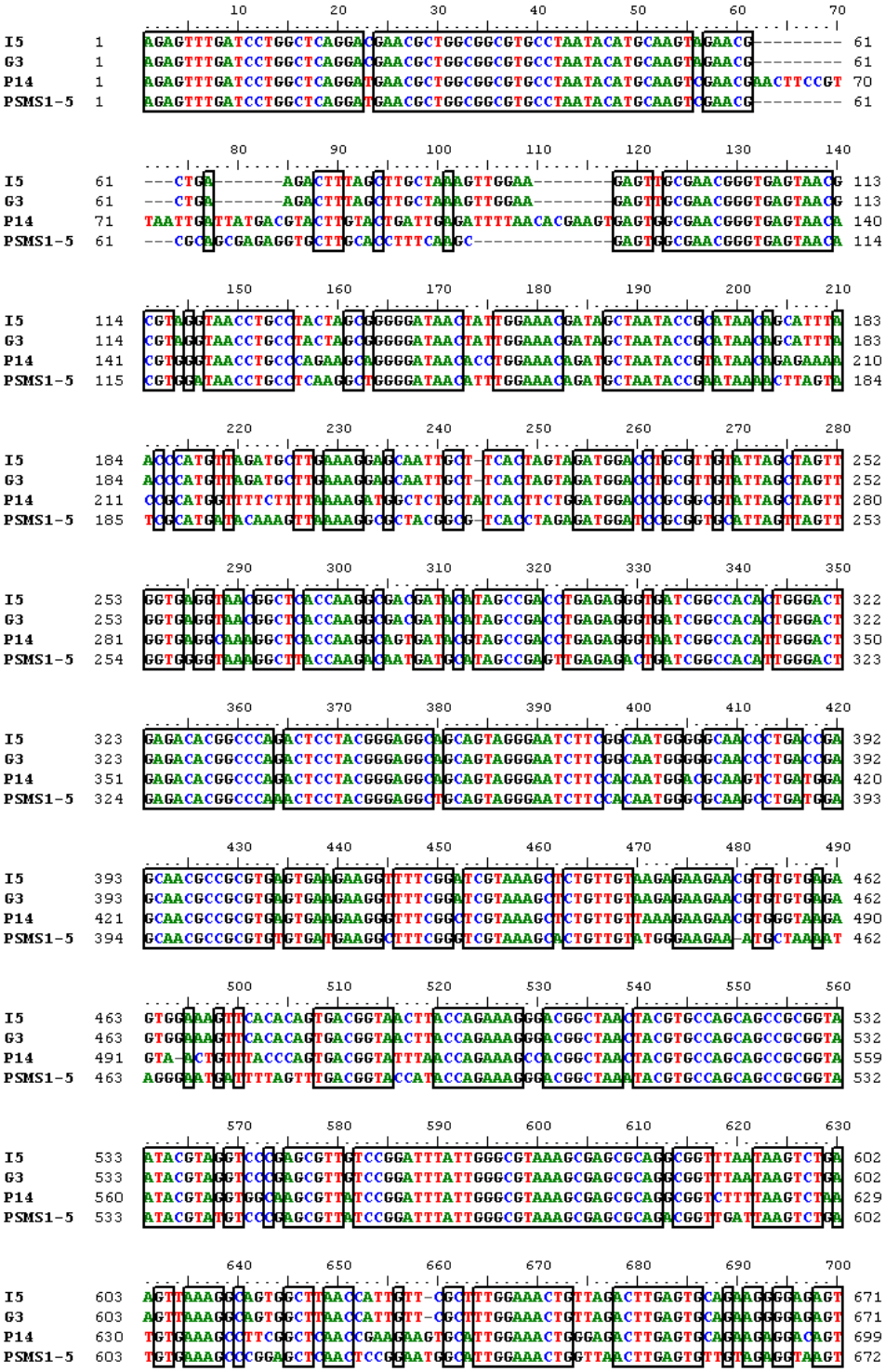

Figure 4.21 Sequence alignment of 16S rRNA gene of Gram-positive lactic acid bacterial cocci: isolates I5, G3, P14, and PSMS1-5, by using ClustalW and BioEdit programs. Blocks indicate the conserved nucleotides.

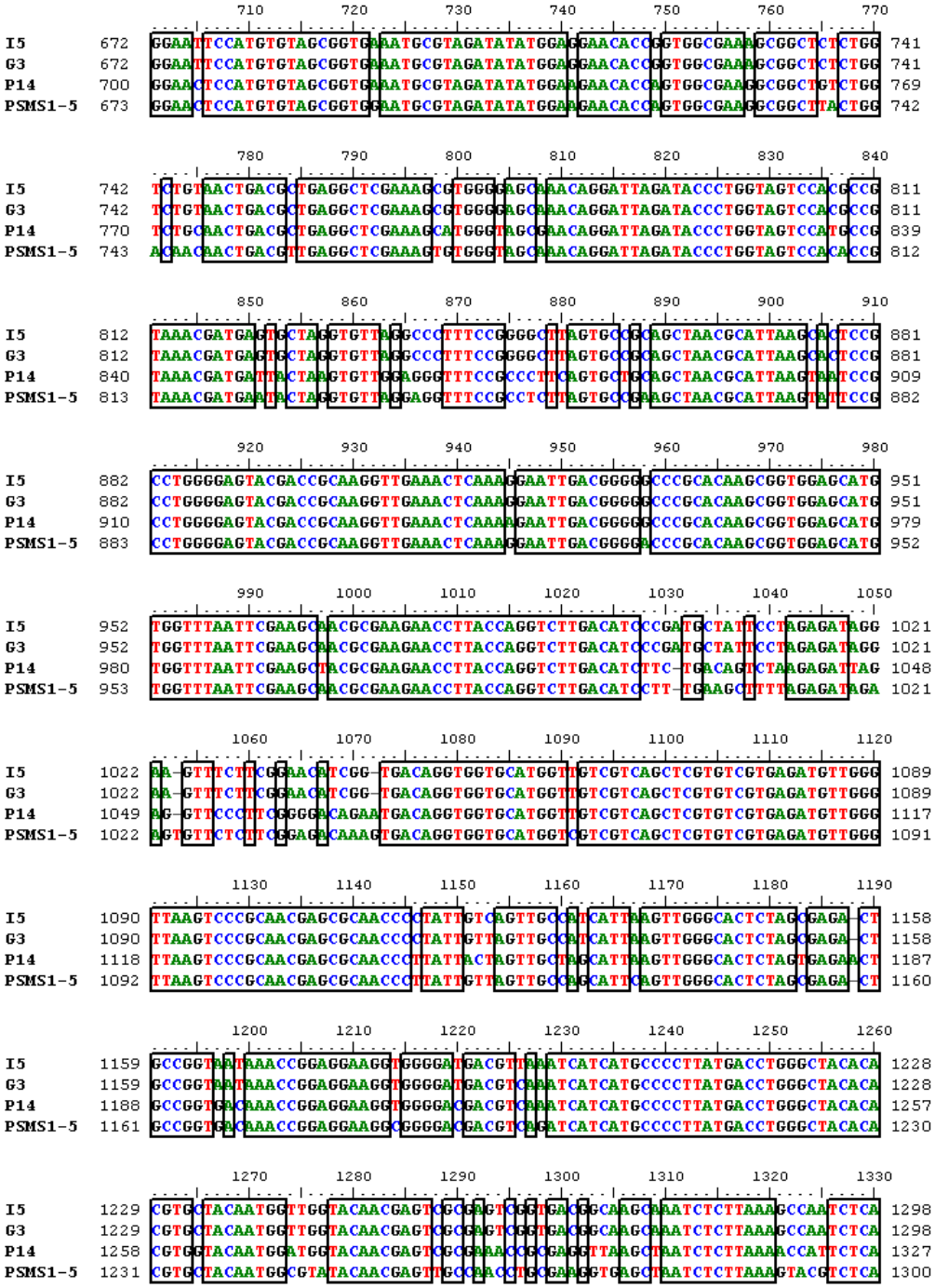

Figure 4.21 (Continued) Sequence alignment of 16S rRNA gene of Gram-positive lactic acid bacterial cocci: isolates I5, G3, P14, and PSMS1-5, by using ClustalW and BioEdit programs. Blocks indicate the conserved nucleotides.

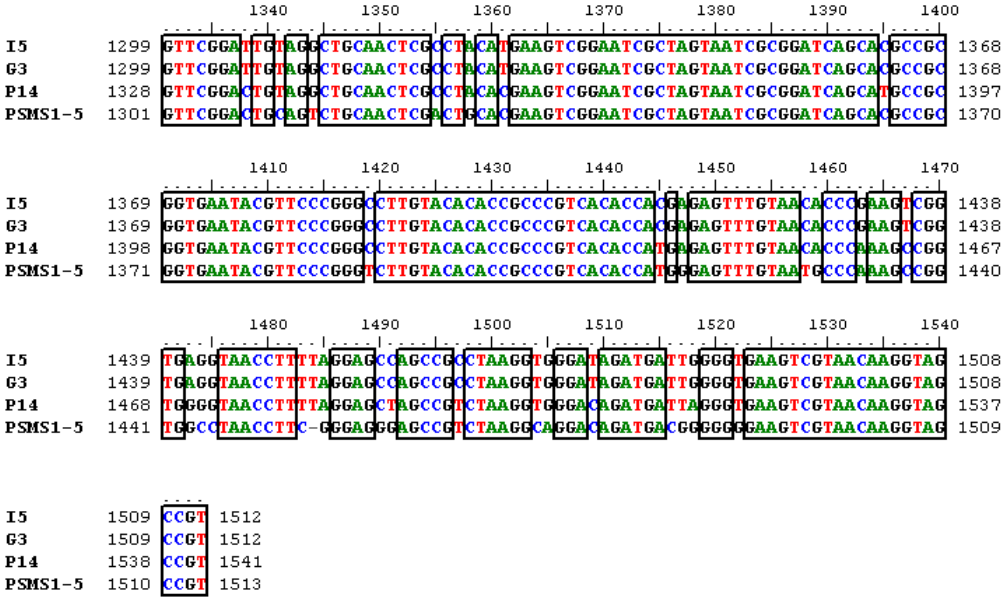

Figure 4.21 (Continued) Sequence alignment of 16S rRNA gene of Gram-positive lactic acid bacterial cocci: isolates I5, G3, P14, and PSMS1-5, by using ClustalW and BioEdit programs. Blocks indicate the conserved nucleotides.

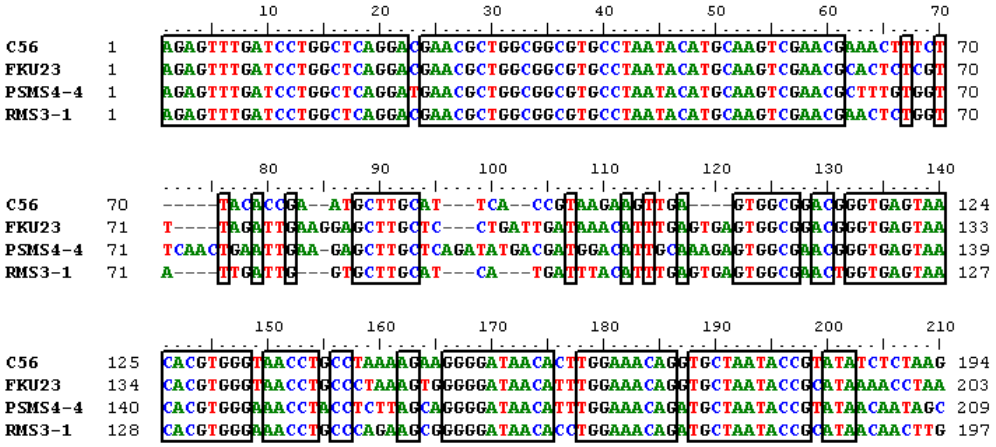

Figure 4.22 Sequence alignment of 16S rRNA gene of Gram-positive lactic acid bacterial rods: isolates C56, FKU23, PSMS4-4, and RMS3-1, by using ClustalW and BioEdit programs. Blocks indicate the conserved nucleotides.

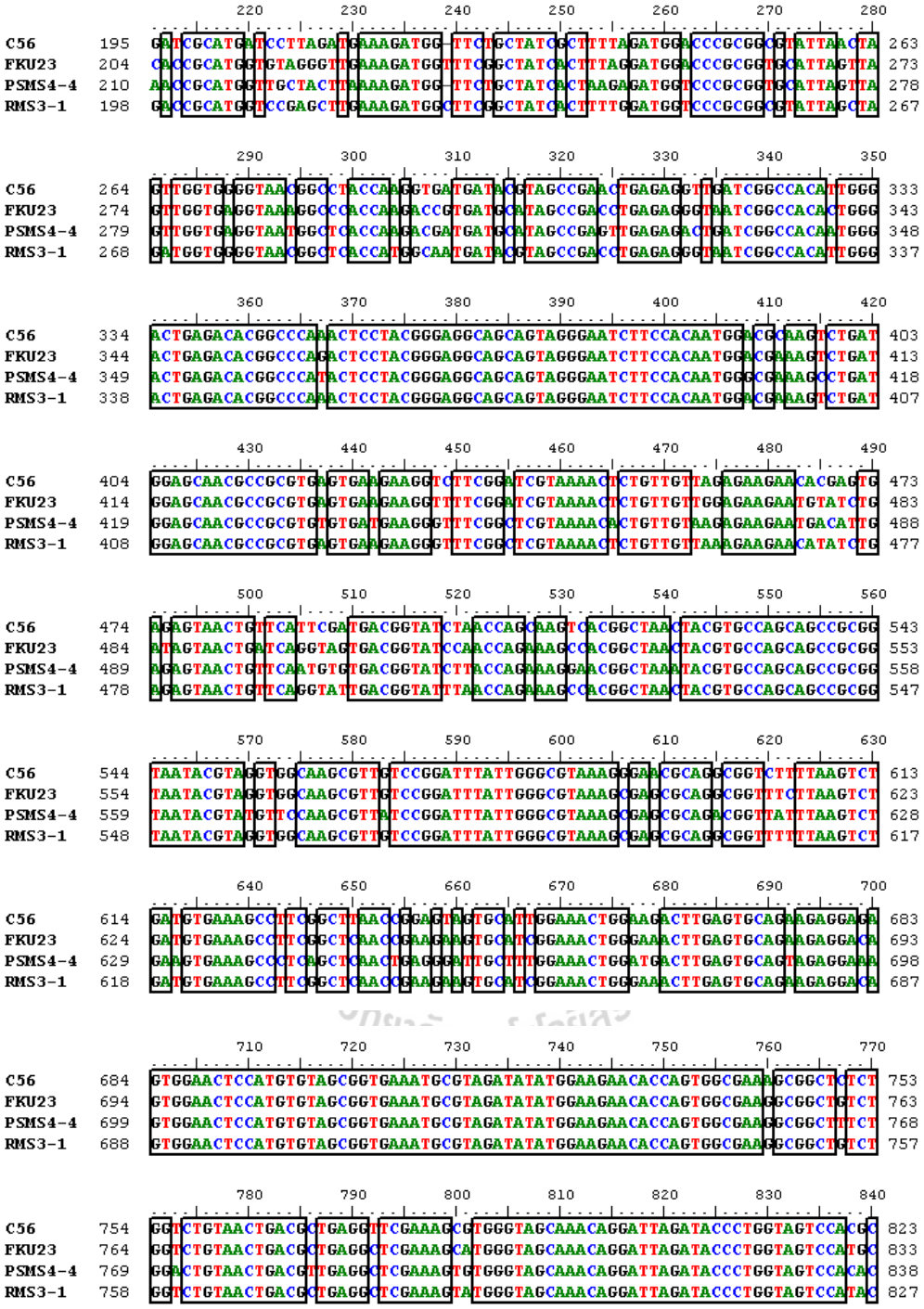

Figure 4.22 (Continued) Sequence alignment of 16S rRNA gene of Gram-positive lactic acid bacterial rods: isolates C56, FKU23, PSMS4-4, and RMS3-1, by using ClustalW and BioEdit programs. Blocks indicate the conserved nucleotides.

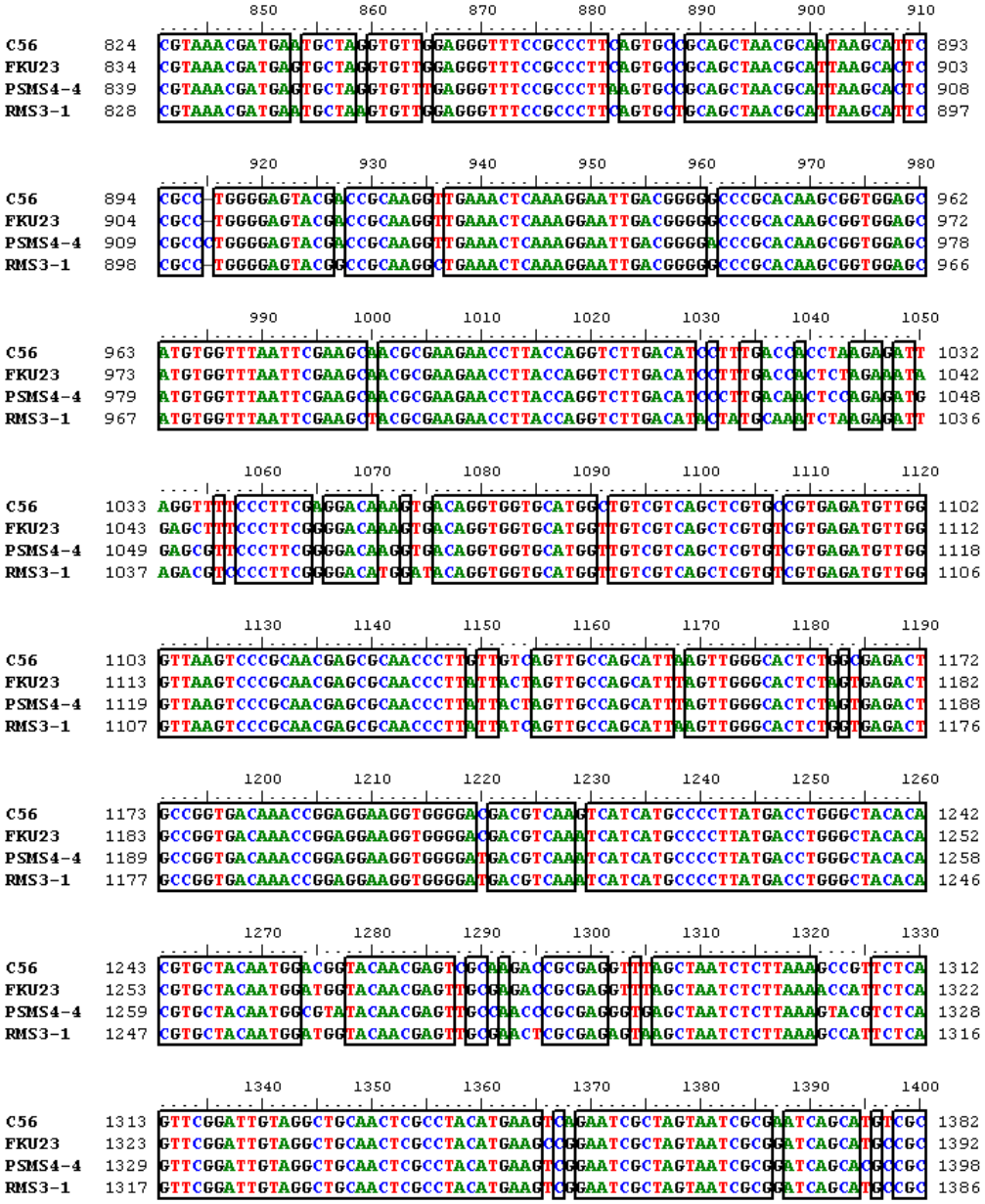

Figure 4.22 (Continued) Sequence alignment of 16S rRNA gene of Gram-positive lactic acid bacterial rods: isolates C56, FKU23, PSMS4-4, and RMS3-1, by using ClustalW and BioEdit programs. Blocks indicate the conserved nucleotides.

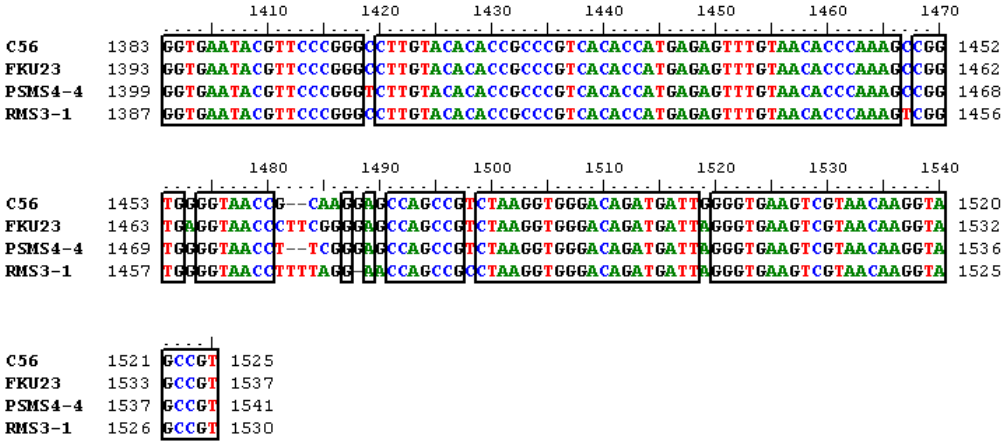

Figure 4.22 (Continued) Sequence alignment of 16S rRNA gene of Gram-positive lactic acid bacterial rods: isolates C56, FKU23, PSMS4-4, and RMS3-1, by using ClustalW and BioEdit programs. Blocks indicate the conserved nucleotides.

Table 4.12 The length of 16S ribosomal RNA gene sequences of eight potential EPS-producing isolates obtained from DNA sequence analysis.

Bacterial isolates	Length of 16S ribosomal RNA gene sequences (nt)	GenBank accession number
C56	1525	FJ611792
FKU23	1537	FJ611791
G3	1512	FJ611790
I5	1512	FJ611789
P14	1541	FJ611788
PSMS1-5	1513	FJ611787
PSMS4-4	1541	FJ611786
RMS3-1	1530	FJ611785

After sequencing, the amplified 16S rRNA gene fragments were aligned (Figure 4.21 and 4.22). The length of the DNA sequences is summarized in Table 4.12. The percentages of 16S rDNA sequence similarity among isolates were 84-99% (Table 4.13 and 4.14). The isolate I5 showed the highest similarity with G3 at relation value of 99%. Then, the 16S rDNA sequences were compared to those available in GenBank databases using standard nucleotide-nucleotide BLAST program to ascertain their closest relatives. Results of similarity and strain homology are shown in Tables 4.13 and 4.14. The isolate G3 had 100, 100 and 99% 16S rRNA gene sequence similarity, when compared to *Streptococcus infantarius* subsp. *infantarius* strains CIP106106, CIP106107, and *Streptococcus luteiensis* CIP 106849^{T} respectively. The isolate I5 had 99% 16S rRNA gene sequence similarity, when compared to *Streptococcus bovis*, *S. equinus*, *S. infantarius* and *S. luteiensis* (Table 4.14). The isolate P14 was identified as *Pediococcus pentosaceus* according to API-system (95.8% identity). Based on 16S rRNA gene sequence, isolate P14 belonged to the *Pediococcus* as shown in Table 4.16. Isolate PSMS1-5 could be identified as belonging to *Leuconostoc mesenteriodes* according to API-system with 99.1% identity. Sequencing of 16S rRNA gene showed that the isolate PSMS1-5 belonged to the *Leuconostoc*. For Gram-positive rod, C56 was similar to *Lactobacillus salivarius* at 99.7% identity and 99% similarity based on API-system and 16S rRNA gene sequence, respectively. Other isolates including FKU23, RMS3-1, and PSMS4-4 appeared to closely related with *Leuconostoc mesenteriodes* (98.4%), *Lactobacillus brevis* (99.9%), and *Weissella confusa* (99.2%) according to API50CH/CHL database, respectively. Based on 16S rRNA gene sequence shown in table 4.15, the isolate FKU23 was closed to *Lactobacillus sakei* DSM 20017^{T} (99% similarity),

Lactobacillus graminis DSM 20719^T (98% similarity), and *Lactobacillus curvatus* DSM 20019^T (98% similarity). The isolate RMS3-1 was closed to *Lactobacillus pentosus* NRIC 1833, *Lactobacillus plantarum* NRIC 1838, and *Lactobacillus pentosus* NRIC 1837 (99% similarity). The isolate PSMS4-4 was closed to *Weissella confusa* JCM 1093 and *Weissella cibaria* LMG 17699^T (99% similarity). The phylogenetic trees were constructed based on the 16S rDNA sequences using the neighbour-joining method with software MEGA version 4.0 (Tamura *et al.*, 2007) (Figure 4.23 and 4.24). Both unrooted trees were divided into two clades; clade I, a homofermentative group and clade II, a heterofermentative group. For Gram-positive cocci, the isolate PSMS1-5 was placed in clade I, which represented homofermentative group. The species of genus *Pediococcus* and *Streptococcus* were placed in clade II, which were heterofermentatives, and demonstrated the close relationship between those genera. For Gram-positive rods, clade I represented homofermentative group, composed of the isolate C56. RMS3-1, FKU23, and PSMS 4-4 were placed in clade II, which were heterofermentatives.

Table 4.13 16S rRNA gene sequence similarity of Gram-positive cocci and related species.

Bacterial isolates	I5	G3	P14	PSMS 1-5	1	2	3	4	5	6	7	8	9	10	11	12	13	14
I5	100																	
G3	99	100																
P14	86	86	100															
PSMS1-5	84	84	85	100														
1	99	99	86	84	100													
2	99	99	85	84	99	100												
3	99	100	86	84	99	99	100											
4	99	99	84	84	99	99	99	100										
5	99	100	86	84	99	99	100	99	100									
6	99	99	86	84	99	99	99	99	99	100								
7	99	99	86	84	99	99	99	99	99	99	100							
8	86	86	99	85	86	86	86	83	86	86	86	100						
9	86	86	97	85	86	86	86	83	86	86	86	98	100					
10	85	85	97	84	85	84	86	83	86	86	84	98	97	100				
11	82	82	96	82	82	82	82	83	82	82	82	96	96	96	100			
12	84	84	85	99	84	84	84	83	84	84	83	85	85	85	83	100		
13	83	83	82	99	83	83	83	83	83	83	83	82	85	82	83	98	100	
14	83	84	82	99	83	84	84	84	84	84	83	82	85	82	83	99	99	100

I5: *Streptococcus* sp. I5, G3: *Streptococcus* sp. G3, P14: *Pediococcus* sp. P14, PSMS1-5: *Leuconostoc* sp. PSMS1-5, 1: *Streptococcus bovis* strain ATCC 27960 (AB002481), 2: *Streptococcus bovis* strain NCFB 2476 (AF396922), 3: *Streptococcus infantarius* subsp. *infantarius* strain CIP 106106 (DQ232529), 4: *Streptococcus equinus* strain NCDO 1037^{T} (AF429765), 5: *Streptococcus infantarius* subsp. *infantarius* strain CIP 106107 (DQ232530), 6: *Streptococcus luteiensis* strain CIP 106849^{T} (DQ232532), 7: *Streptococcus bovis* NCTC 11436 (AJ305257), 8: *Pediococcus pentosaceus* strain DSM 20336^{T} (AJ305321), 9: *Pediococcus acidilactici* strain DSM 20284^{T} (AJ305320), 10: *Pediococcus stilesii* strain LMG 23082^{T} (AJ973157), 11: *Pediococcus claussenii* strain DSM 14800^{T} (AJ621555), 12: *Leuconostoc citreum* strain NRIC 1776 (AB022923), 13: *Leuconostoc mesenteroides* strain NRRL B1355 (EU574896), 14: *Leuconostoc holzapfelii* strain LMG 23990^{T} (AM600682).

Table 4.14 16S rRNA gene sequence similarity of Gram-positive rods and related species.

Bacterial isolates	C56	FKU23	PSMS4-4	RMS3-1	1	2	3	4	5	6	7	8	9	10	11
C56	100														
FKU23	91	100													
PSMS4-4	88	90	100												
RMS3-1	90	93	89	100											
1	99	91	87	90	100										
2	99	91	88	90	99	100									
3	99	91	88	90	99	99	100								
4	91	99	90	93	90	91	91	100							
5	91	98	90	93	91	91	91	99	100						
6	91	98	89	93	90	91	91	99	99	100					
7	87	89	99	88	87	87	87	90	89	89	100				
8	87	89	99	88	87	88	88	90	90	90	99	100			
9	90	92	88	99	90	91	90	92	93	93	8	88	100		
10	90	92	88	99	90	91	90	92	93	93	88	88	100	100	
11	90	92	88	99	90	91	90	92	93	93	88	88	99	99	100

C56: *Lactobacillus* sp. C56, FKU23: *Lactobacillus* sp. FKU23, PSMS4-4: *Weissella* sp. PSMS4-4, RMS3-1: *Lactobacillus* sp. RMS 3-1, 1: *Lactobacillus salivarius* strain ATCC 11741^{T} (AF089108), 2: *Lactobacillus salivarius* strain NBRC 102160^{T} (AB326353), 3: *Lactobacillus salivarius* strain JCM1231^{T} (AB370881), 4: *Lactobacillus sakei* strain DSM 20017^{T} (AM113784), 5: *Lactobacillus graminis* strain DSM 20719^{T} (AM113778), 6: *Lactobacillus curvatus* strain DSM 20019^{T} (AM113777), 7: *Weissella confusa* strain JCM 1093 (AB023241), 8: *Weissella cibaria* strain LMG 17699^{T} (AJ295989), 9: *Lactobacillus pentosus* strain NRIC 1837 (AB362758), 10: *Lactobacillus pentosus* strain NRIC 1833 (AB362754), 11: *Lactobacillus plantarum* strain NRIC 1838 (AB362759).

Table 4.15 Similarity of 16S rRNA gene sequence of eight exopolysaccharide-producing bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI).

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details				
		Closest relative	Length of sequence (bp)	Sequence homology (%)	GenBank accession number	Isolation source/ remark of closest relative
C56	1525	*Lactobacillus salivarius* ATCC 11741^T	1570	99	AF089108	Oral
		Lactobacillus salivarius NBRC 102160^T	1487	99	AB326353	Human intestine
		Lactobacillus salivarius JCM 1231^T	1486	99	AB370881	Human saliva
FKU23	1537	*Lactobacillus sakei* DSM 20017^T	1561	99	AM113784	"Moto" starter of sake
		Lactobacillus graminis DSM 20719^T	1548	98	AM113778	Grass silage
		Lactobacillus curvatus DSM 20019^T	1559	98	AM113777	Milk
G3	1512	*Streptococcus luteiensis* CIP 106849^T	1470	99	DQ232532	Human
		Streptococcus equinus NCDO 1037^T	1463	99	AF429765	Horse faeces
		Streptococcus bovis ATCC 27960	1500	99	AB002481	Swine
		Streptococcus bovis NCFB 2476	1539	99	AF396922	Ruminants
		Streptococcus infantarius subsp. *infantarius* CIP 106106	1470	100	DQ232529	Infant faeces
		Streptococcus infantarius subsp. *infantarius* CIP 106107	1470	100	DQ232530	Human blood

Table 4.15 (continued) Similarity of 16S rRNA gene sequence of eight exopolysaccharide-producing bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI).

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details				
		Closest relative	Length of sequence (bp)	Sequence homology (%)	GenBank accession number	Isolation source/ remark of closest relative
I5	1512	*Streptococcus equinus* NCDO 1037^T	1463	99	AF429765	Horse faeces
		Streptococcus luteiensis CIP 106849^T	1470	99	DQ232532	Human
		Streptococcus bovis ATCC 27960	1500	99	AB002481	Swine
		Streptococcus bovis NCFB 2476	1539	99	AF396922	Ruminants
		Streptococcus bovis NCTC 11436	1517	99	AJ305257	Human blood
		Streptococcus infantarius subsp. *infantarius* CIP 106106	1470	99	DQ232529	Infant faeces
		Streptococcus infantarius subsp. *infantarius* CIP 106107	1470	99	DQ232530	Human blood
P14	1541	*Pediococcus pentosaceus* DSM 20336^T	1569	99	AJ305321	Dried American beer yeast
		Pediococcus claussenii DSM 14800^T	1472	96	AJ621555	Spoiled beer
		Pediococcus acidilactici DSM 20284^T	1569	97	AJ305320	Barley
		Pediococcus stilesii LMG 23082^T	1529	97	AJ973157	White maize grains

Table 4.15 (continued) Similarity of 16S rRNA gene sequence of eight exopolysaccharide-producing bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI).

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details				
		Closest relative	Length of sequence (bp)	Sequence homology (%)	GenBank accession number	Isolation source/ remark of closest relative
PSMS1-5	1513	*Leuconostoc holzapfelii* LMG 23990^T	1415	99	AM600682	Coffee fermentation
		Leuconostoc citreum NRIC 1776	1448	99	AB022923	Honeydew of rye ear
		Leuconostoc mesenteriodes NRRL B-1355	1445	99	EU574896	Alternan sucrase-producing strian
PSMS4-4	1541	*Weissella confusa* JCM 1093	1477	99	AB023241	Sugar cane
		Weissella cibaria LMG 17699^T	1529	99	AJ295989	Chilli Bo
RMS3-1	1530	*Lactobacillus pentosus* NRIC 1833	1561	99	AB362754	Phak-sian-dong (Pickle)
		Lactobacillus plantarum NRIC 1838	1561	99	AB362759	Khom mak (Sweeten rice)
		Lactobacillus pentosus NRIC 1837	1561	99	AB362758	Hom-dong (Pickle spring onion)

Note: ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM, Japan Collection of Microorganisms; LMG, BCCM/LMG Bacteria Collection; NBRC, NITE Biological Resource Center; NCDO, National Collection of Dairy Organisms; NCFB, National Collection of Food Bacteria; NCTC, National Collection of Type Cultures; NRIC, Nodai Research Institute Culture Collection; NRRL, Agricultural Research Service Culture Collection.

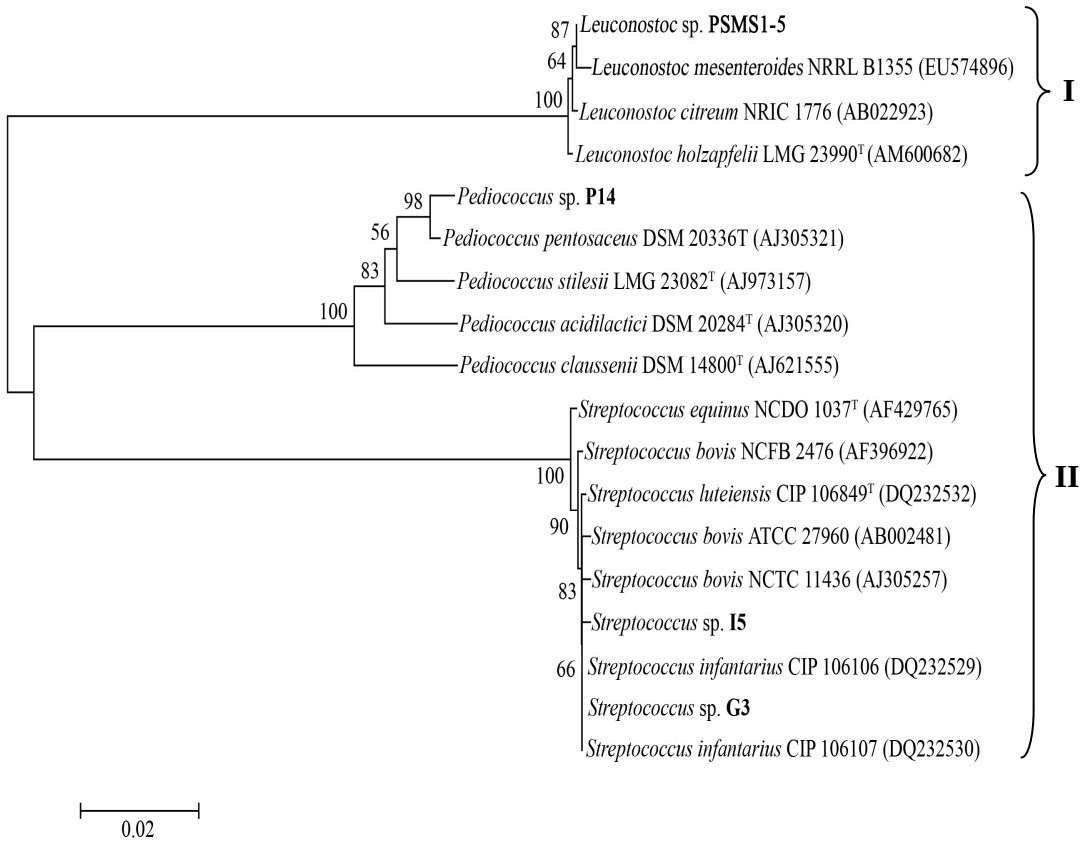

Figure 4.23 Phylogenetic tree of EPS-producing Gram-positive cocci, based on 16S rRNA gene sequence data using the neighbour-joining method. The unrooted tree was derived by using BioEdit, and Mega4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. Two subgroups are indicated: I, Heterofermentive group; II, Homofermentative group. The scale bar represents (calculated) distance.

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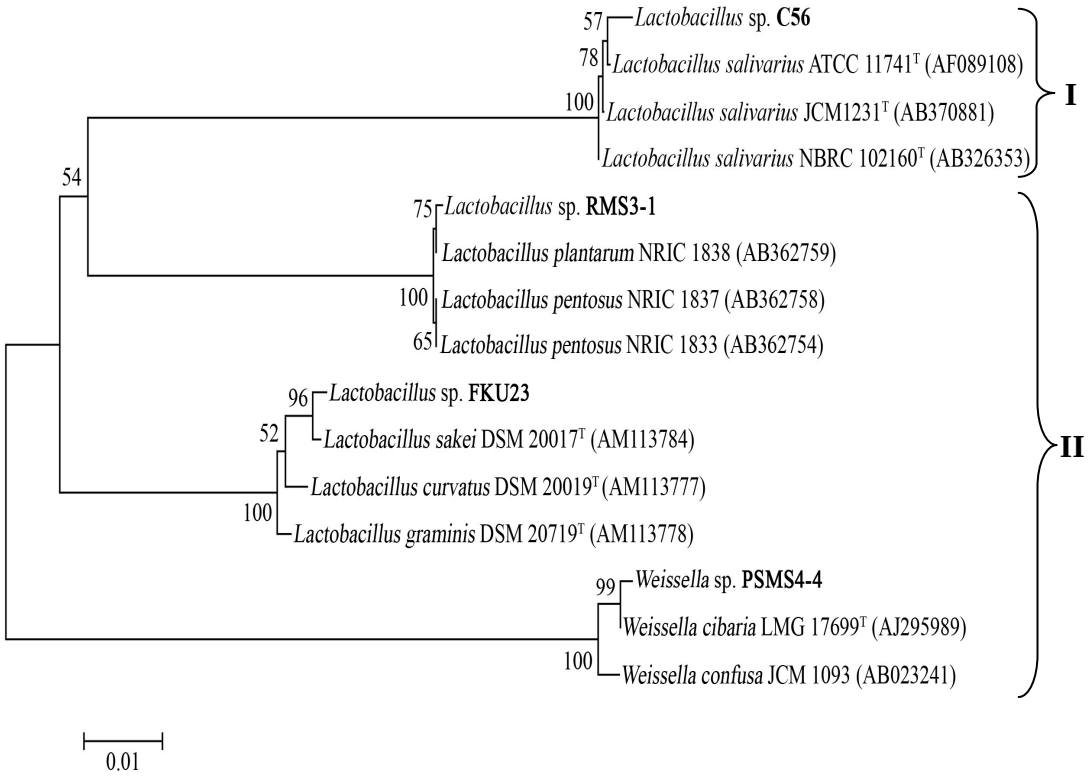

Figure 4.24 Phylogenetic tree of EPS-producing Gram-positive rods, based on 16S rRNA gene sequence data using the neighbour-joining method. The unrooted tree was derived by using BioEdit, and Mega4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. Two subgroups are indicated: I, Hemofermentative group; II, Heterofermentative group. The scale bar represents (calculated) distance.

Table 4.16 Identification results of selected lactic acid bacterial isolates based on API 50CH/CHL system (Biome´rieux) compared to 16S rRNA gene sequence.

Bacterial isolate code	Cell shape	Cell size (µm)	CO_2 production	Identification (% identity)	
				API 50CH/CHL system (Biome´rieux, France)	16S rRNA gene sequence
C56	Rods, short chains	0.30-0.45 x 1.12-1.31	-	*Lactobacillus salivarius* (99.7%)	*Lactobacillus salivarius* ATCC 11741^T (99%) *Lactobacillus salivarius* NBRC 102160^T (99%) *Lactobacillus salivarius* JCM 1231^T (99%)
FKU23	Rods, short chains	0.43-0.63 x 0.76-1.41	+	*Leuconostoc mesenteriodes* (98.4%)	*Lactobacillus sakei* DSM 20017^T (99%) *Lactobacillus graminis* DSM 20719^T (98%) *Lactobacillus curvatus* DSM 20019^T (98%)
G3	Cocci, short chains	0.50-0.59 x 0.59-0.80	-	*Lactobacillus crispatus* (99.9%)	*Streptococcus luteiensis* CIP 106849^T (99%) *Streptococcus infantarius* subsp. *infantarius* CIP 106106 (100%) *Streptococcus infantarius* subsp. *infantarius* CIP 106107 (100%)
I5	Cocci, short chains	0.43-0.52 x 0.52-0.78	-	*Lactobacillus acidophilus* 2 (99.6%)	*Streptococcus equinus* NCDO 1037^T (99%) *Streptococcus luteiensis* CIP 106849^T (99%) *Streptococcus bovis* ATCC 27960 (99%) *Streptococcus bovis* NCFB 2476 (99%) *Streptococcus bovis* NCTC 11436 (99%)

Table 4.16 (Continued) Identification results of selected lactic acid bacterial isolates based on API 50CH/CHL system (Biome´rieux) compared to 16S rRNA gene sequence.

Bacterial isolate code	Cell shape	Cell size (µm)	CO_2 production	Identification (% identity)	
				API 50CH/CHL system (Biome´rieux, France)	16S rRNA gene sequence
P14	Cocci, short chains	0.78-0.88 x 0.98-1.26	-	*Lactococcus lactis* subsp. *lactis* 1 (98.6%)	*Pediococcus pentosaceus* DSM 20336^T (99%)
				Pediococcus pentosaceus 2 (95.8%)	*Pediococcus claussenii* DSM 14800^T (96%)
				Pediococcus pentosaceus 1 (91.2%)	*Pediococcus acidilactici* DSM 20284^T (97%)
					Pediococcus stilesii LMG 23082^T (97%)
PSMS 1-5	Ovoids, short chains	0.31-0.42 x 0.56-1.17	+	*Leuconostoc mesenteriodes* subsp. *mesenteriodes/dextranicum* 2 (99.1%)	*Leuconostoc holzapfelii* LMG 23990^T (99%)
				Lactobacillus brevis 1 (99.5%)	*Leuconostoc citreum* NRIC 1776 (99%)
				Leuconostoc citreum (93.7%)	*Leuconostoc mesenteriodes* NRRL B-1355 (99%)
PSMS 4-4	Rods, short chains	0.66-0.74 x 1.19-1.25	+	*Weissella confusa* (99.2%)	*Weissella cibaria* LMG 17699^T (99%)
				Lactobacillus acidophilus 3 (83.6%)	*Weissella confusa* JCM 1093 (99%)
				Lactobacillus brevis 1 (63.5%)	
RMS 3-1	Rods, short chains	0.54-0.69 x 0.89-1.53	+	*Lactobacillus brevis*1 (99.9%)	*Lactobacillus pentosus* NRIC 1833 (99%)
					Lactobacillus plantarum NRIC 1838 (99%)
					Lactobacillus pentosus NRIC 1837 (99%)

CHAPTER V

CONCLUSIONS

Lactic acid bacterial isolates were screened for EPS production using glucose and sucrose as carbon sources. Approximately 10 and 19 percent of a total of 566 lactic acid bacterial strains isolated from agricultural products and traditional Thai fermented foods produced slimy colonies on MRS agar containing 2% of glucose and sucrose respectively. The colonies were 0.2-0.9 and 0.2-2.1 cm diameter on MRS agar containing 2% of glucose and sucrose respectively. EPS yields of 0.1-0.6 g and 0.1-6.9 g equivalent glucose/l were obtained when these isolates were cultivated in MRS broth containing 2% of glucose and sucrose respectively.

Twenty lactic acid bacterial isolates that had specific EPS production values ranging from 0.26-44.5 pg/cell were selected for optimization of their EPS production conditions. Types and concentrations of carbon sources, the initial pH of the culture medium, and cultivation temperature were investigated to achieve the maximum EPS production. Five carbon sources: soluble starch, cassava starch, rice flour, molasses, and white sugar from sugar cane, were used for EPS production by the selected isolates, and compared to analytical grade sucrose using MRS based-media. White sugar from sugar cane was found to provide the highest EPS-specific production ranging from 0.025-106.94 pg/cell. The sugar was then chosen to be used as carbon source. Five isolates of lactic acid bacteria: FKU23, I5, PSMS1-5, PSMS4-4, and RMS3-1 inhabiting pla-som, tapioca waste, and rice grains respectively, were selected

based on their promising EPS production ability (47.00, 43.19, 106.94, 75.92, and 0.03 pg/cell respectively) and diversity of bacterial habitats. Concentrations of 45, 35, 45, 50, and 30 g/l of white sugar from sugar cane were suitable for EPS production by isolates FKU23, I5, PSMS1-5, PSMS4-4, and RMS3-1 respectively. Initial pH of medium and cultivation temperature affected the production of polymer depending on isolates. Initial pH of 7.0 at 30°C was suitable for EPS production by isolate FKU23. The optimum conditions for EPS production by isolate I5 were at initial pH of 6.0 and 40°C, and initial pH of 8.0 and 30°C. Initial pH of 5.0 and cultivation temperature of 30 and 37°C were suitable for EPS production by isolate PSMS1-5. The optimum initial pH and cultivation temperature for EPS production by isolate PSMS4-4 were 5.0 and 7.0 at 30°C. For isolate RMS3-1, initial pH of 5.0 at 37°C and 8.0 at 35°C were optimum conditions for production of the polymer.

Selected isolates were then cultured under optimum conditions. The highest specific EPS productions of 1.42, 3.94, 7.34, 10.25, and 0.48 g EPS/g dry weight were obtained after 12, 12, 30, 18, and 36 h of fermentation for the isolates FKU23, I5, PSMS1-5, PSMS4-4, and RMS3-1 respectively.

The polymers were purified by protease digestion using both Proteinase K and Pronase E, and trichloroacetic acid precipitation resulting in the reduction of protein. Residual protein content was lower than 1% (w/w).

Characterization of EPS was then performed using acid hydrolysis, and analyzed by HPLC. EPS were found to be either homopolysaccharide or heteropolysaccharides. Homopolysaccharide composes of glucose whereas heteropolysaccharides contain glucose and mannose in varying proportions. Eight EPS-producing isolates were identified using morphological and physiological

characteristics, and 16S rRNA gene sequences. EPS-producing isolates were identified using the API-system (Biome´rieux, France) as *Lactobacillus salivarius* (C56), *Pediococcus pentosaceus* (P14), *Weissella cibaria* (PSMS4-4), and *Lactobacillus plantarum* (RMS3-1); two isolates of *Leuconostoc mesenteriodes* (FKU23 and PSMS 1-5); and two misidentification results of *Lactobacillus* for isolates I5 and G3 respectively. Sequencing of 16S ribosomal RNA gene confirmed that the isolate C56 was *Lactobacillus salivarius* (99% similarity). Other EPS-producing isolates were identified as belonging to genera *Weissella* (PSMS4-4), *Pediococcus* (P14), *Leuconostoc* (PSMS1-5), *Lactobacillus* (FKU23 and RMS3-1), and *Streptococcus* (I5 and G3).

The purified EPS were studied for their impact on the immune system. Eight EPS produced by the selected isolates stimulated splenocyte proliferation. Four of the eight EPS stimulated lymphocyte proliferation, and activated the cells to secrete the anti-inflammatory cytokine IL-10. The highest proliferative response (stimulation index of 13.96) was obtained from mannose-rich EPS (86.91% mannose and 13.09% glucose) produced by isolate I5 when cultured in MRS medium containing 35 g/l of white sugar from sugar cane at 40°C and pH 6.0, and IL-10 (218.49 pg/ml) was produced in response to the EPS. These results suggest the possibility of modulations of the immune system in mouse splenocytes after exposure to EPS from lactic acid bacteria.

Isolate I5 was selected to test for EPS production in a 5 l controlled fermenter containing MRS medium with 150 g/l of white sugar from sugar cane. The fermentation temperature and pH were kept constant at 40°C and pH 6.0, respectively. The maximum EPS production of 53.45 g/l (% EPS yield of 42.66) was achieved after

30 h of fermentation. This yield was 1.7 times higher than those reported for heteropolysaccharide production in other lactic acid bacteria.

Since lactic acid bacteria isolates obtained from this study could produce large amount of EPS, they may be useful for food industry applications. Considering the productivity and yield of the product, EPS production conditions of *Streptococcus* sp. I5 was optimized. EPS production of 53.45 g/l could be useful commercially. From an economic point of view, the use of white sugar from sugar cane as a cheap carbon source to produce EPS was of particular importance. Substitution of the analytical grade sucrose by white sugar from sugar cane resulted in the reduction of the raw material cost by 98%.

In addition to industrial benefits, certain EPS produced by selected lactic acid bacterial isolates stimulated proliferation of immune cells and interleukin-10 production. The EPS may be useful for medical applications as anti-inflammatory materials. This knowledge could be important with respect to a functional effect on human health if the EPS was incorporated into a food matrix.

Studies of the rheological properties of EPS and also the structure-function relationship of EPS in a food matrix are necessary to improve visual appeal, taste, and to fully understand the physical functionality of EPS in food products. To employ EPS produced by LAB as potential functional food components with health-promoting properties, *in vivo* mouse experiments with oral administration of EPS are required. The EPS that stimulated IL-10 production could next be tested for *in vivo* anti-inflammatory activity using experimental animal models of inflammatory bowel diseases.

In conclusion, the information obtained from this study is potentially useful for food industry and medical applications.

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APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENTS

1. Buffer for exopolysaccharide purification

Phosphate buffer saline (PBS)

NaCl	8.00	g
KCl	0.20	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.20	g
Sodium bisulpite (0.02 M)	22.80	g

The ingredients were dissolved and the final volume was adjusted to 1,000 ml with distilled water. Then, the pH was adjusted to 7.4 with HCl. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches, after preparation.

2. Chemicals, reagents and culture media used for 16S ribosomal RNA gene sequencing

2.1 RNAase (10 mg/ml)

RNAase	10.00	mg

The RNAase was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl 1 ml and stored at -20°C.

2.2 Tris-EDTA (TE) Buffer

Tris Base	1.21	g
EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	0.37	g

The ingredients were dissolved and the final volume was adjusted to 1,000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches, after preparation.

2.3 Tris-borate (TBE) buffer (5X)

Tris Base	54.00	g
Boric acid	27.50	g
EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	0.37	g

The ingredients were dissolved and the volume was adjusted to 1,000 ml with deionized water.

2.4 Gel loading buffer (6X)

Bromophenol blue	25.00	g

The dye was dissolved and the volume was adjusted to 10 ml with 40% sucrose in water.

2.5 Ethidium bromide (10 mg/ml)

Ethidium bromide	1.00	g

The chemical was dissolved and the volume was adjusted to 10 ml with sterilized deionized water.

2.6 Luana Bertani media (LB medium)

Tryptone	10	g
Yeast extract	5	g
NaCl	5	g
Agar	15	g
Distilled water	1,000	ml

Sterilization was done by autoclaving for 15 min at 121°C. The medium was then cooled down to room temperature, and added 500 µl of 25 mg/ml Kanamycin to LB medium.

2.7 Luana Bertani broth (LB broth)

Tryptone	10	g
Yeast extract	5	g
NaCl	5	g
Distilled water	1000	ml

Sterilization was done by autoclaving for 15 min at 121°C. The medium was then cooled down to room temperature, and added 200 µl of 25 mg/ml Kanamycin to LB broth.

2.8 SOC medium

Tryptone	20	g
Yeast Extract	5	g
NaCl	0.5	g
KCl	0.18	g

$MgSO_4$	2.4	g
Distilled water	1000	ml

Sterilization was done by autoclaving for 15 min at 121°C. The medium was then cooled down to room temperature, and added 20 mM sterile glucose.

3. Culture media for lactic acid bacterium cultivation and exopolysaccharide production

3.1 MRS medium (De Man, Rogosa and Sharpe Medium)

Proteose peptone	10.00	g
Beef extract	8.00	g
Yeast extract	4.00	g
Polysorbate 80 ($(NH_4)_3C_6H_5O_7$)	1.00	g
Tri Ammonium citrate ($CH_3COONa.3H_2O$)	2.00	g
Sodium acetate.$3H_2O$	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.H_2O$	0.05	g
K_2HPO_4	2.00	g
Dextose	20.00	g
Agar	10.00	g

Final pH 6.2 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

3.2 Culture medium for cell culture (Complete RPMI-1640 medium)

Fetal calf serum	5%	
Penicillin	100	U/ml
Streptomycin	100	µg/ml
50 mM Mercaptoethanol	0.1	ml/ml

The compositions were suspended in 1,000 ml of RPMI-1640 medium.

APPENDIX B

STANDARD CURVES

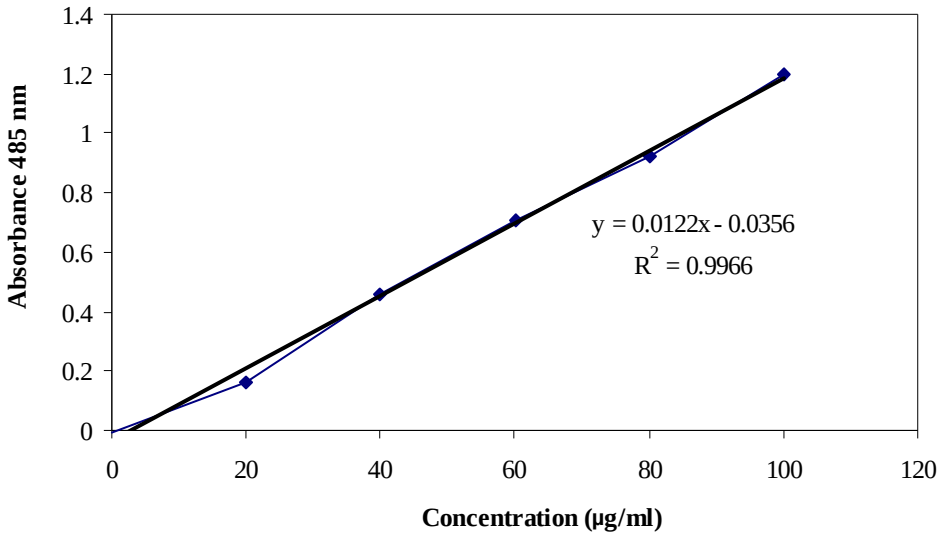

Figure 1B. Standard curve of glucose according to phenol-sulfuric acid method (Dubois *et al*., 1956).

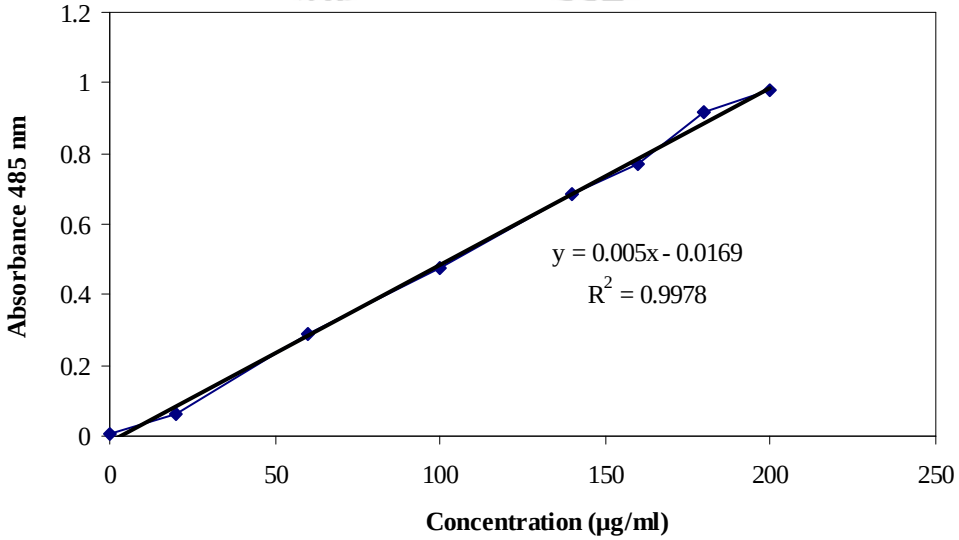

Figure 2B. Standard curve of bovine serum albumin (BSA) according to Bradford (Bradford, 1976).

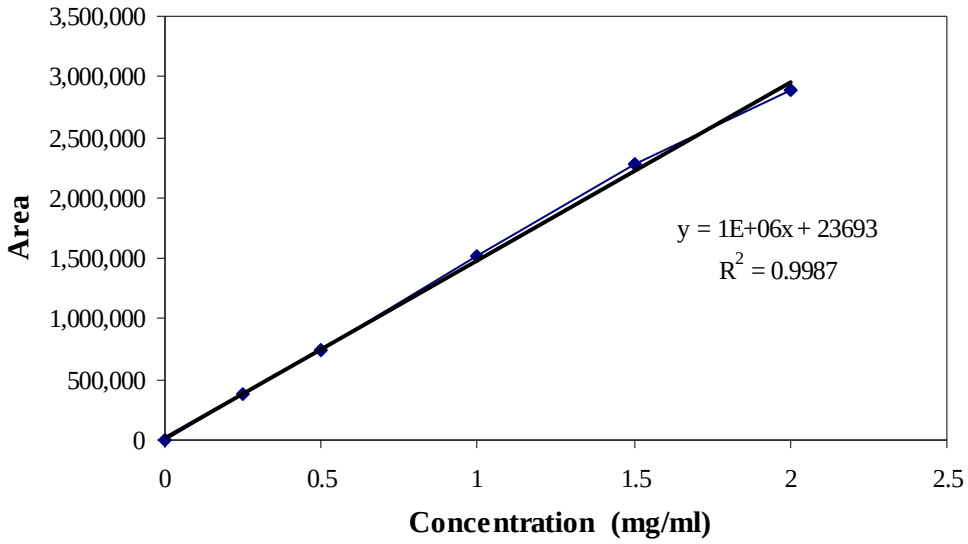

Figure 3B. Standard curve of standard D-glucose using Vertisep™ OA HPLC with a 100% water mobile phase and a refractive index detector.

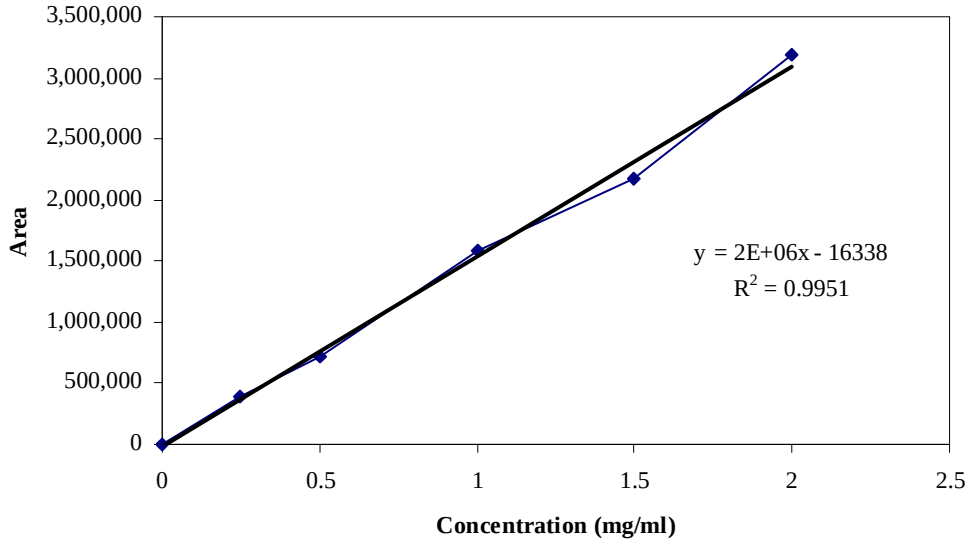

Figure 4B. Standard curve of standard D-mannose using Vertisep™ OA HPLC with a 100% water mobile phase and a refractive index detector.

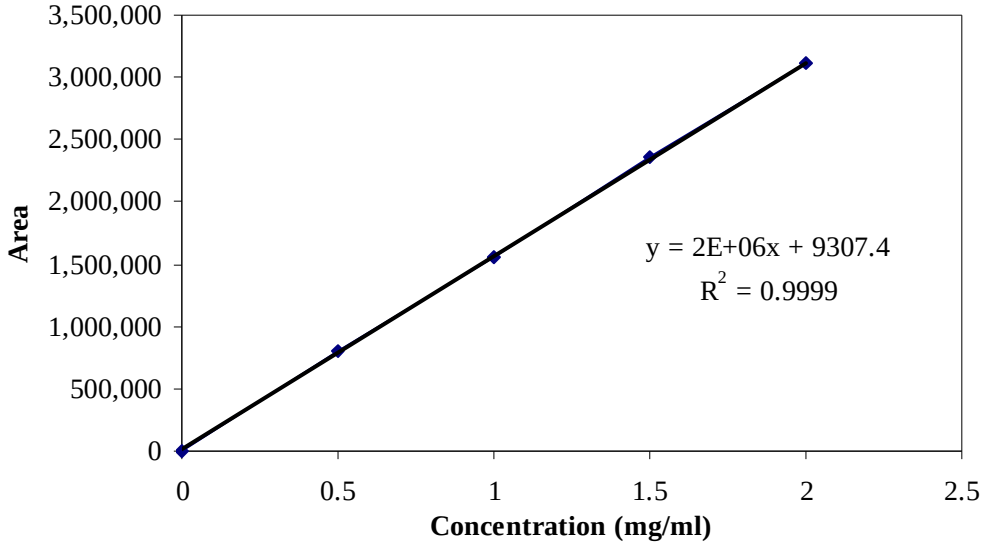

Figure 5B. Standard curve of white sugar from sugar cane using Vertisep™ OA HPLC with a 0.005 M H_2SO_4 mobile phase and a refractive index detector.

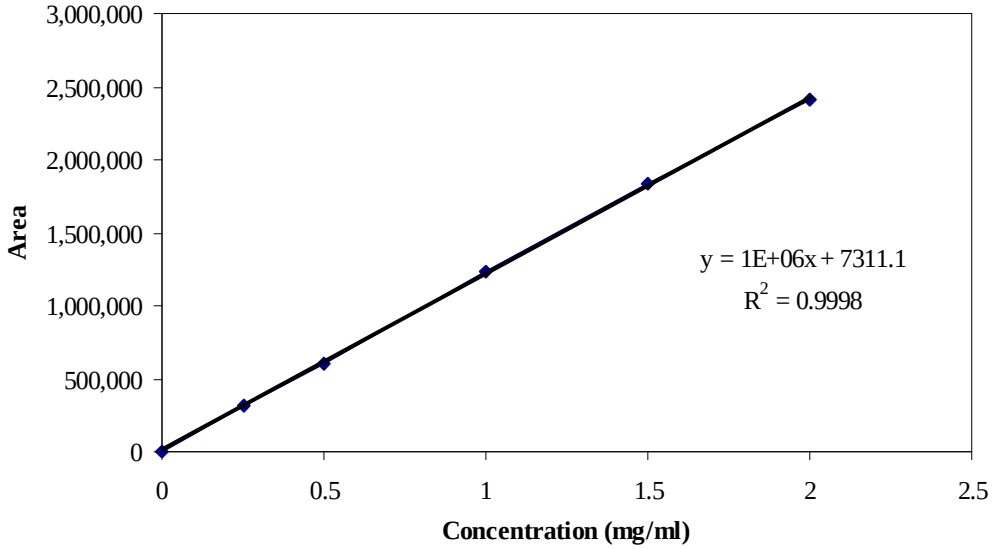

Figure 6B. Standard curve of standard D-lactic acid using Vertisep™ OA HPLC with a 0.005 M H_2SO_4 mobile phase and a refractive index detector.

APPENDIX C

HPLC CHROMATOGRAM

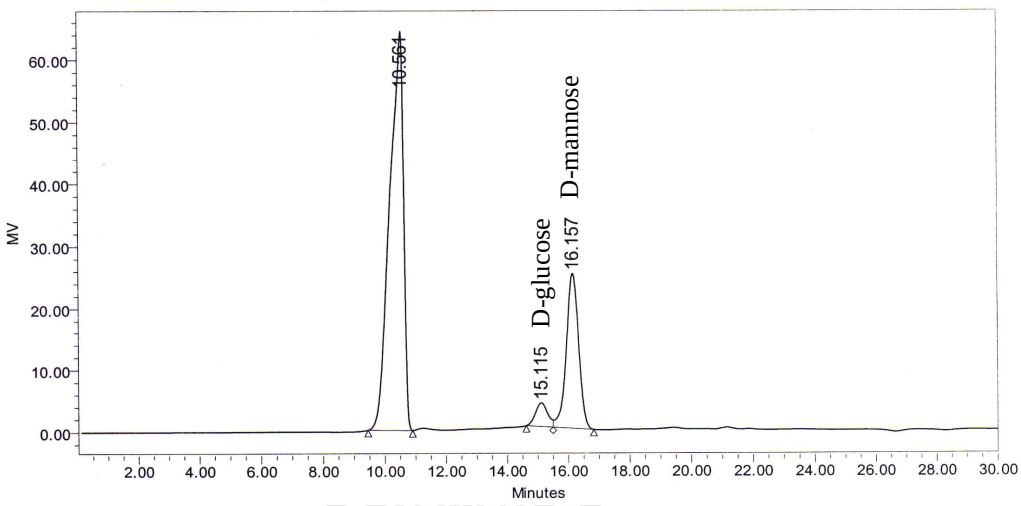

Figure 1C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Lactobacillus salivarius* C56 cultivated in MRS broth containing 2% of glucose with initial pH of 6.2 at 37°C for 48 h. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

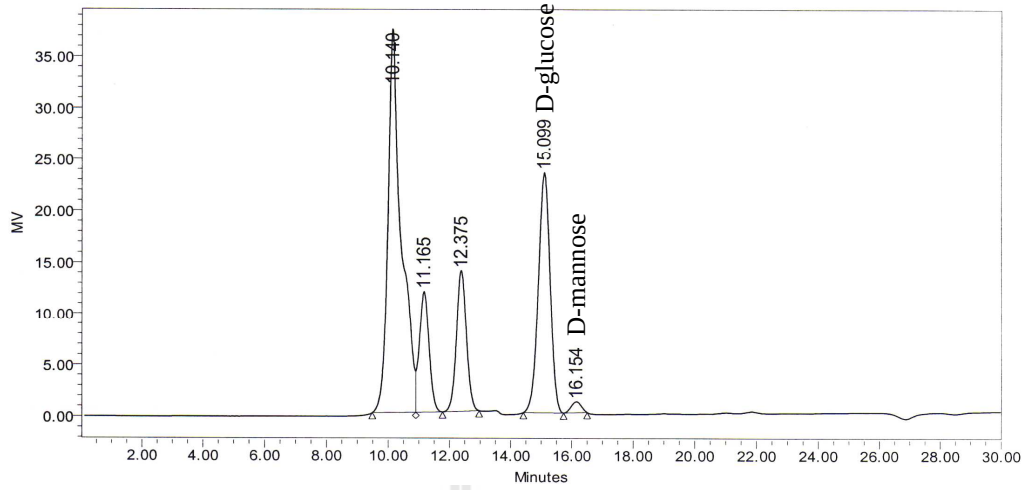

Figure 2C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Lactobacillus* sp. FKU23 cultivated in MRS broth containing 4.5% of white sugar from sugar cane with initial pH of 7.0 at 30°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

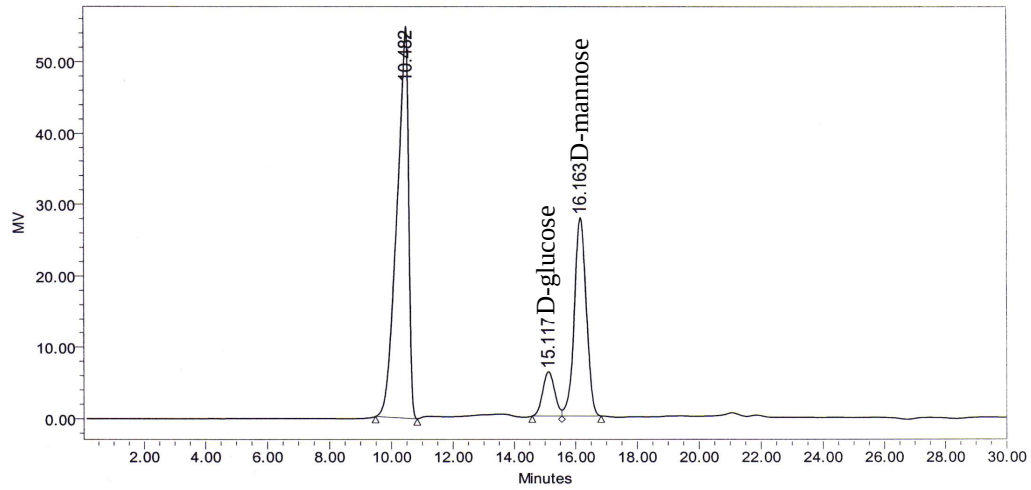

Figure 3C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Streptococcus* sp. G3 cultivated in MRS broth containing 2% of white sugar from sugar cane with initial pH of 6.2 at 30°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

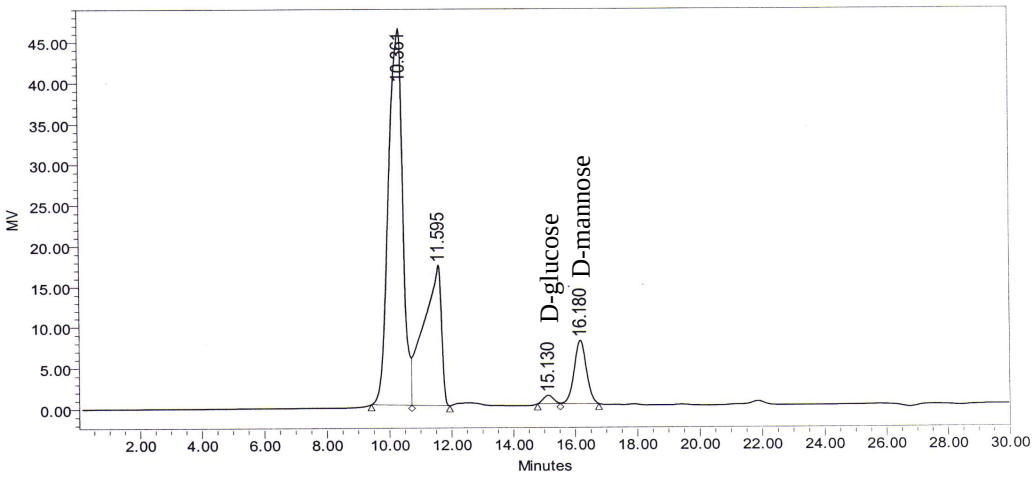

Figure 4C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Streptococcus* sp. I5 cultivated in MRS broth containing 3.5% of white sugar from sugar cane with initial pH of 6.0 at 40°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

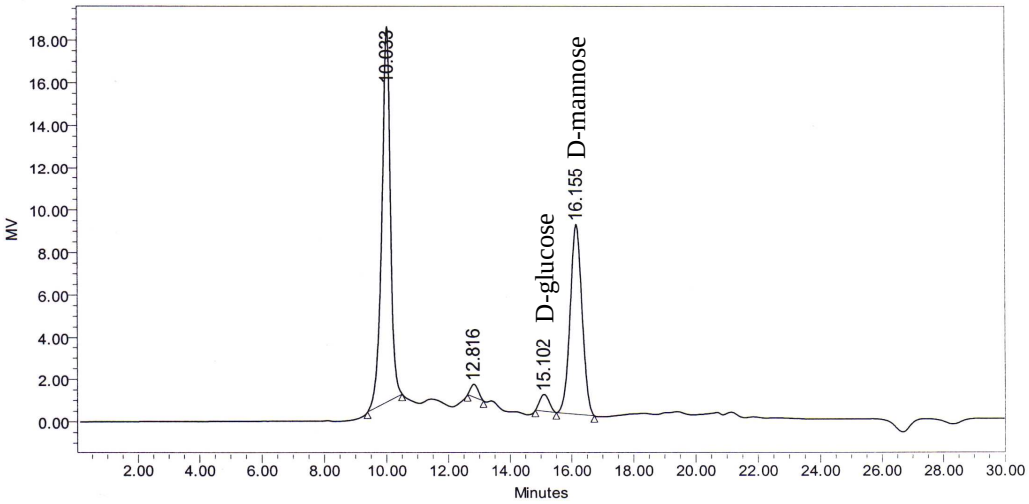

Figure 5C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Streptococcus* sp. I5 cultivated in MRS broth containing 2% of glucose with initial pH of 6.2 at 37°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

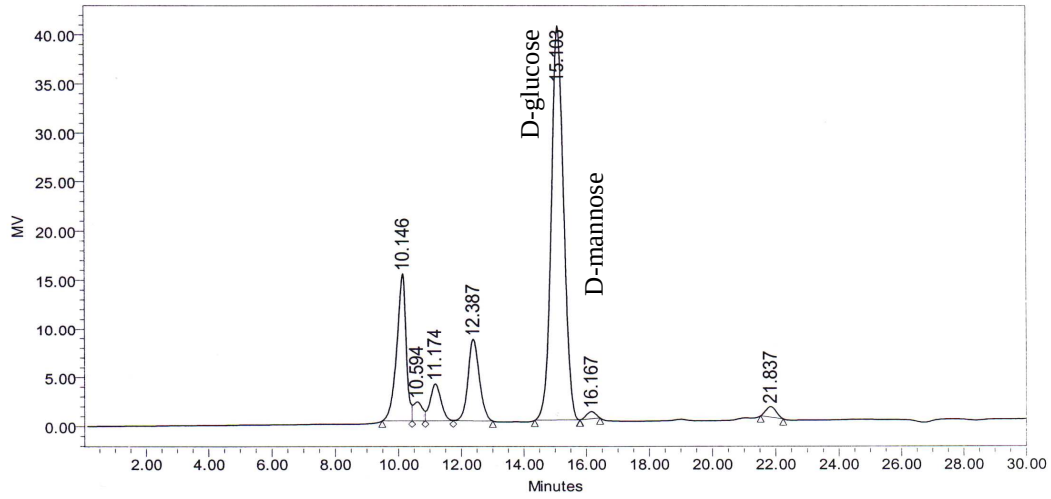

Figure 6C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Pediococcus* sp. P14 cultivated in MRS broth containing 2% of analytical grade sucrose with initial pH of 6.2 at 30°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

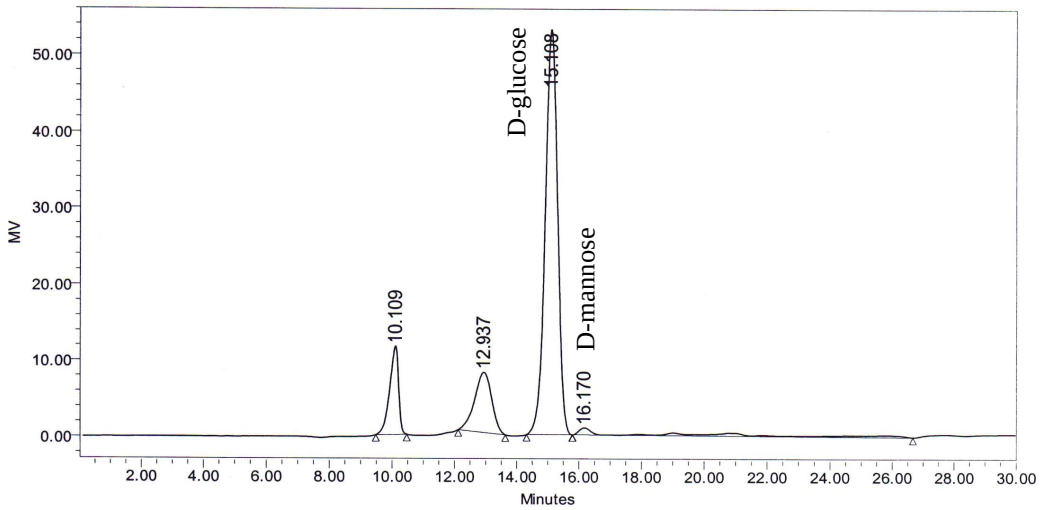

Figure 7C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Leuconostoc* sp. PSMS1-5 cultivated in MRS broth containing 4.5% of white sugar from sugar cane with initial pH of 5.0 at 37°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

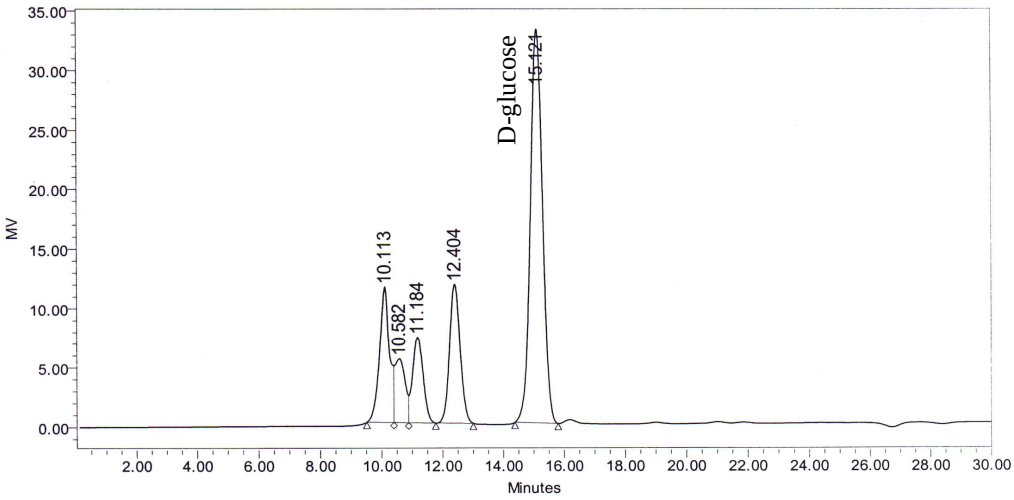

Figure 8C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Weissella* sp. PSMS4-4 cultivated in MRS broth containing 5% of white sugar from sugar cane with initial pH of 7.0 at 30°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

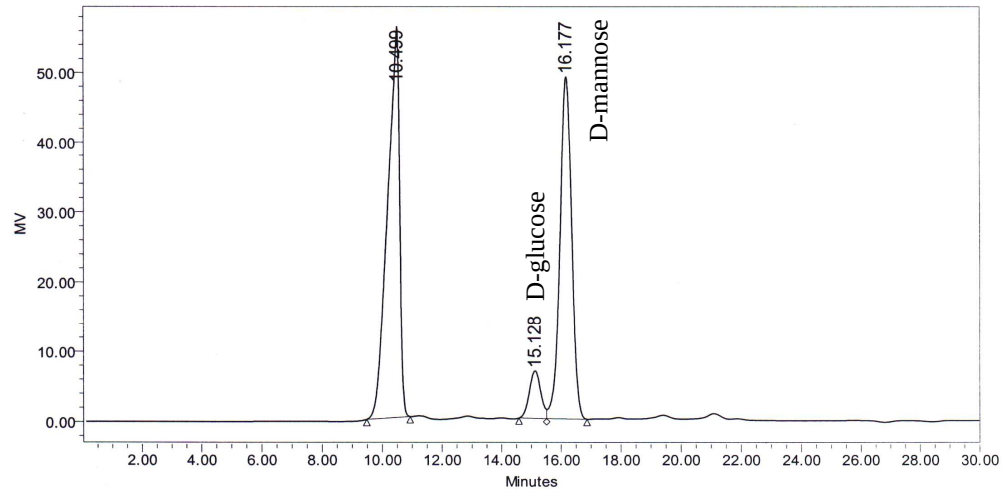

Figure 9C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Weissella* sp. PSMS4-4 cultivated in MRS broth containing 2% of glucose with initial pH of 6.2 at 30°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

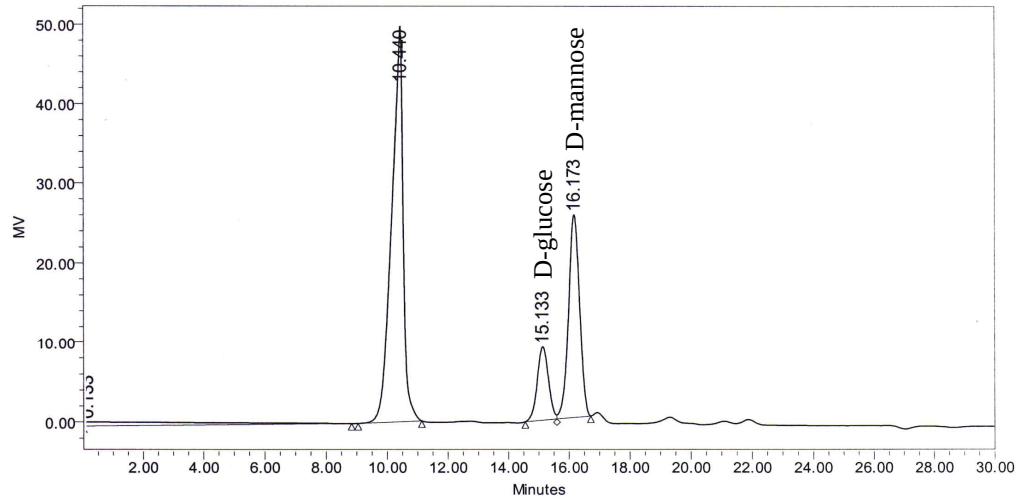

Figure 10C. HPLC Chromatogram of partial hydrolyzed exopolysaccharide from *Lactobacillus* sp. RMS3-1 cultivated in MRS broth containing 3% of white sugar from sugar cane with initial pH of 8.0 at 35°C. Sample injection volume was 100 µl. Chromatographic run time was 30 min.

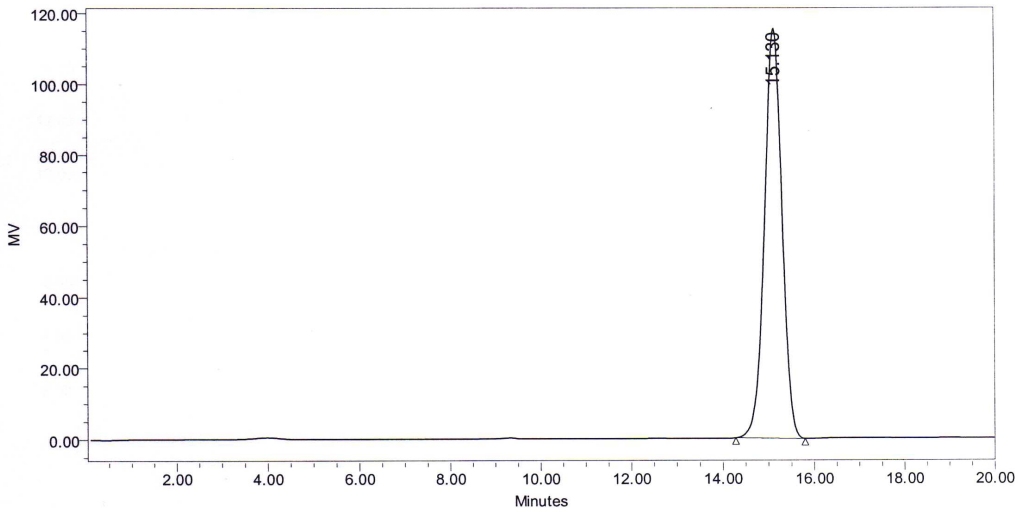

Figure 11C. HPLC Chromatogram of 2 mg/ml standard D-glucose using Vertisep^{TM} OA HPLC with a 100% water mobile phase and a refractive index detector.

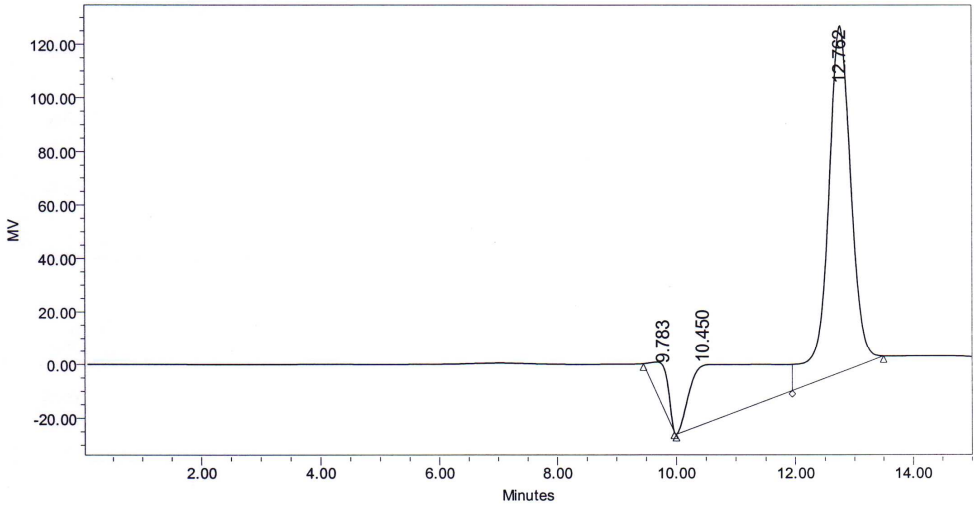

Figure 12C. HPLC Chromatogram of 2 mg/ml white sugar from sugar cane using Vertisep^{TM} OA HPLC with a 0.005M H_2SO_4 mobile phase and a refractive index detector.

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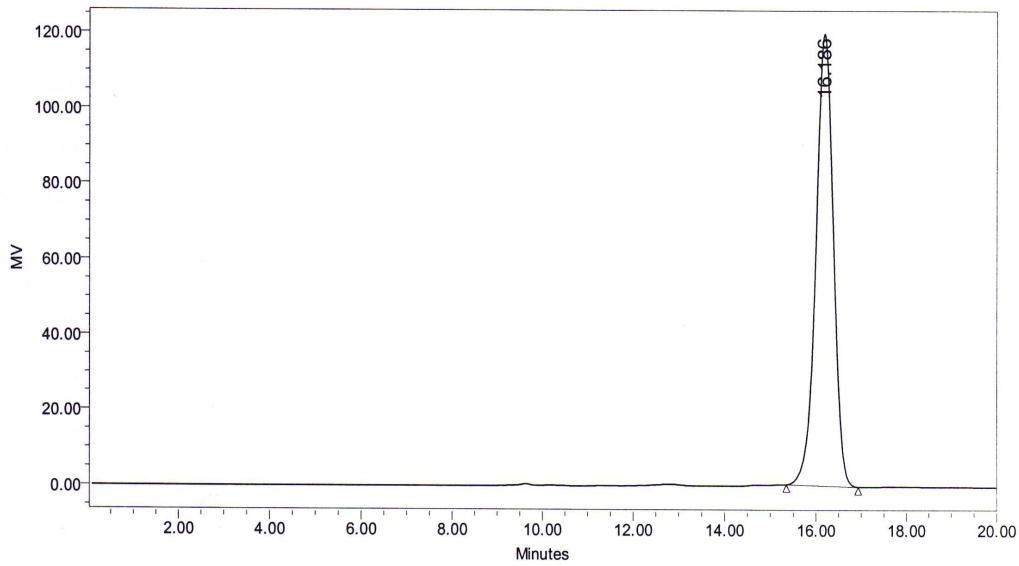

Figure 13C. HPLC Chromatogram of 2 mg/ml standard D-mannose using Vertisep^{TM} OA HPLC with a 100% water mobile phase and a refractive index detector.

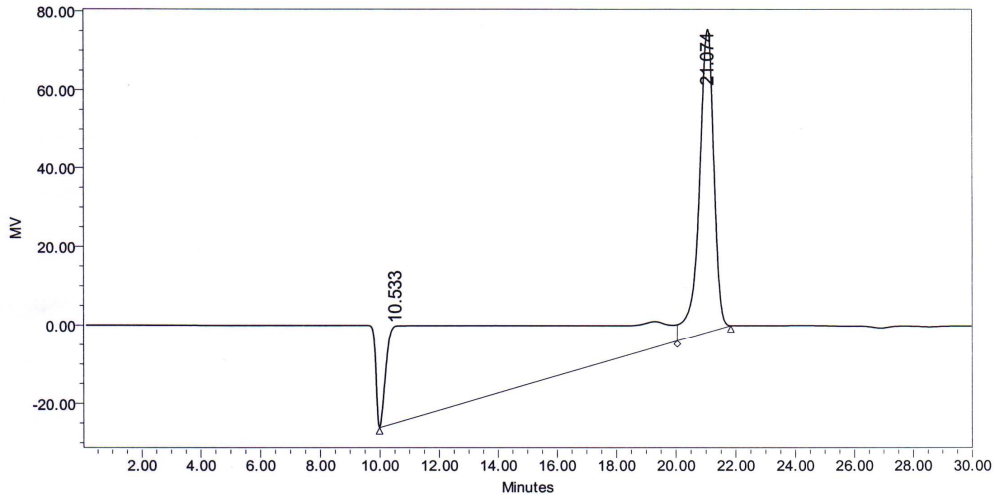

Figure 14C. HPLC Chromatogram of 2 mg/ml standard D-lactic acid Vertisep^{TM} OA HPLC with a 0.005M H_2SO_4 mobile phase and a refractive index detector.

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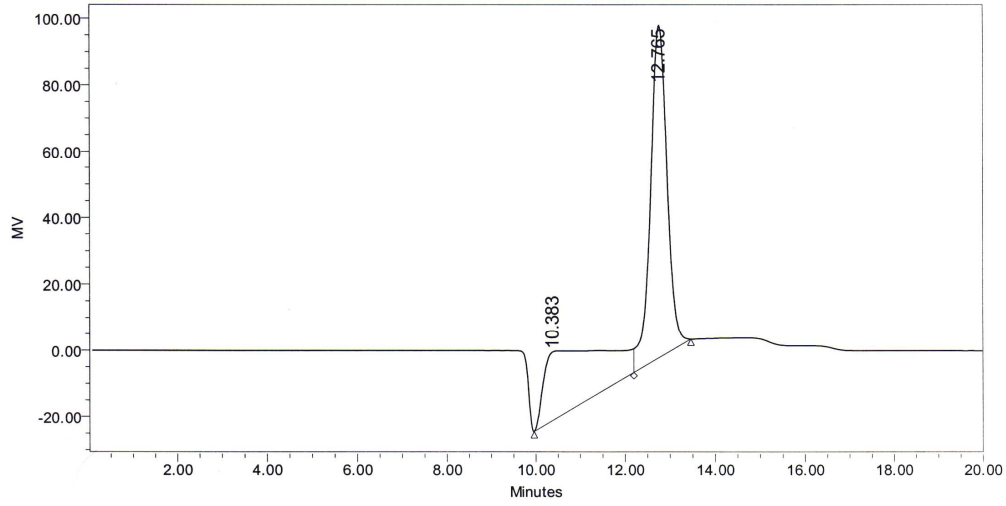

Figure 15C. HPLC Chromatogram of 2 mg/ml standard D-sucrose (analytical grade) using Vertisep^{TM} OA HPLC with a 100% water mobile phase and a refractive index detector.

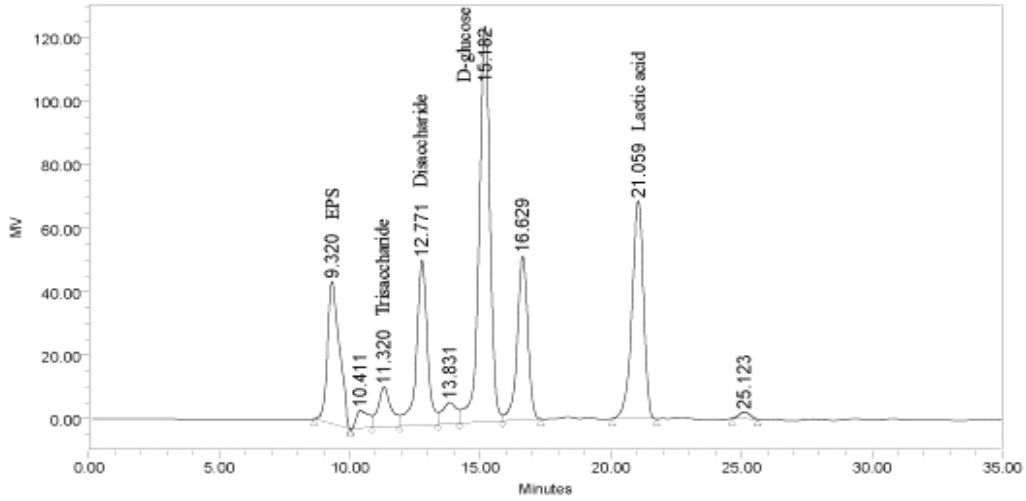

Figure 16C. HPLC Chromatogram of EPS, white sugar from sugar cane and lactic acid in culture broth using Vertisep^{TM} OA HPLC with a 0.005M H_2SO_4 mobile phase and a refractive index detector.

APPENDIX D

RESULTS OF CYTOKINE PRODUCTION ASSAY

Table 1D Effect of EPS produced by lactic acid bacterial isolate C56 when culturing in MRS broth containing 2% of glucose at initial pH of 6.2 and 37°C for 48 h on cytokine production**.**

IL-4	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	12.33	0.00	0.00	266.24	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	59.31	0.00	0.00	665.40	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00					
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	346.28	0.00	0.00	2389.45	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00					
Average	0.00			0.00		
S.D.	0.00			0.00		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	1147.54	0.00	0.00	3164.72	0.00
	0.00			8.83		
	0.00			0.00		
	0.00			0.00		
	2.42			29.34		
Average	0.48			7.63		
S.D.	1.08			12.72		

Table 1D (Continued) Effect of EPS produced by lactic acid bacterial isolate C56 when culturing in MRS broth containing 2% of glucose at initial pH of 6.2 and 37°C for 48 h on cytokine production**.**

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	23.10	6.57	1.07	89.27	1.07
	1.07			6.57		
	1.07			0.00		
	23.10			1.07		
	1.07					
Average	5.26			2.18		
S.D.	9.98			2.97		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	16.46	0.00	0.00	132.64	0.00
	0.00			0.00		
	0.00			0.00		
	0.05			0.00		
	0.00					
Average	0.01			0.00		
S.D.	0.02			0.00		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 2D Effect of EPS produced by lactic acid bacterial isolate FKU23 when culturing in MRS broth containing 4.5% of white sugar from sugar cane at initial pH of 7.0 and 30°C for 48 h on cytokine production.

IL-4	**Content of cytokine (pg/ml)**					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	1.91	12.11	0	0	14.98	0
	0			0		
	0			19.58		
	0			0		
	0			0		
Average	0.38			3.92		
S.D.	0.85			8.76		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	54.96	0	0	59.89	0
	0			0		
	0			69.77		
	0			0		
	0			0		
Average	0.00			13.95		
S.D.	0.00			31.20		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	21.95	128.27	0.76	0.76	136.83	5.39
	14.63			17.12		
	19.55			239		
	19.55			3.94		
	12.1			6.79		
Average	17.56			53.52		
S.D.	4.05			103.87		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	2	575.47	1.34	1.34	457	1.34
	1.67			0		
	1.34			436.82		
	2			0.28		
	0			1.34		
Average	1.40			87.96		
S.D.	0.83			195.02		

Table 2D (Continued) Effect of EPS produced by lactic acid bacterial isolate FKU23 when culturing in MRS broth containing 4.5% of white sugar from sugar cane at initial pH of 7.0 and 30°C for 48 h on cytokine production.

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	7.64	9.57	3.75	3.75	11.5	7.64
	3.75			11.5		
	11.5			11.5		
	7.64			3.75		
	0			7.64		
Average	6.11			7.63		
S.D.	4.38			3.88		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	5.42	3.66	1.94	7.21	7.21	3.66
	3.66			1.94		
	3.66			11.76		
	3.66			0.3		
	7.21			1.94		
Average	4.72			4.63		
S.D.	1.59			4.76		

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium
Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS

Table 3D Effect of EPS produced by lactic acid bacterial isolate G3 when culturing in MRS broth containing 2% of white sugar from sugar cane at initial pH of 6.2 and 30°C for 48 h on cytokine production**.**

IL-4	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	93.84	0	0	104.79	0
	0			0		
	0			0		
	0			0		
	0			0		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	129.24	0	0	186.4	0
	0			0		
	0			0		
	0			0		
	0			0		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	3.94	1080.15	6.97	14.63	1369.71	33.53
	54.3			20.76		
	40.23			14.63		
	35.78			17.12		
	24.32			6.79		
Average	31.71			14.79		
S.D.	18.88			5.13		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	1.34	861.32	1.34	0.65	1178.84	1.34
	0.65			1.34		
	1.34			0.65		
	0.65			1.34		
	0.65			0.65		
Average	0.93			0.93		
S.D.	0.38			0.38		

Table 3D (Continued) Effect of EPS produced by lactic acid bacterial isolate G3 when culturing in MRS broth containing 2% of white sugar from sugar cane at initial pH of 6.2 and 30°C for 48 h on cytokine production**.**

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	3.75	15.33	7.64	7.64	15.33	7.64
	7.64			3.75		
	7.64			3.75		
	7.64			7.64		
	7.64			7.64		
Average	6.86			6.08		
S.D.	1.74			2.13		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	3.66	14.54	5.42	5.42	22.07	3.66
	3.66			1.94		
	5.42			1.94		
	3.66			3.66		
	5.42			1.94		
Average	4.36			2.98		
S.D.	0.96			1.55		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 4D Effect of EPS produced by lactic acid bacterial isolate I5 when culturing in MRS broth containing 2% of glucose at initial pH of 6.2 and 37°C for 48 h on cytokine production**.**

IL-4	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.07	90.40	0.00		79.74	0.00
	0.00			0.00		
	0.00			0.00		
	0.01			0.00		
	0.00			0.00		
Average	0.02			0.00		
S.D.	0.03			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	644.28	9.82		759.48	0.00
	0.00			0.00		
	0.00			0.00		
	88.10			0.00		
	0.00			0.00		
Average	17.62			0.00		
S.D.	39.40			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	2.84	1372.54	0.00	2.84	1779.53	0.00
	0.00			0.00		
	0.00			0.00		
	2.84			2.84		
	0.00			0.00		
Average	1.14			1.14		
S.D.	1.56			1.56		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	890.20	8.83		915.49	0.00
	0.00			0.00		
	0.00			0.00		
	2.42			8.83		
	0.00			0.00		
Average	0.48			2.21		
S.D.	1.08			4.42		

Table 4D (Continued) Effect of EPS produced by lactic acid bacterial isolate I5 when culturing in MRS broth containing 2% of glucose at initial pH of 6.2 and 37°C for 48 h on cytokine production**.**

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	6.57	34.12	6.57		17.59	1.07
	1.07			0.00		
				6.57		
	6.57			12.08		
	0.00			0.00		
Average	3.55			4.66		
S.D.	3.51			5.83		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	45.32	0.05		38.30	0.00
	0.00			0.00		
	0.00			0.05		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.01		
S.D.	0.00			0.03		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 5D Effect of EPS produced by lactic acid bacterial isolate I5 when culturing in MRS broth containing 3.5% of white sugar from sugar cane at initial pH of 6 and 40°C for 48 h on cytokine production**.**

IL-4	**Content of cytokine (pg/ml)**					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	49.30	0.00	0.00	6.69	0.00
	0.00			0.00		
	0.00			0.11		
	0.00			0.00		
	0.01			0.00		
Average	0.00			0.02		
S.D.	0.00			0.05		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	597.84	0.00	0.00	155.08	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	210.17	936.59	0.00	30.28	771.37	0.00
	374.72			15.25		
	175.85			42.27		
	133.93			30.28		
	197.79			0.00		
Average	218.49			23.62		
S.D.	92.02			16.31		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	1652.27	0.00	8.83	1205.37	0.00
	0.00			8.83		
	33.99			0.00		
	0.00			0.00		
	0.00			0.00		
Average	6.80			3.53		
S.D.	15.20			4.84		

Table 5D (Continued) Effect of EPS produced by lactic acid bacterial isolate I5 when culturing in MRS broth containing 3.5% of white sugar from sugar cane at initial pH of 6 and 40°C for 48 h on cytokine production**.**

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	6.57	17.59	12.08	6.57	12.08	0.00
	12.08			1.07		
	1.07			6.57		
	12.08			6.57		
	1.07			1.07		
Average	6.57			4.37		
S.D.	5.51			3.01		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	27.80	0.00	0.05	15.59	0.00
	0.05			0.00		
	0.00			0.05		
	0.00			0.00		
	0.05			0.00		
Average	0.02			0.02		
S.D.	0.03			0.03		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 6D Effect of EPS produced by isolate I5 when culturing in MRS broth containing 3.5% of white sugar from sugar cane (without yeast extract) at initial pH of 6 and 40°C for 48 h on cytokine production.

IL-4	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	48.13	2.25	0.00	159.15	0.00
	1.42			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.28			0.00		
S.D.	0.64			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	792.68	446.94	0.00	536.87	0.00
	416.03			0.00		
	16.18			0.00		
	213.06			0.00		
	0.00			0.00		
Average	129.05			0.00		
S.D.	184.03			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	255.69	459.62	8.15	2.84	478.76	0.00
	550.26			30.28		
	194.68			38.34		
	156.71			57.52		
	32.32			288.30		
Average	237.93			83.46		
S.D.	192.75			116.19		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	1489.92	19.60	0.00	2138.65	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			19.60		
	0.00			0.00		
Average	0.00			3.92		
S.D.	0.00			8.77		

Table 6D (Continued) Effect of EPS produced by isolate I5 when culturing in MRS broth containing 3.5% of white sugar from sugar cane (without yeast extract) at initial pH of 6 and 40°C for 48 h on cytokine production.

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	1.07	17.59	12.08	6.57	28.61	0.00
	12.08			6.57		
	0.00			0.00		
	0.00			6.57		
	1.07			1.07		
Average	2.84			4.16		
S.D.	5.19			3.33		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	17.33	1.75	0.00	48.83	0.00
	3.46			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.69			0.00		
S.D.	1.55			0.00		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS exposed to homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS exposed to ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS exposed to RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS exposed to homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS exposed to ConA
Control group-RPMI, Splenocytes from mice injected with PBS exposed to RPMI medium

Table 7D Effect of EPS produced by lactic acid bacterial isolate P14 when culturing in MRS broth containing 2% of analytical grade sucrose at initial pH of 6.2 and 30°C for 48 h on cytokine production.

IL-4	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	14.23	0	0	15.73	0
	0			0		
	0			0		
	1.73			0		
	0			0		
Average	0.35			0.00		
S.D.	0.77			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	41.2	0	0	55.26	0
	0			0		
	0			0		
	0.97			0		
	0			0		
Average	0.19			0.00		
S.D.	0.43			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	14.63	183.31	3.94	9.49	222.12	3.94
	14.63			3.94		
	12.1			0		
	17.12			3.94		
	9.49			3.94		
Average	13.59			4.26		
S.D.	2.90			3.38		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	1.34	300	1.34	2	357.77	0.65
	2			1.34		
	1.34			0		
	1.34			0.65		
	0.65			1.34		
Average	1.33			1.07		
S.D.	0.48			0.76		

Table 7D (Continued) Effect of EPS produced by lactic acid bacterial isolate P14 when culturing in MRS broth containing 2% of analytical grade sucrose at initial pH of 6.2 and 30°C for 48 h on cytokine production.

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	7.64	11.5	3.75	3.75	11.5	3.75
	7.64			3.75		
	7.64			0		
	7.64			3.75		
	7.64			3.75		
Average	7.64			3.00		
S.D.	0.00			1.68		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	3.66	3.66	1.94	3.66	5.42	1.94
	3.66			1.94		
	3.66			1.1		
	5.42			3.66		
	3.66			3.66		
Average	4.01			2.80		
S.D.	0.79			1.21		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 8D Effect of EPS produced by lactic acid bacterial isolate PSMS1-5 when culturing in MRS broth containing 4.5% of white sugar from sugar cane at initial pH of 5 and 37°C for 48 h on cytokine production.

IL-4	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	11.4	0	0	10.79	0
	0			0		
	0			0		
	0			0		
	0			0		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	54.01	0	0	78	0
	0			0		
	0			0		
	0			0		
	0			0		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	9.49	129.22	3.94	3.94	163.06	3.94
	12.1			0		
	9.49			3.94		
	12.1			5.39		
	9.49			3.94		
Average	10.53			3.44		
S.D.	1.43			2.02		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.65	277.68	1.34	0.65	333.66	0.65
	1.34			2		
	0.65			1.34		
	1.34			1.34		
	0.65			0.65		
Average	0.93			1.20		
S.D.	0.38			0.57		

Table 8D (Continued) Effect of EPS produced by lactic acid bacterial isolate PSMS1-5 when culturing in MRS broth containing 4.5% of white sugar from sugar cane at initial pH of 5 and 37°C for 48 h on cytokine production.

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	7.64	9.57	3.75	3.75	11.5	5.7
	7.64			0		
	7.64			3.75		
	3.75			3.75		
	3.75			7.64		
Average	6.08			3.78		
S.D.	2.13			2.70		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	5.42	5.42	3.66	0	5.42	3.66
	5.42			2.79		
	3.66			1.94		
	5.42			3.66		
	3.66			3.66		
Average	4.72			2.41		
S.D.	0.96			1.52		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 9D Effect of EPS produced by lactic acid bacterial isolate PSMS4-4 when culturing in MRS broth containing 5% of white sugar from sugar cane at initial pH of 7and 30°C for 48 h on cytokine production**.**

IL-4	**Content of cytokine (pg/ml)**					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	17.02	0	0	13.04	0
	0			0		
	0					
	0					
	0					
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	91.91	0	0	86.85	0
	0			0		
	0					
	0					
	0					
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	9.49	330	3.94	14.63	181.48	6.79
	9.49			6.79		
	12.1					
	6.79					
	8.16					
Average	9.21			10.71		
S.D.	1.97			5.54		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	1.34	539	0.65	3.27	346.16	0.65
	0.65			0.65		
	0.65					
	1.34					
	1.34					
Average	1.06			1.96		
S.D.	0.38			1.85		

Table 9D (Continued) Effect of EPS produced by lactic acid bacterial isolate PSMS4-4 when culturing in MRS broth containing 5% of white sugar from sugar cane at initial pH of 7and 30°C for 48 h on cytokine production**.**

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	3.75	11.5	7.64	13.41	11.5	3.75
	7.64			7.64		
	7.64					
	3.75					
	3.75					
Average	5.31			10.53		
S.D.	2.13			4.08		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	3.66	7.21	5.42	5.42	7.21	3.66
	3.66			3.66		
	3.66					
	3.66					
	3.66					
Average	3.66			4.54		
S.D.	0.00			1.24		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 10D Effect of EPS produced by lactic acid bacterial isolate PSMS4-4 when culturing in MRS broth containing 2% of glucose at initial pH of 6.2 and 37°C for 48 h on cytokine production.

IL-4	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	91.18	0.00	0.00	86.27	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	445.41	0.00	0.00	514.08	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	766.29	0.00	0.00	1518.40	0.00
	0.00			0.00		
	0.00			0.00		
	30.28			0.00		
	0.00			0.00		
Average	6.06			0.00		
S.D.	13.54			0.00		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	1101.37	0.00	0.00	912.96	0.00
	0.00			0.00		
	0.00			0.00		
	8.83			0.00		
	0.00			0.00		
Average	1.77			0.00		
S.D.	3.95			0.00		

Table 10D (Continued) Effect of EPS produced by lactic acid bacterial isolate PSMS4-4 when culturing in MRS broth containing 2% of glucose at initial pH of 6.2 and 37°C for 48 h on cytokine production.

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	1.07	34.12	6.57	6.57	28.61	1.07
	6.57			1.07		
	0.00			0.00		
	6.57			0.00		
	1.07			1.07		
Average	3.06			1.74		
S.D.	3.24			2.75		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	36.55	0.00	0.00	38.30	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.00		
S.D.	0.00			0.00		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 11D Effect of EPS produced by lactic acid bacterial isolate RMS3-1 when culturing in MRS broth containing 3% of white sugar from sugar cane at initial pH of 8 and 35°C for 48 h on cytokine production**.**

IL-4	**Content of cytokine (pg/ml)**					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	27.08	0.00	0.01	28.85	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	314.56	0.00	0.00	431.43	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	839.54	0.00	0.00	1349.18	0.00
	26.13			0.00		
	34.34			0.00		
	0.00			0.00		
	38.34			0.00		
Average	19.76			0.00		
S.D.	18.57			0.00		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	1069.59	0.00	0.00	1224.53	0.00
	8.83			8.83		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	1.77			1.77		
S.D.	3.95			3.95		

Table 11D (Continued) Effect of EPS produced by lactic acid bacterial isolate RMS3-1 when culturing in MRS broth containing 3% of white sugar from sugar cane at initial pH of 8 and 35°C for 48 h on cytokine production**.**

TNF-a	Content of cytokine (pg/ml)					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	1.07	17.59	1.07	6.57	12.08	6.57
	1.07			1.07		
	6.57			1.07		
	3.82			3.82		
	6.57			0.00		
Average	3.82			2.51		
S.D.	2.75			2.68		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.00	16.46	0.00	0.00	20.81	0.00
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
	0.00			0.00		
Average	0.00			0.00		
S.D.	0.00			0.00		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

Table 12D Effect of EPS produced by *Lactobacillus reuteri* 100-23 when culturing in MRS broth containing 2% of analytical grade sucrose at initial pH of 6.2 and 37°C for 48 h on cytokine production.

IL-4	**Content of cytokine (pg/ml)**					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0.84	0	0	0	29.39	0
	0			0		
	0			0		
	0			0		
	0			0		
Average	0.17			0.00		
S.D.	0.38			0.00		
IL-5	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	0	0	0	0	120.95	0
	0			0		
	0			0		
	0			0		
	0			0		
Average	0.00			0.00		
S.D.	0.00			0.00		
IL-10	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	24.32	6.79	3.94	17.12	261.88	3.94
	12.1			12.1		
	14.63			12.1		
	15.88			14.63		
	12.1			9.49		
Average	15.81			13.09		
S.D.	5.03			2.90		
IFN-g	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	2.64	0	1.34	1.34	506.53	1.34
	1.34			1.34		
	1.34			0.65		
	1.34			0.65		
	1.34			0.65		
Average	1.60			0.93		
S.D.	0.58			0.38		

Table 12D (Continued) Effect of EPS produced by *Lactobacillus reuteri* 100-23 when culturing in MRS broth containing 2% of analytical grade sucrose at initial pH of 6.2 and 37°C for 48 h on cytokine production.

TNF-a	**Content of cytokine (pg/ml)**					
	Experiment group-EPS	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	7.64	3.75	3.75	7.64	15.33	3.75
	7.64			7.64		
	7.64			3.75		
	3.75			7.64		
	7.64			3.75		
Average	6.86			6.08		
S.D.	1.74			2.13		
IL-12	**Experiment group-EPS**	**Experiment group-ConA**	**Experiment group-RPMI**	**Control group-EPS**	**Control group-ConA**	**Control group-RPMI**
	3.66	0.3	1.94	3.66	9.02	2.79
	5.42			5.42		
	5.42			3.66		
	5.42			1.94		
	4.53			3.66		
Average	4.89			3.67		
S.D.	0.79			1.23		

Remarks:

Experiment group-EPS, Splenocytes from mice injected with EPS were stimulated *in vitro* with homologous EPS
Experiment group-ConA, Splenocytes from mice injected with EPS were stimulated *in vitro* with ConA
Experiment group-RPMI, Splenocytes from mice injected with EPS were stimulated *in vitro* with RPMI medium
Control group-EPS, Splenocytes from mice injected with PBS were stimulated *in vitro* with homologous EPS
Control group-ConA, Splenocytes from mice injected with PBS were stimulated *in vitro* with ConA
Control group-RPMI, Splenocytes from mice injected with PBS were stimulated *in vitro* with RPMI medium

APPENDIX E
LIST OF PRESENTATIONS

Poster Presentation

Tayuan, C., La-ongkham, O., and Rodtong, S. (2008). **Selection of lactic acid bacteria isolated from Thai fermented food for exopolysaccharide production**. The 4th Naresuan Research Conference, July 28-29, 2008, Naresuan University, Pisanulok, Thailand.

Tayuan, C., Ratanachai, K., and Rodtong, S. (2007). **Strain variation of exopolysaccharide-producing *Weissella confusa***. The 24th MST Annual Conference. February 14-16, 2007, Kasaetsart University, Bangkok, Thailand.

Tayuan, C. and Rodtong, S. (2006). **Investigation of potential lactic acid bacteria for exopolysaccharide production.** RGJ–Ph.D. Congress VII. April 20-22, 2006, Chonburi, Thailand.

Oral Presentation

Tayuan, C., La-ongkham, O., and Rodtong, S. (2008). **Exopolysaccharide production from food grade sucrose by *Lactobacillus sakei* FKU23**. The 4th Naresuan Research Conference. July 28-29, 2008, Naresuan University, Pisanulok, Thailand.

Publication

Tayuan, C., La-ongkham, O., and Rodtong, S. (2008). Exopolysaccharide production from food grade sucrose by *Lactobacillus sakei* FKU23. **Proceedings of 4th Naresuan Research Conference**. 857-861.

Tayuan, C., Ratanachai, K., and Rodtong, S. (2007). Strain variation of exopolysaccharide-producing *Weissella confusa*. **Journal of Microscopy Society of Thailand**. 21: 185-189.

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