HALOPHILIC AND LACTIC ACID BACTERIA IN THAI FERMENTED FOODS

AMNAT PAKDEETO

TOKYO UNIVERSITY OF AGRICULTURE

TOKYO, JAPAN

2016

HALOPHILIC AND LACTIC ACID BACTERIA IN THAI FERMENTED FOODS

by

AMNAT PAKDEETO

A THESIS

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

…….

DEPARTMENT OF APPLIED BIOLOGY AND CHEMISTRY

TOKYO UNIVERSITY OF AGRICULTURE

TOKYO, JAPAN

2016

Abstract

The objectives of this study were to isolate and identify halophilic and lactic acid bacteria based on phenotypic, chemotaxonomic, DNA-DNA relatedness and 16S rRNA gene sequencing and to screen and study on diacetyl production of lactic acid bacteria from Thai fermented foods. Seventeen strains of halophilic lactic acid bacteria were isolated from fermented shrimp paste (*ka-pi*) samples collected at the market in the southern part of Thailand. They were divided into 2 groups. The representative strains in Group I were identified as *T. halophilus* while Group II isolates were identified as *T. muriaticus.* Their phenotypic characteristics, DNA-DNA relatedness and growth on 5% and 10% NaCl were useful for identification of *Tetragenococcus* species. Thirty-two strains of moderately halophilic bacteria were isolated from 6 samples of *Ka-pi* and 3 samples of fish sauce (*nam-pla*) from Thai fish sauce factories at various stages. They were divided in to three groups. Group I (15 strains) were isolated from *Nam-pla* , that were closely related to *Lentibacillus juripiscarius* JCM 12147^T (97.3 % similarity) but they showed low levels of DNA-DNA relatedness (17%) with *Ln. juripiscarius* JCM 12147^T. Therefore, the strains represent a novel species and the name *Lentibacillus halophilus* sp. nov. is proposed. Two strains (Group II**)** from *Ka-pi* produced red pigment and non-motile, were closely related to *Lentibacillus salarius* KCTC 3911^T (96.5% similarity). These strains represent a novel species and the name *Lentibacillus kapialis* sp. nov. is proposed. Fifteen strains (Group III) from *Ka-pi* were closely related to *Salinicoccus roseus* JCM 14630^T (97.3% similarity). They showed low DNA-DNA relatedness to *S. roseus* JCM 14630^T (21.7%). Consequently, these strains represent a novel species and the name *Salinicoccus siamensis* sp. nov. is proposed. Twenty-five of lactic acid bacteria were isolated from fermented tea leaves (*miang*) produced in the northern part of Thailand. They were divided into seven groups. Groups I to VI belonged to *Lactobacillus* and Group VII to *Pediococcus*. Six strains in Group I were identified as *Lactobacillus pantheris*, five strains in Group II as *Lactobacillus pentosus* and four strains in Group V as *Lactobacillus suebicus*. Two strains in Group VI were identified as *Lactobacillus fermentum*. Five strains in Group III are proposed as *Lactobacillus thailandensis* sp. nov., two strains in Group IV are proposed as *Lactobacillus camelliae* sp. nov., and one strain in Group VII is proposed as *Pediococcus siamensis* sp. nov.

After screening of diacetyl-producing lactic acid bacteria by using the colorimetric method. The result showed that the most of homofermentative lactic acid bacteria could produce diacetyl higher than the heterofermentative lactic acid bacteria. Seven high producing-diacetyl lactic acid bacteria were selected to study on diacetyl production in MMRS broth. The result showed all selected strains could produce the maximum of diacetyl concentration at 18 to 36 hours of fermentation time under stationary phase and showed the maximum growth at 12 hours and could produce high diacetyl concentration in the medium containing citrate under static conditions which was different from previous reports. Moreover, diacetyl production under static condition of strains SR4-2, PM3-14 and AP2-1 could produce high diacetyl were 3.35, 3.27 and 3.16 mM at pH 5.74, 6.41 and 7.51 after fermentation at 30, 18 and 30 hours respectively. On the basis of the phenotypic and chemotaxonomic characteristics and DNA-DNA hybridization, the strains SR4-2 isolated from soy sauce mash was identified as *Lactobacillus pentosus*, the strain AP2-1 from pork sausage (*mu-yor*) was identified as *Weissella confusa* and the strains PM3-14 (from pasteurized milk) was identified as *Enterococcus faecium.*

ACKNOWLEDGEMENTS

I would like to thank many concerned person that made my study be possible and be successful here to:

Professor Dr. Sanae Okada, Professor Dr. Masataka Uchino, and Professor Dr. Tanaka Naoto, Department of Applied Biology and Chemistry, Tokyo; Dr. Takashi Itoh and Dr. Takuji Kudo, Japan Collection of Microorganisms, Tsukuba; Professor Dr. Somboon Tanasupawat, Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Assoc. Professor Dr. Nuanphan Naranong and Assist. Professor Dr. Chitti Thawai, Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok; Assist. Professor Bussakorn Utarapichat, Department of Biology, Faculty of Science, Thaksin University, Songkhla; Dr. Somporn Moonmangmee, Thailand Institute of Scientific and Technological Research, Pathumthani; Assist. Professor Dr. Sirilak Namwong Department of Biotechnology, Faculty of Sciences and Technology, Suan Sunandha Rajabhat University, Bangkok; and Dr. Pattaraporn Yukphan, BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani; Thesis committee chairperson and all thesis committee members and their recommendations for the research. Finally, I wish to thank my parents and my family for their care support, patience, understanding, and encouragement those gave me strength to persevere during trying times and overcome all the difficulties in this work.

CONTENTS

CHAPTER I

INTRODUCTION

Thai traditional fermented foods are produced by natural fermentation in processes which vary from simple to complicated (Phithakpol et al., 1995). Fermented foods are products that prepared involving a step where microorganisms that has enzymes that change or modify the properties of the food for a better characteristics and nutrition. The purpose of fermenting food is often to get a better taste or texture and also for the important reason is that for keeping longer. The purpose of producing fermented foods for foods storage problem occurs in seasonal periods; dry season, saving of expenditure, improve flavor and texture, eliminate undesired of raw material and producing variety of foods for consumption. In Thailand, there are numerous Traditional fermented foods such as fish base fermented foods, meat based fermented foods, vegetable based fermented foods, fruit based fermented foods, rice based fermented foods and plant based fermented foods. The traditional fermented foods play an important role for Thai peoples that most consume the fermented products as daily diet. The foods usually produced at home for family consumption since ancient times. Currently some fermented foods have move to industrialize production. (Fungsin, 2014)

A number of fermented foods are home-made but some are produced at commercial factories. For some products, such as fish sauce, raw materials, processing methods and composition, are very similar to products found in other Asian countries. Fishery products can be categorized according to main processing techniques and ingredients. *Nam-pla* (fish sauce), *ka-pi* (shrimp paste) and *bu-du* are produced from fish with a large proportion of salt. Most of them are made from marine fish in the coastal provinces, especially the east coast. There are many kinds of fermented fish with salt and carbohydrate, such as *pla-ra, pla-som, pla-chao, som-fak*, and *pla-chom*. (Tanasupawat and Vissessanguan, 2008)

1.1 Traditional Thai Fermented foods

1.1.1 Fermented shrimp paste (*ka-pi*)

Ka-pi is a dark-coloured strong smelling paste that varied depending on the shrimp used (*Acetes erythraeus, Koei-dtaa-daeng* or *Acetes* sp., *Koei-maletkhao-saan-som-oh)*. It contained high concentration of NaCl (14-40.1%). The production of *Ka-pi* shown in Fig. 1 (Phithakpol et al., 1995; Tanasupawat and Komagata, 1995). Ingredients, shrimp and salt are mixed and left for 1-2 days. After the contents are drained of liquid, pound the mixture or mince it to paste until it becomes sticky, pack it tightly in a wide-mouthed earthenware and left fermenting for 4-6 months.

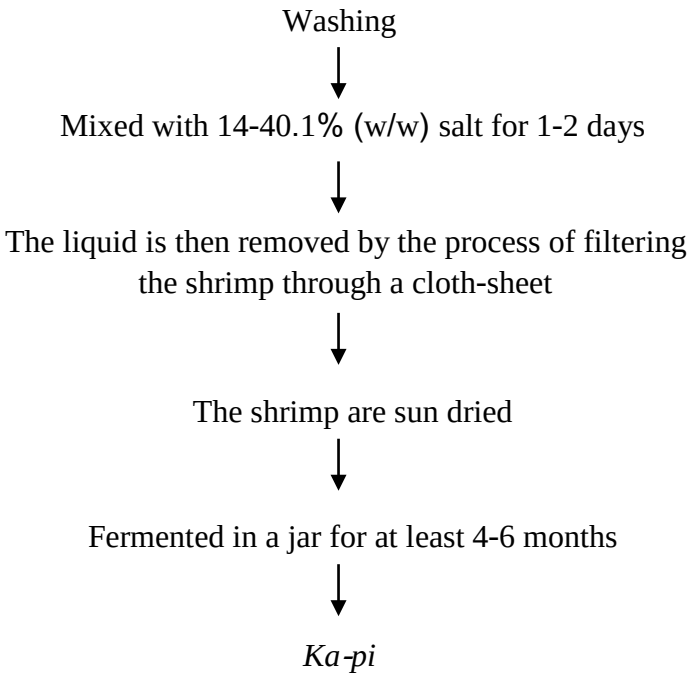


Fig. 1 Flow chart for production of fermented shrimp paste (*ka-pi*)

1.1.2 Fermented fish sauce (*nam-pla*)

Nam-pla is a clear brown liquid traditionally produced and widely used in Southeast Asia and to some extent in other parts of the world. (Saisithi et al., 1994; Tanasupawat and Daengsubha (1983); Tanasupawat and Komagata (1995) reported the strain " *Peddiococcus halophilus*" have been found in the product contain high

concentrations of NaCl (*Nam-pla*) Satomi et al.(1997); Kobayashi et al. (2000) reported the strains *Tetragenococcus halophilus* and *T. muriaticus* have been found in Japanese squid liver sauce and puffer fish ovaries.In addition, Thongsanit et al.(2002) reported the strains " *T. halophilus* and *T. muriaticus* have been found in fish sauce fermentation in Thailand. The production of *Nam-pla* shown in Fig. 2 (Namwong, 2005).

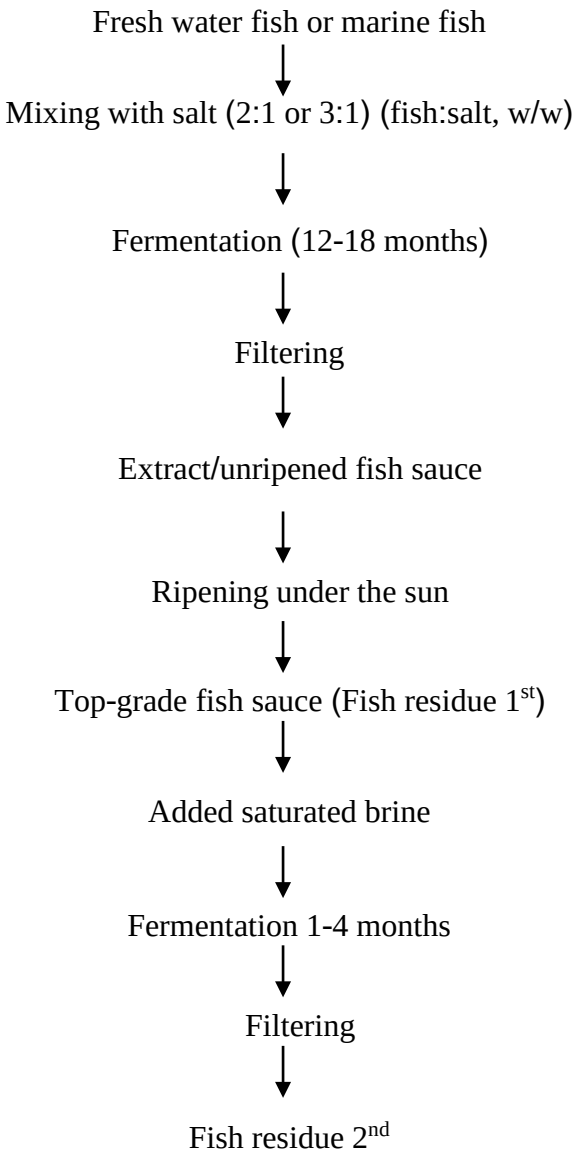


Fig. 2 Flow chart for production of fish sauce (*nam-pla*)

1.1.3 Fermented tea leaves (*miang)*

Miang is one of the fermented products in the northern part of Thailand, non-salted and fermented tea leaves, the fermented product are soft and have a bitter and acidic taste with good flavor and contain a considerable amount of tannic acid which is known to be a microbial growth inhibitor. The most strains of the lactic acid bacteria were inhibited by tea tannic acid, but several strains were not. The strains *Lactobacillus plantarum*, *L. pentosus*, *L. vaccinostercus*, *Lactobacillus* sp., *Enterococcus casseliflavus*, and *Enterococcus* sp. were reported that the non-sensitive from tannic acid. It is consumed as a snack. The production of *Miang* as shown in Fig. 3 (Okada et al., 1979, 1986; Tanasupawat et al., 1992a, b; Tanasupawat and Komagata, 1995).

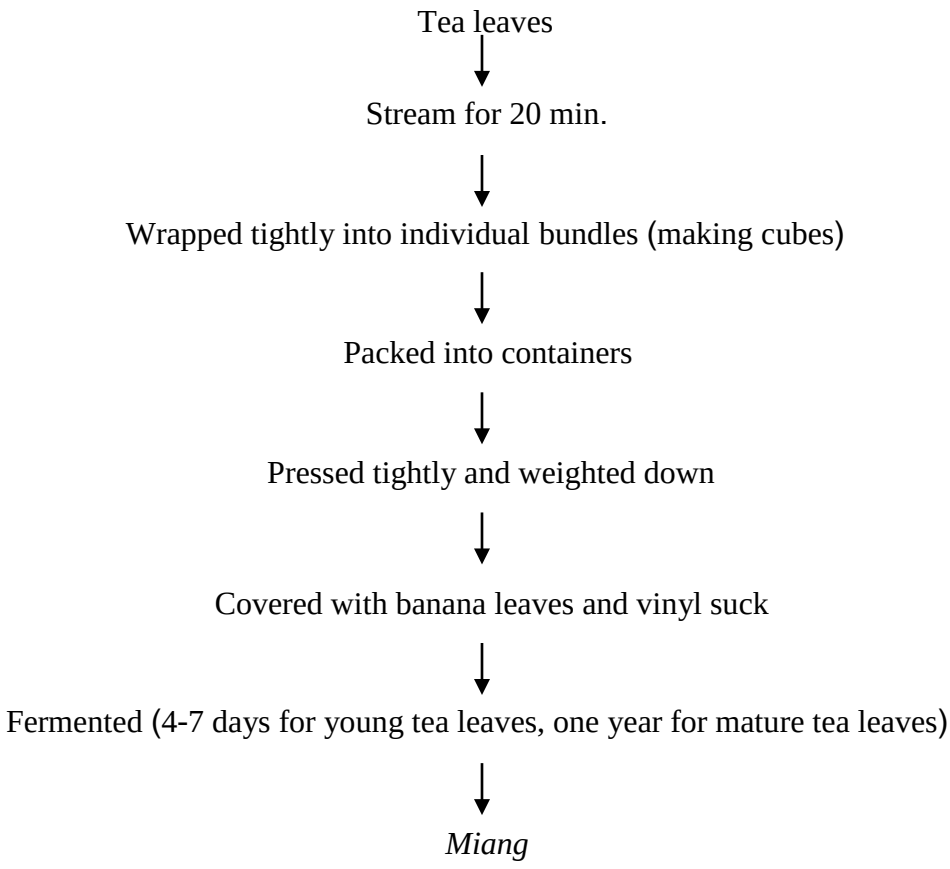


Fig. 3 Flow chart for production of fermented tea leaves (*miang*)

1.2 Bacteria involved in Thai fermented foods

1.2.1 Lactic acid bacteria (LAB) and metabolic products

A group of Gram-positive bacteria, cocci or rods, nonsporing, anaerobic, microaerophilic, or aero-tolerant, and catalase negative. They produce lactic acid as the major end product during the fermentation of carbohydrates. LAB have a GRAS status (generally recognized as safe) or food-grade organisms. The genera of lactic acid bacteria were shown in Table 1. The classification of lactic acid bacteria into different genera is largely based on their morphology, growth at different temperature, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. They are mesophilic. Some can grow below 5 ^{o}C and some as high as 45 ^{o}C, with respect to growth pH, some can grow as low as 3.2, some as high as 9.6, and most grow in the pH range 4.0-4.5 depending on the species, they synthesize either the L(+) or D(-) isomer of lactic acid or both. Two main sugar fermentation pathways can be distinguished among lactic acid bacteria. Embden-Meyerhof pathway (Glycolysis) results in almost exclusively lactic acid as end-product under standard conditions, and the metabolism is refered to as homolactic fermentation.

The homofermenters are *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcu*s, and some species of *Lactobacillus.* In the case of heterolactic fermention, 6-phosphogluconate/ phosphoketolase pathway used by *Leuconostoc*, *Oenococcus*, *Weissella*, and some species of *Lactobacillus*, are mainly sugar fermentation pathway that results in significant amounts of other and-products such as ethanol, acetate, and carbon dioxide in addition to lactic acid. In additionally, lactic acid bacteria can produce a variety of antimicrobial compounds, which provide these organisms with a competitive advantage over other microorganisms. The antimicrobial compounds, include lactic acid, acetic acid, hydrogen peroxide, carbon dioxide, diacetyl (Fig. 4), as well as bacteriocins (Gould, 1995; Herbin, et al. , 1997; Mishara and Lambert, 1996; Lee and Paik, 2001) including other fermentation end products of lactic acid bacteria in Thai fermented foods such as γ-aminobutyric acid GABA, protease, and polysaccharide (Tanasupawat, 2009).

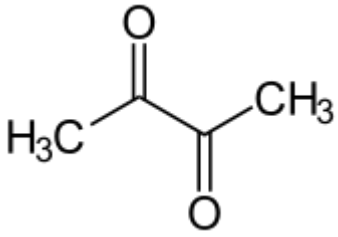


Fig. 4 Structure of diacetyl

Diacetyl ($C_4H_6O_2$ or biacetyl; 2,3-butanedione; dimethyl diketone; 2,3-diketobutane) is a major flavor compound essential in many dairy products such as butter, cream, and some cheeses. It is GRAS (generally recognized as safe) substance. In addition to butter and other dairy products, it is found in red and white wines, brandy, roasted coffee, ensilage, and many other fermented foods (Jay, 1982). Its production was shown to depend on the strains used as starter cultures and the conditions of fermentation such as pH, oxygen, and temperature (Bassit et al., 1993; Gasson, 1983; Monnet et al., 1994). Moreover, it is a metabolic and products that is synthesized from pyruvate aerobically as well as anerobically (Condon, 1987) that is actually produced by citrate fermenting lactic acid bacteria (Hugenholtz, 1993). The genera of diacetyl-producing lactic acid bacteria such as *Lactobacillus bulgaricus, L. lactis, L. helveticus, Leuconostoc cremolis, L. dextranicum, L. Lactis, Streptococcus thermophilus, Pediococcus acidilactici* and *Lactococcus lactis, L. cremoris, L. diacetylactis* can produce diacetyl as well as other organisms (Cathy, 1994; Jay, 1982; Kaneko et al., 1990, 1991). In food, diacetyl is important not only because it is responsible for the desirable flavor in many foods, but also because it has antimicrobial properties (Ray, 1992). In addition, diacetyl and acetoin are two compounds that give butter its characteristic taste. Because of this, manufacturers of artificial butter flavoring, margarines or similar oil-based products typically add diacetyl and acetoin (along with beta carotene for the yellow color) to make the final product butter-flavored, because it would otherwise be relatively tasteless (Pavia et al., 2006). The production of diacetyl from citrate of lactic acid bacteria as shown in Fig. 5 (Hugenholtz and Starrenburg,1992; Cogan,1981)

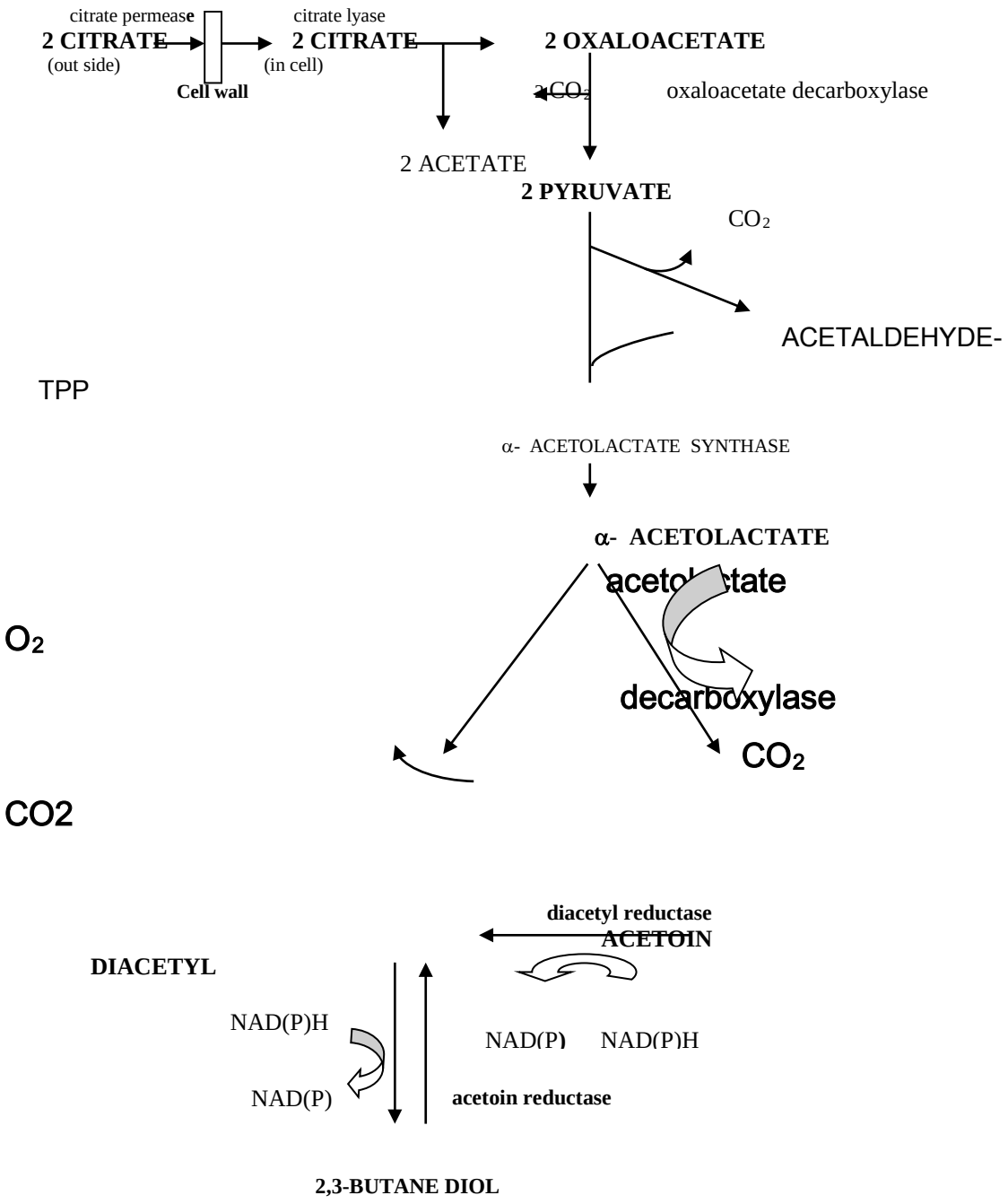


Fig. 5. The synthesis of diacetyl from citrate of lactic acid bacteria

Table 1. The characteristics of genera of lactic acid bacteria (LAB)

Characteristics	Rods	◄ Cocci ►								
	Carnobacterium	*Lactobacillus*	*Weissella* [a]	*Aerococcus*	*Enterococcus*	*Lactococcus*	*Vagococcus*	*Leuconostoc*	*Oenococcus*	*Pediococcus*	*Streptococcus*	*Tetragenococcus*
Tetrad shape	-	-	-	+	-	-		-	+	-	+	
CO_2 from glucose	-	+/-	+	-	-	-		+	-	-	-	
Growth at temperature≀ 10 °C	+	+/-	+	+	+	+		+	+/-	-	+	
Growth at temperature 45 °C	-	+/-	-	-	+	-		-	+/-	+/-	-	
Growth in NaCl 6.5 %	ND	+/-	+/-	+	+	-		+/-	+/-	-	+	
Growth in NaCl 18 %	-	-	-	-	-	-		-	-	-	+	
Growth at pH 4.4	ND	+/-	+/-	-	+	+/-		+/-	+	-	-	
Growth at pH 9.6	-	-	-	+	+	-		-	-	-	+	
Isomer of lactic acid	L	D, L, DL[b]	D, DL[b]	L	L	L		D	L, DL[b]	L	L	

+ , positive - , negative +/-, difference between species ND, no data

[a] , some species rods shape [b] , type of lactic acid difference with species

(Source: Axelsson,1993; 1998; Collins et al. ,1993; Dicks et al.,1995)

1.2.1.1 *Lactobacillus*

Gram positive rods, cells vary from long and slender, sometime bent rods to short, often coryneform coccobacilli, chain formation common. Motile

uncommon. Nonsporing-forming. Facultatively anaerobic, surface growth on solid media, generally enhanced by anaerobiosis or reduced oxygen pressure and 5-10% CO_2. Strictly aerobic conditions are commonly growth inhibitory. Some strains exhibit bipolar bodies, internal granulation, or a barred appearance with the Gram-reaction or methylene blue stain. Nitrate reduction, gelatin not liquefied. Casein not digested. Indole and H_2S not produced. Catalase negative. Nutrition requirement for amino acid, peptides, nucleic acid derivatives, vitamins, salts, fatty acids, or fatty acid esters, and fermentable carbohydrates. Nutritional requirements are generally characteristic for each species, often particular strains only. Growth temperature use range 2-53 ^oC, optimum generally 30-40^oC, optimal pH usually 5.5-6.2; growth generally occurs at pH 5.0 or less; the growth rate is often reduced in neutral or initially alkaline conditions. Found in dairy products, grain products, meat and fish products, beer, wine, fruits and fruit juices, pickled vegetables, mash, sauerkraut, silage, sourdough, water, soil, and sewage; they are a part of the normal flora in the mouth, intestinal tract, and vagina humans and many animals. DNA G+C content (mol%) is 32-55 (Sharpe, 1981; Kandler and Weiss, 1986; Axelsson, 1998)

1.2.1.2 *Enterococcus*

Gram-positive cocci, facultative anaerobic organisms that often occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci on physical characteristics alone. Two species are common commensal organisms in the intestines of humans: *E. faecalis* (90-95%) and *E. faecium* (5-10%). Rare clusters of infections occur with other species, including *E. casseliflavus, E. gallinarum,* and *E. raffinosus*. (Gilmore et al., 2002). They are not capable of forming spores, enterococci are tolerant of a wide range of environmental conditions such as extreme temperature (10-45°C), pH (4.5-10.0) and high sodium chloride concentrations (Fisher and Phillips, 2009).). They are typically exhibit gamma-hemolysis on sheep's blood agar (Ryan and Ray, 2004).

1.2.1.3 *Weissella*

Gram-positive rods, non-spore-forming, the cells are generally short rods with rounded to tapered ends or coccoid in shape, occurring singly, in pairs or in short chains. Heterofermentative and non-motile. With the exception of *W. paramesenteroides* and *W. hellenica*, all species of the genus generally produce DL-lactic acid from glucose. The peptidoglycan subunit contains lysine and the interpeptide bridge contains alanine or serine and alanine as typical constituents. The species of genus *Weissella* such as *Weissella confusa, W. halotolerans, W. hellenica, W. kandleri, W. minor, W. paramesenteroides, W. thailandensis* and *W. viridescens* (Collins et al., 1993).

1.2.1.4 *Pediococcus*

Gram-positive cocci lactic acid bacteria, facultatively aerobic and purely homofermentative which and produce lactic acid as major end product of glucose fermentation, usually occur in pairs or tetrads, and divide along two planes of symmetry, as do the other lactic acid cocci genera *Aerococcus* and *Tetragenococcus*, responsible for the fermentation of cabbage, making it sauerkraut. In this process, the sugars in fresh cabbage are fermented to lactic acid, which gives sauerkraut a sour flavour and good keeping qualities. They are usually considered contaminants of beer and wine, can produce diacetyl which gives a buttery or butter scotch aroma to some wines and a few styles of beer. They are occur in a wide variety of environmental niches including plant material, fermented beverages, meat, vegetable, dairy products as well as in animals and humans. They are often used in silage inoculants and used as probiotics, and are commonly added as beneficial microbes in the creation of cheeses and yogurts (Haakensen, et al., 2009).

1.2.1.5 *Tetragenococcus*

A group of Gram positive cocci halophilic lactic acid bacteria, colonies are small and non pigmentation, non-spore forming. Catalase and oxidase are both negative, optimum pH and temperature are 7.0-8.0 and 25-35 ^{o}C. They produce L(+)-lactic acid from glucose, but D(-)-lactic acid depends on species specific. Cellular fatty acid contains long-chain saturated monounsaturated, cis-vaccenic acid (ω7 isomer), and

cycropropane-ring types (Collins et al., 1990). Type of peptodoglycan is Lys-D-Asp (Satomi et al, 1997). The G+C contents are 34-36 mol%. This genus was created by Collins et al. (1990) after reclassification of *Pediococcus halophilus* as *T. halophilus*. The both species, *T. halophilus* and *T. muriaticus* are homofermentative with no CO_2 production from glucose. This genus was found in soy sauce, miso, soy paste, brined anchovies, fish sauce, shrimp paste, fermented mustard, kim-chi and sugar thick juice samples. Differential characteristics of *Tetragenococcus* species are showed in Table 2.

The fermented products were produced in Thailand such as fermented shrimp paste (*ka-pi*) and fermented fish (fish sauce; *nam-pla*), contains a high concentration of NaCl which permits the growth of various halophilic lactic acid bacteria (halo-LAB) (Tanasupawat and Komagata, 1995; Tanasupawat, et al., 1998) and halophilic bacteria (HB) (Phithakpol et al., 1995; Tanasupawat and Komagata, 2001). Moderately halophilic lactic acid bacteria, *Tetragenococcus halophilus*, formerly known as *Pediococcus halophilus*, were isolated from various fermented fish in Thailand. (Tanasupawat and Daengsubha, 1983; Anonymous. 1994; Thongsanit, et al., 2002). This bacterium was reclassified in the genus *Tetragenococcus* bases on 16S rRNA studies. (Collins et al., 1990). In 1997, the existence of second species, *Tetragenococcus muriaticus*, has been proposed. (Satomi et al., 1997). In Addition, isolation and characterizationof halophilic lactic acid bacteria isolated from "terasi" shrimp paste: a traditional fermented seafood product in Indonesia had been reported by Kobayashi et al. (2003) and many researchers found the distribution of this species in other fermented products and it was widely distributed in mixed populations with *T. halophilus* in fermented products like Japanese fermented puffer fish ovaries. Also they found that the ability of this strain to produce histamine (Kimura et al., 2001) such as previously, Karnop (1988) isolated a halophilic bacterium as the mian histamine former in semi-preserved anchovies and identified it as *Pediococcus halophilus*, which reclassified to *T. halophilus*. Therefore, both of *T. halophilus and T. muriaticus* play important roles in histamine formation in salted food products.

Table 2. Characteristics of species belonging to the genus *Tetragenococcus*

Characteristics	*T. halophilus* ATCC 33315^T	*T. koreensis* ATCC 35924^T	*T. muriaticus* JCM 10006^T	*T. osmophilus* IAM 1676^T	*T. solitaries* DSM 5634^T
Cell morphology	Spherical, pairs	Cocci	Tetrads, pairs	Cocci	Ovoid
Peptidoglycan	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	nd	Nd
Oxidase	-	-	-	-	-
Growth at 45 (ºC)	-	-	-	-	+
Optimum NaCl (%)	5-10	2-5	7-10	nd	7-10
NaCl range (%)	nd	0-8	1-25	0-25	6.5
Nitrate reduction	-	-	-	nd	Nd
Lactic acid production (homofermentative)	He	Ho	Ho	He	Ho
Growth on MRS	-	+	-	-	+
Acid production from:					
L-Arabinose	+	-	-	-	-
D-Cellulose	+	-	-	nd	+
D-Galactose	+	+	-	-	+
Maltose	+	+	-	+	+
D-Xylose	+	+	-	-	-
Mannitol	-	+	+	+	+
Xylitol	-	+	-	-	-
DNA G+C content (mol%)	34-36	38.3	36.5	36.7	38

Source: Satomi et al., 1997; Ennahar and Cai, 2005; Lee et al., 2005; Justé et al. 2012;
+, positive; -, negative; nd, no data; Ho, homofermentative; He, heterofermentative

1.2.2 Halophilic bacteria (HB)

A group of gram-positive or gram-negative bacteria, rods or cocci, a multitude of pleomorphic forms including flat disks, triangles or squares, non-motile or motile by tufts of flagella. Aerobic or facultative anaerobic with or without nitrate. Optimum temperature for growth is 35-50 ^{o}C. Occur ubiquitously in nature where the salt concentration is high, such as in salt lakes, soda lakes, and salterns. (Grant and Larsen,1989) The Three main groups of halophilic bacteria : Slight halophiles (growth at 2–5% NaCl), moderate halophile (growth at 5 –20% NaCl) and extreme halophiles (growth above 20–30% NaCl). Moderately halophilic, alkaliphilic, and related aerobic endospore-forming, Gram-positive, rod-shaped bacteria have been isolated from various salty environments such as saline lake. These isolates have included members of the genera *Marinococcus* (Hao et al., 1984), *Bacillus* (Ventosa et al., 1989), *Amphibacillus* (Niimura et al., 1990; Zhilina et al., 2001), *Halobacillus* (Spring et al., 1996), *Virgibacillus* (Heyndrickx et al., 1998), *Gracilibacillus* (Wain∅ et al., 1999), *Filobacillus* (Schlesner et al., 2001), *Oceanobacillus* (Lu et al., 2001), *Lentibacillus* (Yoon et al., 2002), *Paraliobacillus*, *Halolactibacillus* (Ishikawa et al., 2002, 2005), *Cerasibacillus* (Nakamura et al., 2004), *Pontibacillus* (Lim et al., 2005a), *Tenuibacillus* (Ren and Zhou, 2005a), *Salinibacillus* (Ren and Zhou, 2005b), *Alkalibacillus* (Jeon et al., 2005b) and *Thalassobacillus* (García et al., 2005). Several novel species of the genus *Lentibacillus* have been described recently, *Lentibacillus salicampi*, *Lentibacillus juripiscarius, Lentibacillus salarius*, and *Lentibacillus lacisalsi* (Yoon et al., 2002; Namwong et al., 2005; Jeon et al., 2005a; Lim et al., 2005b). In addition, there are also moderately halophilic Gram-positive cocci have been recognized within several bacterial genera, and include *Marinococcus albus*, *M. halophilus* and *M. halotolerans* (Hao et al., 1984; Li et al., 2005), *Salinicoccus roseus*, *S. hispanicus* and *S. alkaliphilus* (Ventosa et al., 1990, 1992; Zhang et al., 2002), *Nesterenkonia halobia* (Stackebrandt et al., 1995), *Tetragenococcus muriaticus* (Satomi et al., 1997), and *Jeotgalicoccus halotolerans* and *J. psychrophilus* (Yoon et al., 2003).

1.2.2.1 Genus *Lentibacillus*

Gram-variable rods shaped cells, aerobic, forming spherical or oval endospores at terminal position in swollen sporangia. Motile or non motile. Colonies are white to cream-coloured, smooth, circular to slightly irregular and raised. Catalase positive and oxidase variable, and urease-negative. Unable to hydrolyze starch, tyrosine or xanthine. No acid production from D-melibiose, raffinose, or L-rhamnose. The cell wall peptidoglycan contains *meso*- diaminopimelic acid (DAP) at position 3 of the peptide subunit. The predominant menaquinone is MK-7. The major polar lipids are diphosphatidylglycerol and phosphatidylglycerol, the major fatty acids are anteiso-$C_{15:0}$ and iso-$C_{16:0}$ and the G+C content of the DNA is in the range 42–49 mol% . (Yoon et al., 2002). This genus was described by Yoon et al. (2002) to accommodate a single species, *Lentibacillus salicampi*, isolated from a salt field of the Yellow Sea in Korea. Namwong et al. (2005) described *Lentibacillus juripiscarius*, isolated from sauce produced in Thailand by the fermentation of fish. Addition, Jeon et al. (2005a) described a third species within this genus, *Lentibacillus salarius*, isolated from saline sediment of a salt lake in China. In addition, the other species of the genus *Lentibacillus* were isolated from soil sediments of different hypersaline habitats such as *L. salarius*, *L. lacisalsi* (Lim et al., 2005b), *L. halodurans* (Yuan et al., 2007), *L. salinarum* (Lee et al., 2008a) and *L. salis* (Lee et al., 2008b). The differential characteristics of *Lentibacillus* species are showed in Table 3. All these species are considered moderately halophilic (Ventosa et al., 1998) and grow optimally at 4–15% NaCl.

1.2.2.2 Genus *Salinicoccus*

Gram-positive cocci, moderately halophilic, strictly aerobic, catalase and oxidase positive, non-motile, non-spore forming. Colonies are round, smooth, and form pink-red, or orange, non diffusible pigments. The predominant lipoquinone is MK-6. The cell wall contains murein of the L-Lys–Gly_5 type. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, and a glycolipid of unknown structure. DNA G+C content (mol%) is 46–51. (Ventosa et al., 1992). Optimum NaCl concentration for growth is 4-10 %; growth is 4-49 ^{o}C; optimal temperature is 30-37 ^{o}C.

pH range for growth is 6-11.5; optimal pH is 7-9.5. This genus such as *Salinicoccus roseus* and *S. hispanicus* (Ventosa et al., 1990, 1992), *S. alkaliphilus* (Zhang et al., 2002), *S. salsiraiae* (Franca et al., 2006), *S. jeotgali* (Aslam et al., 2007), *S. luteus* (Zhang et al., 2007) and *S. kunmingensis* (Chen et al., 2007). Their physiological adaptations to highly saline conditions such as salt mines, salted fish and fermented seafood and their ecology have attracted the interest of researchers. Differential characteristics of *Salinicoccus* species are showed in Table 4.

Table 3. Differential characteristics of the genus *Lentibacillus* species

Characteristics	*L. juripiscarius*[a] JCM 12147^T	*L. salarius*[b] KCTC 3911^T	*L. salicampi*[c] JCM 11462^T	*L. lacisalsi*[d] KCTC 3915^T
Spore shape	Oval	Spherical /oval	Spherical /oval	Spherical
Motility	-	+	+	+
Colony diameter (mm)	1.3-3.2	NA	0.2-1.3	NA
Maximum temp. for growth(°C)	45	50	40	40
NaCl range for growth (%)	3-30	1-20	3-25	5-25
Oxidase	+	-	+	+
Nitrate reduction	+	+	+	+
Hydrolysis of :				
Aesculin	-	+	-	-
Casein	+	-	+	-
Tween 80	+	-	+	-
Acid production from:				
Cellobiose	-	NA	w	NA
D-Glucose	+	+	+	-
D-Galactose	-	NA	w	NA
D-Fructose	+	+	-	+
Maltose	-	+	w	-
D-Mannitol	-	W	-	-
D-Mannose	-	+	w	-
D-Ribose	+	+	-	+
Salicin	-	-	w	-

Sucrose	w	NA	-	NA
D-Trehalose	-	W	-	-
D-Xylose	+	+	-	w
Major fatty acids	anteiso-$C_{15:0}$ or iso-$C_{16:0}$	anteiso-$C_{15:0}$or iso-$C_{16:0}$	anteiso-$C_{15:0}$ or anteiso-$C_{17:0}$	anteiso-$C_{15:0}$ or anteiso-$C_{17:0}$
DNA G+C content (mol%)	43	43	42	44

[a] Data from Namwong et al. (2005); [b]Data from Jeon et al. (2005a); [c]Data from Yoon et al. (2002) and Namwong et al. (2005); [d]Data from Lim et al. (2005b); Symbol ; +, positive; - ,negative; w, weak; NA, not data available.

Table 4. Differential characteristics of the genus *Salinicoccus*

Characteristics	*S. roseus*[a] DSM 5351^T	*S. hispanicus*[b] DSM 5352^T	*S. alkaliphilus*[c] JCM 11311^T	*J. halotolerans*[d] JCM 11198^T
Cell size (μm)	1-2.5	1-2.5	0.5-0.8	0.6-1.1
Colony pigmentation	Pink-red	Reddish orange	Pinkish	Light yellow
pH range for growth	5-9	6-9	6.5-11.5	7-8
Optimum pH for growth	7.5	8.0	9.5	NA
NaCl range for growth (%)	0.5-25	0.9-25	0-25	0-20
Temp. range for growth (°C)	15-37	15-40	10-49	4-42
Optimum temp. for growth	37	37	32	30-35
Methyl red test	-	+	-	NA
Urease test	-	+	+	-
Nitrate reduction	-	+	+	+
Hydrolysis of:				
Casein	+	-	-	-
Aesculin	-	+	+	-
Gelatin	+	+	-	NA
Tween 80	+	-	-	-
Starch	+	-	-	-
Acid production from:				
D-Fructose	-	+	-	-
D-Galactose	-	+	-	-
D-Glucose	-	+	+	-
Maltose	-	+	-	-
D-Mannitol	-	+	+	+
Major fatty acids	anteiso-$C_{15:0}$ or anteiso-$C_{17:0}$	iso-$C_{15:0}$ or anteiso-$C_{15:0}$	iso-$C_{15:0}$ or anteiso-$C_{15:0}$	iso-$C_{15:0}$ or anteiso-$C_{15:0}$
DNA G+C content (mol%)	51.2	45.6-49.3	49.6 [f]	42

[a]Data from Ventosa et al., 1990, [b]Data from Marquez et al.,1990, [c]Data from Zhang et al., 2002, [d]Data from Yoon et al., 2003, [e]The G+C content of the type strain is 46 mol%, [f]Range of values for five strains given by Marquez et al. (1990). The value for the type strain is 45.7 mol%. +, positive; -, negative; NA, no data available.

Research objectives

1. To isolate and identify halophilic and lactic acid bacteria from Thai fermented foods based on phenotypic, chemotaxonomic, DNA-DNA relatedness and 16S rRNA gene sequencing
2. To screen and study on diacetyl production of lactic acid bacteria from Thai fermented foods

CHAPTER II

MATERIALS AND METHODS

2.1. Sample collection and isolation

2.1.1 Halophilic lactic acid bacteria

The fermented fish (*ka-pi*) samples were obtained from the markets in Trang, Nakhonsithammarat, Songkhla, Ranong, Krabi, Pattani and Phungnga Provinces, and lactic acid bacteria were isolated and counted (CFU/g) by a poured plate technique using MRS agar, with 5% NaCl incubating at 30°C for 3-5 days. (De Man et al., 1960)

2.1.2 Halophilic bacteria

Fish sauce (*nam-pla*) samples were collected from fish-sauce factories in Thailand during the early, middle and late stages of the fermentation process and fermented shrimp paste (*ka-pi*) samples which were collected from a market in Nakhonsrithammarat Province in southern part of Thailand. Halophilic bacteria were isolated from the samples by using the spread-plate technique on agar plates of JCM medium no. 168 (containing, l^{-1}, 200 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g glutamic acid, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4.7H_2O$, 36 mg $FeCl_2.4H_2O$, 0.36 mg $MnCl_2.4H_2O$ and 20 g agar, per litre distilled water; pH 7.2) or JCM medium no. 377 (containing, l^{-1}, 100 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g glutamic acid, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4.7H_2O$, 36 mg $FeCl_2.4H_2O$, 0.36 mg $MnCl_2.4H_2O$ and 20 g agar, per litre distilled water; pH 7.2) with incubation at 37°C for 7 days. Liquid cultures were cultivated in Erlenmeyer flasks containing the same medium without agar and were incubated on a rotary shaker. All media contained 10-20 % (w/v) NaCl, except those used to investigate NaCl tolerance.

2.1.3 Lactic acid bacteria

Lactic acid bacteria were isolated by an enrichment culture approach from six samples of fermented tea leaves (*miang*) collected at the markets in Bangkok and Chiang Mai provinces produced in the northern part of Thailand and isolated from five

samples of pasteurized milk and twenty-one of fermented foods obtained at the markets, and from six samples of raw cow's milk at the Dairy Farming Promotion Organization. GYPB-0.3% $CaCO_3$ or MRS-0.3% $CaCO_3$ agar plate was used for isolation (Tanasupawat et al., 1998). Pure cultures were obtained by streaking cultured cells on MRS-$CaCO_3$ agar plates (De Man et al., 1960).

2.2. Identification methods

2.2.1 Halophilic lactic acid bacteria

All isolates and each of the strains of *Tetragenococcus halophilus* ATCC 33315^T and *T. muriaticus* JCM 10006^T were used in this study. All tests were carried out by incubating the cultures at 30^{o}C, except for the investigation of effects of temperature. Cell, form, cell size, cell arrangement, and colonial appearance were examined on the cell grown on MRS agar with 5% NaCl incubated for 5 days. Hucker-Conn modification was used for Gram stain. Spore formation was examined in Gramstained specimens. Motility was detected by the appearance of stab cultures in soft agar. Catalase (with hematin in the medium), oxidase, nitrate reduction, hydrolysis of arginine, casein, starch, gelatin tributyrin, oxidation-fermentation test, hydrogen sulfide formation; and Methyl red - Voges-Proskauer reaction were tested as reported (Barrow and Feltham, 1993; Tanasupawat et al., 1992a; Tanasupawat and Komagata, 1995). The effect of temperature (40, 50^{o}C), and different concentrations of NaCl (0, 10, 15, 20, and 25 %) were tested by using MRS broth as a basal medium. Acid formation from carbohydrates was determined as reported previously (Tanasupawat and Komagata, 1995). DNAs were isolated from cells grown in MRS broth with 5-10% NaCl after incubating for 1-2 days and were purified by the method of Saito and Miura (1963). For strains with difficult in isolation of DNA, the medium was supplemented with 0.5% glycine. Photobiotin labelling DNA-DNA similarity was carried out in 2xSSC (saline trisodium citrate) and 50% formamide solution at 40^{o}C for 15 hours and detected by colorimetric method. The growth of selected *Tetragenococcus* strains and the type strains of each group in MRS broth (200 ml) with 5 and 10% NaCl were determined by spectrophotometer at 600 nm when incubating at 30^{o}C for 72 hours.

2.2.2 Halophilic bacteria

Cell shape, cell size and cell arrangement were examined on JCM medium no. 168 agar at 37^{o}C for 5 days. The Hucker–Conn modificationwas used for Gram staining (Hucker and Conn, 1923). Spore formation was examined on Gram-stained specimens. Critical-point-dried cells were observed under a scanning electron microscope. Flagella were examined as described by Forbes (1981) and observed by transmission electron microscopy. Catalase activity, oxidase activity and the hydrolysis of aesculin were investigated as described by Barrow and Feltham (1993), while urease activity and the hydrolysis of gelatin, casein, starch, Tween 80, tyrosine, phenylalanine, xanthine and hypoxanthine were tested as described by Namwong et al. (2005). Arginine hydrolysis was investigated by using the medium reported by Thornley (1960). Acid production from carbohydrate was determined in the medium described by Leifson (1963), supplemented with 20 % NaCl. Growth under anaerobic conditions on agar plates with or without KNO_3 (1 %, w/v) was performed in a Gaspak (BBL) anaerobic jar. Growth at various temperatures (10–50 ^{o}C), pH values (5, 6, 7, 7.5, 8 and 9) and NaCl concentrations (0–30 %, w/v) was tested by using JCM medium no. 168 as a basal medium (Namwong et al., 2005).

The diaminopimelic acid in the peptidoglycan and the menaquinone composition were determined as described previously (Komagata and Suzuki, 1987). Polar lipids were investigated according to the methods of Minnikin et al. (1984) and Albert et al. (2005). A loop of cell mass was used for the extraction and quantitative analysis of the cellular fatty acids by means of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer and Kroppenstedt, 1996). DNA was isolated from cells grown in JCM medium no. 168 broth and purified according to the method of Saito and Miura (1963). The DNA G+C content was determined by the method of Tamaoka and Komagata (1984), using reversed-phase HPLC. DNA–DNA hybridization was conducted in microdilution-well plates, as reported by Ezaki et al. (1989), and was detected by using the colorimetric method described by Tanasupawat et al. (2000). The 16S rRNA gene of the isolate was amplified, purified and sequenced as described previously

(Seearunruangchai et al., 2004). The sequence determined (1,410 bases) was aligned with selected sequences (obtained from the GenBank/ EMBL/DDBJ database) by using CLUSTAL W, version 1.81 (Thompson et al., 1994). The alignment was manually edited to remove gaps and ambiguous nucleotides prior to the construction of the phylogenetic tree. The phylogenetic tree was constructed by using the neighbour-joining method (Saitou and Nei, 1987) in MEGA, version 2.1 (Kumar et al., 2001). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings.

2.2.3 Lactic acid bacteria

The morphological, cultural, physiological, and biochemical characteristics and the isomers of lactic acid produced were determined as reported previously (Okada et al., 1978; Tanasupawat et al., 1998). Cell wall peptidoglycan type: The *meso*-diaminopimelic acid (*meso*-DAP) and lysine of cell wall were determined as described previously (Komagata and Suzuki,1987; Tanasupawat et al., 1993). DNA base composition: DNAs were isolated and purified as reported previously (Saito and Miura, 1963). The medium supplemented with 0.8–1.5% glycine was used for strains with difficulty in isolation of DNA (Yamada and Komagata, 1970). The purified DNA was hydrolyzed to nucleosides as described previously and the hydrolysate was applied to reversed phase high-performance liquid chromatography (HPLC) (Tamaoka and Komagata, 1984). DNA-DNA hybridization: DNAs were isolated and purified by the method reported previously (Saito and Miura, 1963; Yamada and Komagata, 1970). Photobiotin labeling DNA-DNA hybridization was carried out in 2xSSC (Saline trisodium citrate) and 50% formamide solution at 40 or 45^{o}C for 15 hours (Ezaki et al., 1989). DNA-DNA similarity was determined by using the colorimetric method as reported (Tanasupawat et al., 2000). 16S rRNA sequence and phylogenetic analysis: The 16S rRNA gene sequence was determined as described previously (Tanasupawat et al., 2004). The sequences determined (1,417–1,535 bases) were aligned with the selected sequences obtained from theGenBank/EMBL/DDBJ database employing CLUSTAL_X (Thompson et al., 1997). The alignment was edited to remove gaps and ambiguous

nucleotides prior to construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei, 1987) in the MEGA program version 2.1 (Kumar et al., 2001). The confidence values of branches of the phylogenetic tree were determined by using bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings.

2.3 Study on diacetyl production of lactic acid bacteria

All the isolates obtained from various sources were screened for diacetyl/acetoin production in 15 ml MMRS broth (Phalip et al., 1994) by using the colorimetric method, as described by Mattessich and Cooper (1989). The selected strains that showed high diacetyl/acetoin production were further studied for their ability to produce diacetyl in MMRS medium. Cell grown in MRS broth were harvested at the end of exponential growth phase, and the absorbance of culture at 575 nm (Boumerdassi et al., 1997) was measured with a spectrophotometer (UV-160: Shimadzu, Kyoto, Japan) for preparing the inoculum. A 1% (v/v) inoculation was transferred into 400 ml MMRS broth in a 1,000 ml Erlenmeyer flask and incubated at 30^{o}C with shaking (200 rpm) and static condition (Kaneko et al., 1991). The fermentation broth was sample every 6 hours for observing the growth (O.D. at 575 nm) by spectrophotometer and for measuring the pH by Beckman pH-meter. Diacetyl and acetoin were determined by gas-liquid chromatography (Chrompack CP 9000) as described by Thornhill and Cogan (1984).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Bacterial strains and sources of isolation

3.1.1 Halophilic lactic acid bacteria

A total of seventeen strains isolated from 17 samples of *Ka-pi* samples were obtained from the markets in Trang, Nakhonsithammarat, Songkhla, Ranong, Krabi, Pattani and Phungnga Provinces. Halophilic Lactic acid bacterial cells count on MRS medium with 5% NaCl are 3.3×10^3 to 3.0×10^6 CFU/g shown in Table 5.

Table 5. Source of samples, lactic acid bacterial cells count and isolate number

Province	Number of samples	Bacterial count (CFU/g)	Strains no.	Number of strains
Trang	4	1.1×10^6 - 3.0×10^6	SP2-1, SP12-1, SP28-1, SP29-1	4
Nakhonsithammarat	6	6.0×10^4 - 3.0×10^6	SP4-1, SP5-2, SP7-1, SP15-1, SP20-1, SP25-1	6
Songkhla	3	1.5×10^4 - 2.0×10^6	SP10-2, SP11-1, SP11-2	3
Ranong	1	2.9×10^6	SP23-1	1
Krabi	1	2.7×10^6	SP24-1	1
Pattani	1	3.3×10^3	SP31-2	1
Phangnga	1	3.0×10^6	SP36-1	1
Total	17	3.3×10^3 - 3.0×10^6		17

3.1.2 Halophilic bacteria

A total of thirty-two strains isolated from 9 samples. Fifteen aerobic rods, extremely halophilic bacteria were isolated from the fish sauce (*nam-pla*) samples, two aerobic rods and fifteen aerobic cocci halophilic bacteria from fermented shrimp paste (*ka-pi*) were shown in Table 6.

Table 6. Source of samples, types of samples, number of samples and strains.

Province	Types of samples	Number of samples	Number of strains	Shape of strains	
				Rods	cocci
Samutprakarn Samutsongkram Chonburi	Fish sauce* (*Nam-pla*)	3	15	15	-
Nakhonsithammarat	Shrimp paste *(Ka-pi)*	6	17	2	15
Total		9	32	17	15

*Obtained from Thai Fish Sauce Factory

3.1.3 Lactic acid bacteria

A total of twenty-five strains isolated from 6 samples of fermented tea leaves (*miang*) 24 were rod-shaped and 1 sphere shaped. Therefore, the rod-shaped and sphere-shaped strains were widely distributed in *Miang* (Table 7). In addition, a total of 137 strains isolated from 5 samples of pasteurized milk were 5 rods and 44 cocci, 6 of raw cow's milk were 14 rods and 26 cocci and 21 of fermented foods were 38 rods and 10 cocci, respectively as shown in Table 8. Most of the rod-shaped strains were found in fermented foods (Tanasupawat et al., 1993, 1995, 1998) while the coccal strains were distributed in pasteurized milk and raw cow's milk (Devriese et al., 1991).

Table 7. Source of samples, strain no., number of strains and shape of lactic acid bacteria isolated from *Miang*

Province	Strain no.	Number of strains	Shape
Chiang Mai	FP37-1	1	rods
Banglkok (Chatuchak market)	MCH1-1, MCH1-2, MCH1-3 MCH1-4, MCH1-6, MCH1-7	6	rods
Chiang Mai (Tapair market)	MCH2-5, MCH2-1, MCH2-2, MCH2-3, MCH2-4	5	rods
Chiang Mai (Tapair market)	MCH3-1, MCH3-3	2	rods
	MCH3-2	1	cocci
Chiang Mai (Varoroch market)	MCH4-3, MCH4-1, MCH4-4, MCH4-2, MCH4-5	5	rods
Chiang Mai (Varoroch market)	MCH5-5, MCH5-6, MCH5-2, MCH5-4, MCH5-9	5	rods
Total		25	

Table 8. Source of samples, number of sample, number of strains and shape of lactic acid bacteria isolated from milk and fermented foods

Types of samples	Number of samples	Shape	
		cocci	rods
Pasteurized milk[a]	5	44	5
Fermented foods[a]	21	10	38
Raw cow's milk[b]	6	26	14
Total	32	80	57

[a]Collected from markets; [b]Obtained from Daily Farming Promotion Organization of Thailand

3.2 Identification of strains

3.2.1 Halophilic LAB isolated from *Ka-pi*

All the strains were Gram-positive cocci 0.6-1.0μm in size, and they appeared singly, in pairs, and in tetrads. Cells were nonmotile and nonsporing. Colonies on MRS agar plate were circular, low convex with entire margin, and nonpigmented (Table 9). All the strains were homofermentative and microaerophilic. They showed negative reactions to oxidase; Voges-Proskauer reaction; hydrolysis of starch, gelatin, tributyrin; hydrogen sulfide formation and nitrate reduction. All strains produced catalase in the medium containing hematin and showed methyl red reaction. Few strains hydrolysed arginine. All strains grew at pH 5.0 to 9.0, in 10 to 25% NaCl , and at 40 ^{o}C but not at 50^{o}C. Variable reactions were shown in Table 9. All produced acid from D-glucose, D-fructose, glycerol, D-maltose, D-mannose, D-ribose, salicin and D-sorbitol, and variable reactions were shown in Table 20. In addition, Growth in MRS broth with 5 and 10% NaCl of *T. halophilus* SP36-1 and *T. muriaticus* SP11-1 including the type strains were shown in Figs 6 to 7. *T. halophilus* strains grew more faster and better than *T. muriaticus* strains in 5% NaCl broth. *T. halophilus* ATCC 33315^{T} grew better than *T. halophilus* SP36-1 while, *T. muriaticus* strains preferred growth in 10% NaCl broth.This characteristics also be useful for differentiation of *T. halophilus* and *T. muriaticus.*

As mentioned above, shrimp pasted (ka-pi) contained over 14% of NaCl (Phithakpol et al., 1995), the significant amounts of salt that are present in the product may be the cause of the predominant of *Tetragenococcus* species, *T. halophilus* and *T. muriaticus* the same result as reported by Kobayashi et al., 2003. The both species are halophilic bacteria that could grew in 25% NaCl therefore and found them distributed from 3.3x103 x 3.0x106 CFU/g of shrimp paste.

The selected isolates were divided into 2 groups on the basis of DNA-DNA relatedness as shown in Table 11. Four strains in Group I (SP2-1, SP7-1, SP28-1, and SP36-1) showed high DNA-DNA relatedness (83.6-89.1 %) with *Tetragenococcus halophilus* ATCC 33315^{T}. Two strains in Group II (SP10-2 and SP11-1) showed high

DNA-DNA relatedness (94.7-117.4 %) with *Tetragenococcus muriaticus* JCM 10006^{T}**.** Seventeen isolates were included in the genus *Tetragenococcus* (Dellaglio et al., 1981; Garvie, 1986 and Wayne et al., 1987). On the basis of phenotypic characteristics and DNA-DNA relatedness as shown in Tables 9 to 11.

Group I contained 4 isolates, as shown in Table 11 were similar to *Tetragenococcus halophilus* ATCC 33315 ^{T}. They were identified as *T. halophilus* (Satomi et al, 1997).

Group II contained 2 isolates, as shown in Table 11 were similar to *Tetragenococcus muriaticus* JCM 10006 ^{T}. They were identified as *T. muriaticus* (Satomi et al, 1997).

Tetragenococcus halophilus strains had been reported so far by Nakagawa and Kitahara (1959), Coster and White (1964), Whittenbury (1965), Sakaguchi, and Mori 1969), Uchida (1982), Tanasupawat and Daengsubha (1983), and Villar et al.(1985). Total of eleven strains of *T. muriaticus* were isolated from fermented squid liver sauce and proposed as the new species (Satomi et al, 1997). This species was described limited on only the strains from one source. In this study, we found some isolates from shrimp paste and they showed the different characteristics with the type strains as shown in Tables 9 and 10.

The differential characteristics of *T. halophilus* and *T. muriaticus* are the growth in MRS broth with no NaCl and the hydrolysis of casein. The hydrolysis of arginine, acids production from L-arabinose and mannitol are not significant characteristics to separate these two species as discussed by Satomi et al.(1997). As a mention the phenotypic characteristics of seventeen isolates in Tables 9 and 10, this result was not significant to separate them at the species level. Photobiotin labelling DNA-DNA relatedness is useful to differentiate the two *Tetragenococcus* species.

Table 9. Characteristics of *Tetragenococcus* isolates.

Characteristics		*T. halophilus* ATCC 33315^T	*T. muriaticus* JCM 10006^T	Isolates (17)
Cell shape		……..……Cocci……..		
Cell size (μm)		.....……..0.6-1.0…….		
Cell arrangment		….....Occurring singly, in pairs and in tetrads…….		
Colony form		..…..…… White, raised, circular and entire…….		
Oxidase		-	-	-
Catalase with hematin		+	+	+
Hydrolysis of arginine		+	-	+(+3)
Hydrolysis of casein		-	+	-
Nitrate reduction		-	-	-
MR				
VP				
Oxidative-Fermentative		F	F	F
H_2S production		-	-	-
Growth at:	40°C	-	+	+
	50°C			
Growth at pH:	4.2	-	-	-
	5.0	+	+	+
	9.0	+	+	+
Growth in:	0 % NaCl	+	-	+
	10 % NaCl	+	+	+
	25% NaCl	+	+	+

+ , positive reaction; -, negative reaction. Numbers in parentheses indicate the number of isolates or numbers of isolates showing the reaction. ATCC, American Type Culture Collection, Manassas, VA, USA; JCM, Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Japan.

Table 10. Acid from carbohydrates of *Tetragenococcus* isolates.

Carbohydrate	*T. halophilus* ATCC 33315^T	*T. muriaticus* JCM 10006^T	Isolates (17)
Amygdalin	+	+	+(-4)
L-Arabinose	-	+	+(-4)
D-Cellobiose	+	-	+(-2)
Dextrin	-	+	+(-2)
D-Fructose	+	+	+
D-Galactose	+	+	+(-3)
Glycerol	+	-	+
Lactose	-	-	+(-7)
Maltose	+	+	+
D-Mannitol	-	+	+(-2)
D-Mannose	+	+	+
D-Melibiose	-	+	+(-6)
D-Melezitose	+	-	+(-4)
α-Methyl-D-glucoside	+	+	+(-1)
L-Raffinose	-	-	+(-6)
L-Rhamnose	-	+	+(-7)
D-Ribose	+	+	+
Salicin	+	+	+
D-Sorbitol	-	+	+
Sucrose	+	-	+(-1)
D-Trehalose	+	+	+(-1)
D-Xylose	+	+	+(-5)

+, positive reaction; -, negative reaction. Numbers in parentheses indicate the number of isolates or numbers of isolates showing the reaction.

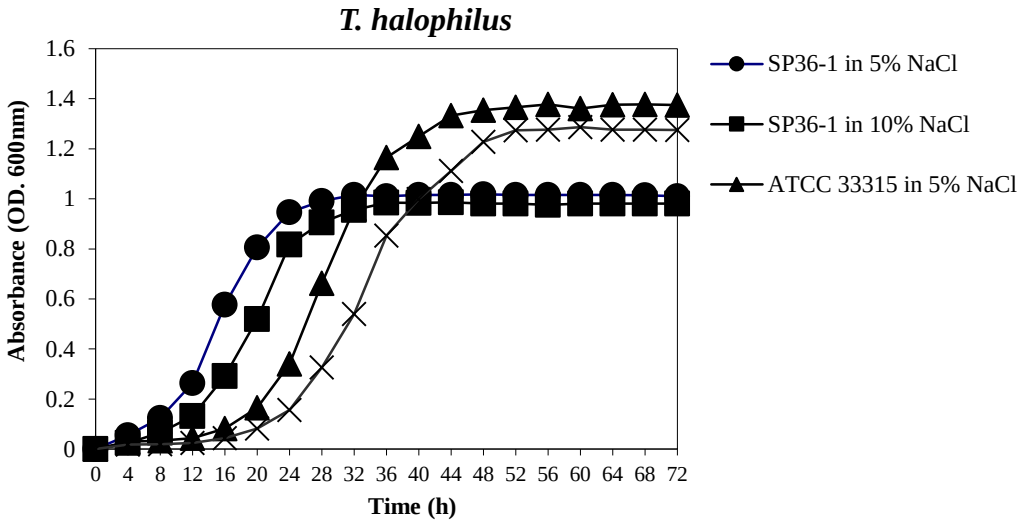


Fig. 6. Growth of *T. halophilus* SP36-1 and ATCC 33315^T in MRS broth with 5 and 10% NaCl

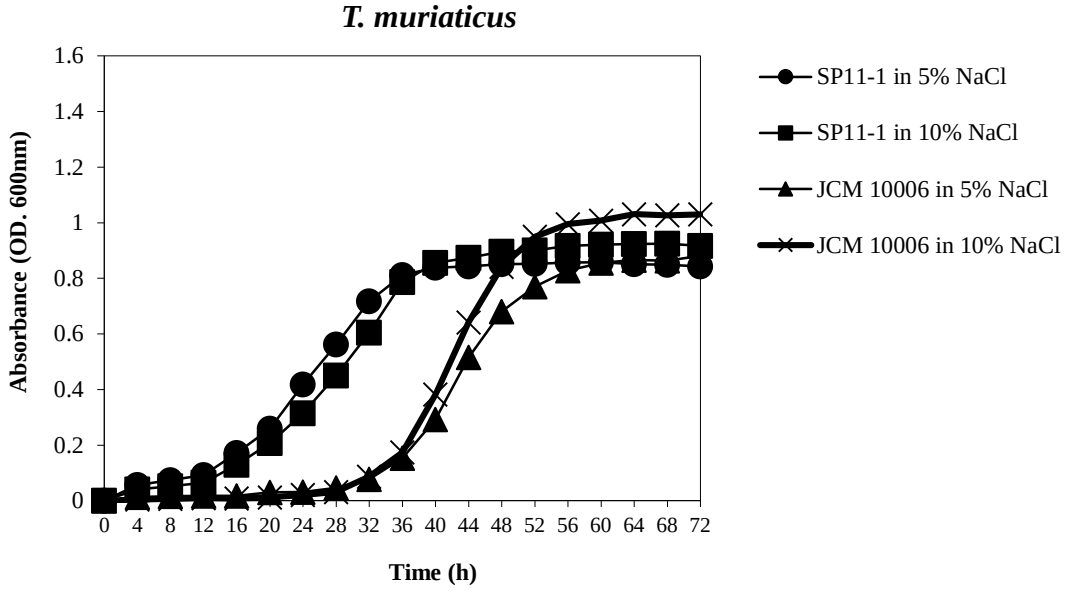


Fig. 7. Growth of *T. muriaticus* SP11-1 and JCM 10006^T in MRS broth with 5 and 10% NaCl

Table 11. DNA–DNA relatedness of *Tetragenococcus* strains

	Strain	% Similarity with labelled strains	
		ATCC 33315 ^{T}	JCM 10006 ^{T}
Group I	SP2-1	84.4	49.7
	SP7-1	83.6	41.9
	SP28-1	84.5	49.6
	SP36-1	89.1	3.2
Group II	SP10-2	59.3	117.4
	SP11-1	61.5	94.7
T. halophilus	ATCC 33315^{T}	100	19.5
T. muriaticus	JCM 10006^{T}	30.7	100

3.2.2 Halophilic bacteria isolated from *Nam-pla* and *Ka-pi*

Group I contained fifteen strains. They were aerobic extremely halophilic bacteria, Gram-positive rods, motile and are mostly 0.4- 0.6 μm wide and 1.0-3.0 μm long. Longer cells (up to 6 μm) or short filaments are observed. Spherical endospores are formed terminally in swollen sporangia (Fig. 8). Colonies on JCM no.168 agar plate are white to cream and low convex or raised, smooth, and circular (0.1-0.8 mm in diameter). They produced catalase and oxidase but not urease. Optimal growth temperature is 30-37 °C. Growth occures between 15 ^{o}C (weekly) and 42 ^{o}C but not at 10, 40 or 50 ^{o}C. Growth is observed at pH 6 and 8 (optimum, pH 7.0-7.5) but not at pH 5 or 9. Extremely halophilic, growing in the presence of 12-30% (w/v) NaCl but not at or below10% (w/v) NaCl (optimum, 20-26% NaCl, w/v). Anaerobic growth is not observed in the presence of 1% KNO_3 (w/v) or other media. Aesculin, arginine, casein, gelatin, Tween 80, tyrosine, starch, phenylalanine, xanthine and hypoxanthine are not hydrolysed. Acid is not produced from D-glucose, glycerol, D-ribose, D-xylose, L-arabinose, cellobiose, D-fructose, D-galactose, lactose, maltose, mannitol, D-mannose, melibiose,

melezitose, *myo*-inositol, raffinose, L-rhamnose, salicin, sorbitol, sucrose and trehalose. Contains *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. MK-7 is the major menaquinone. The cellular fatty acids and polar lipids of four of these strains, namely PS11-2^T, CB0-1, DS26-3 and DB9-1, were analysed. The four strains contained the following: MK-7 as a major menaquinone; anteiso-$C_{15:0}$ (58.0-62.7 %), anteiso-$C_{17:0}$ (24.6-33.8 %), iso- $C_{15:0}$ (2.4-7.8%), $C_{16:0}$ (1.7-3.0 %), iso- $C_{16:0}$ (1.8-2.3%), iso- $C_{17:0}$ (0.5-1.4 %), $C_{14:0}$ (0-0.9 %), $C_{14:0}$ (0-0.7%), $C_{15:0}$ (0-0.6 %) and $C_{12:0}$ (0-0.5 %). as a cellular fatty acid. The DNA G+C content of the these isolates ranged from 42.1 to 43.1 mol% (Table 12.). The type strain PS11-2^T contained anteiso-$C_{15:0}$ (53.9%), anteiso-$C_{17:0}$ (25.3%), and $C_{16:0}$ (5.4%) as a major cellular fatty acid (Table 13), phosphatidylglycerol (PG) and diphoshatidylglycerol (DPG) as major polar lipids and two unidentified glycolipids as major polar lipids (Fig. 9).

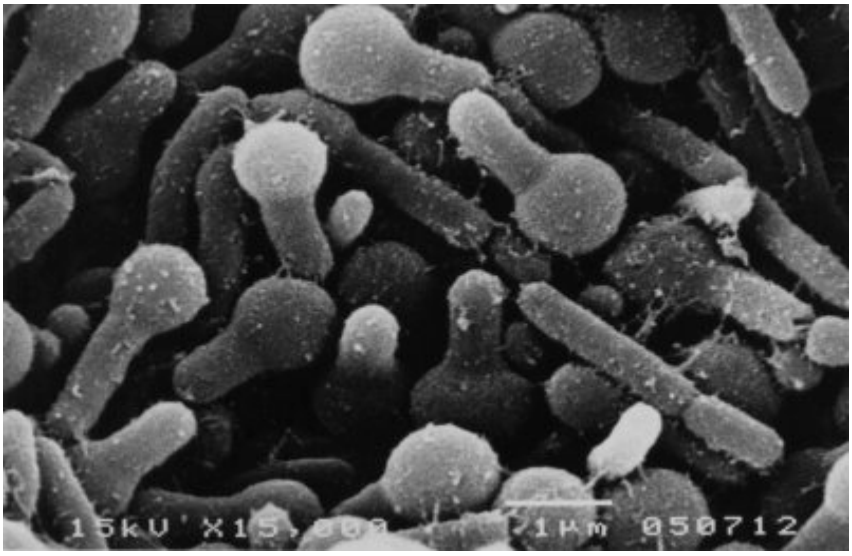


Fig. 8. Scanning electron micrograph of strain PS11-2 ^{T} grown on JCM no. 168 medium at 37 °C. Bar, 1 µm

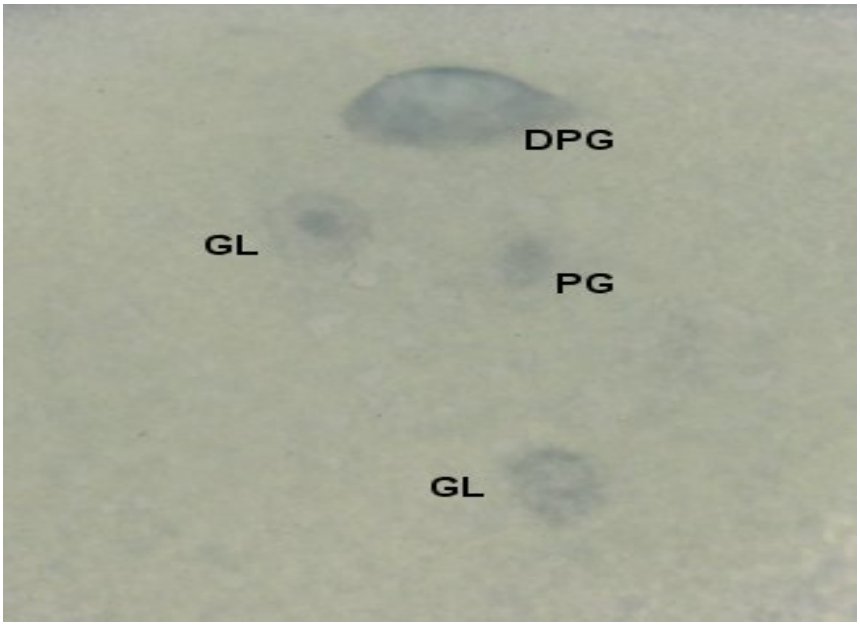


Fig. 9. Thin-layer chromatogram of the total polar lipids of strain PS11-2 ^{T}
DPG, diphoshatidylglycerol; PG, phosphatidylglycerol; GL, glycolipids

In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain PS11-2^{T}was positioned in a monophyletic cluster consisting of the members of the genus *Lentibacillus* (Fig. 10). The 16S rRNA gene sequence similarity values between PS11-2^{T} and *L. juripiscarius* JCM 12147^{T}, *L. salarius* KCTC 3911^{T}, *L. lacisalsi* KCTC 3915^{T} and *L. salicampi* JCM 11462^{T} were 97.3, 95.5, 95.4 and 95.3 %, respectively. Furthermore, strain PS11-2^{T} showed 93.3–94.2 % 16S rRNA gene sequence similarity to members of the genus *Virgibacillus*. Hybridization studies revealed high levels of DNA–DNA relatedness between PS11-2^{T} and PB7-3 and to the other isolates (>70 %), but only low levels with respect to *L. salicampi* JCM 11462^{T} (4.9–19.4%) and *L. juripiscarius* JCM 12147^{T} (17.0–17.1%), as shown in Table14).

Table 12. Differential characteristic of strain PS11-2^T from related *Lentibacillus* species

Characteristics	PS11-2^T	*L. juripiscarius*[a] JCM 12147^T	*L. salarius*[b] KCTC 3911^T	*L. salicampi*[c] JCM 11462^T	*L. lacisalsi*[d] KCTC 3915^T
Spore shape	Spherical	Oval	Spherical /oval	Spherical /oval	Spherical
Motility	+	-	+	+	+
Colony diameter (mm)	0.2-0.6	1.3-3.2	NA	0.2-1.3	NA
Maximum temp. for growth(^{o}C)	42	45	50	40	40
NaCl range for growth (%)	12-30	3-30	1-20	3-25	5-25
Oxidase	+	+	-	+	+
Nitrate reduction	-	+	+	+	+
Hydrolysis of :					
Aesculin	-	-	+	-	-
Casein	-	+	-	+	-
Tween 80	-	+	-	+	-
Acid production from:					
Cellobiose	-	-	NA	W	NA
D-Glucose	-	+	+	+	-
D-Galactose	-	-	NA	W	NA
D-Fructose	-	+	+	-	+
Maltose	-	-	+	W	-
D-Mannitol	-	-	W	-	-
D-Mannose	-	-	+	W	-
D-Ribose	-	+	+	-	+
Salicin	-	-	-	W	-
Sucrose	-	w	NA	-	NA
D-Trehalose	-	-	W	-	-
D-Xylose	-	+	+	-	w
Major fatty acids	anteiso-$C_{15:0}$ or anteiso-$C_{17:0}$	anteiso-$C_{15:0}$ or iso-$C_{16:0}$	anteiso-$C_{15:0}$or iso-$C_{16:0}$	anteiso-$C_{15:0}$ or anteiso-$C_{17:0}$	anteiso-$C_{15:0}$ or anteiso-$C_{17:0}$
DNA G+C content (mol%)	42	43	43	42	44

[a] Data from Namwong et al. (2005); [b]Data from Jeon et al. (2005a); [c]Data from Yoon et al. (2002) and Namwong et al. (2005); [d]Data from Lim et al. (2005b); Symbol ; +, positive; - ,negative; w, weak; NA, not data available.

Table 13. Cellular fatty acid compositions of strain PS11-2^T and related taxa

Fatty acid	PS11-2^T	*L. juripiscarius*[a] JCM 12147^T	*L. salarius*[b] KCTC 3911^T	*L. salicampi*[c] JCM 11462^T	*L. lacisalsi*[d] KCTC 3915^T
Saturated fatty acids					
$C_{12:0}$	1.3	NA	NA	NA	NA
$C_{14:0}$	1.0	0.2	0.2	0.2	NA
$C_{15:0}$	0.4	0.1	0.3	0.1	NA
$C_{16:0}$	5.4	0.8	1.0	0.9	1.3
Unsaturated fatty acids					
$C_{16:1}$ *ω7c* alcohol	ND	0.6	ND	ND	1.5
Branched fatty acids					
iso-$C_{14:0}$	1.0	10.2	13.9	7.9	5.7
iso-$C_{15:0}$	4.7	4.9	16.5	4.1	8.0
anteiso-$C_{15:0}$	53.9	45.1	25.3	48.6	50.8
iso-$C_{16:0}$	4.3	20.2	26.5	16.3	12.0
iso-$C_{17:0}$	0.8	0.5	4.4	0.9	1.9
anteiso-$C_{17:0}$	25.3	16.7	11.5	20.9	18.2

[a]Data from Jeon et al. (2005b); [b]Data from Lim et al. (2005b); NA, not available; ND, not detected.

The 16S rRNA gene sequence-based phylogenetic analysis clearly indicated that representative strain PS11-2^T belongs to the genus *Lentibacillus*. The chemotaxonomic properties (i.e. diamino acid content in the peptidoglycan, menaquinone content, the polar lipids and the cellular fatty acid profiles) of the isolates were in accordance with those of the genus *Lentibacillus* (Yoon et al., 2002; Namwong et al., 2005; Jeon et al., 2005a; Lim et al., 2005b). The fatty acid profiles of the four strains examined were qualitatively similar to those of other *Lentibacillus* species, although the levels of iso-$C_{14:0}$ and iso-$C_{16:0}$ were significantly lower than those of the other *Lentibacillus* species reported (≤ 0.7 % iso-$C_{16:0}$ and 1.8–2.3 % iso-$C_{16:0}$ and 16.3–26.5 % iso-$C_{16:0}$ for the isolates; 5.7–13.9 % iso-$C_{16:0}$ for *Lentibacillus* species). When the cellular fatty acid profile of *L. juripiscarius* JCM 12147^T grown on JCM medium no. 168 (containing 20 % NaCl) was compared with the profile from cells grown on the *Lentibacillus* medium (JCM medium no. 377, containing 10 % NaCl), the levels of iso-$C_{14:0}$ and iso-$C_{16:0}$ were significantly lower 2.1 and 6.3 %, respectively (Table 13.). Thus, the levels of iso-$C_{14:0}$ and iso-$C_{16:0}$ were affected by NaCl concentration during growth. The morphological, cultural, physiological and biochemical characteristics that differentiate the isolates from *Lentibacillus* species are shown in Table 12. It is

noteworthy that all of the isolates are extreme halophiles requiring at least 12 % NaCl for growth. In addition, the isolates do not reduce nitrate and do not produce acid from sugars, unlike *Lentibacillus* species. The 16S rRNA gene sequence of PS11-2^T shows a relatively high level of similarity to that of the type strain of *L. juripiscarius* (97.3 %) (Fig. 10). However, the low levels of DNA–DNA relatedness (≤ 19.4 %) demonstrate that the novel strains do not belong to the species *L. juripiscarius*. On the other hand, inclusion of the 15 strains in the same species is supported by the fact that they share the same phenotypic properties and demonstrate high levels of DNA–DNA relatedness to each other (>70 %) (Wayne et al., 1987). Therefore, these 15 isolates represent a novel species in the genus *Lentibacillus*, for which we propose the name *Lentibacillus halophilus* sp. nov. (ha.lo' phi.lus. Gr. n. *hals, halos* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving). The type strain is PS11-2^T (=JCM 12149^T=TISTR 1549^T=PCU 240^T), was isolated from a fish sauce fermentation in Thailand.

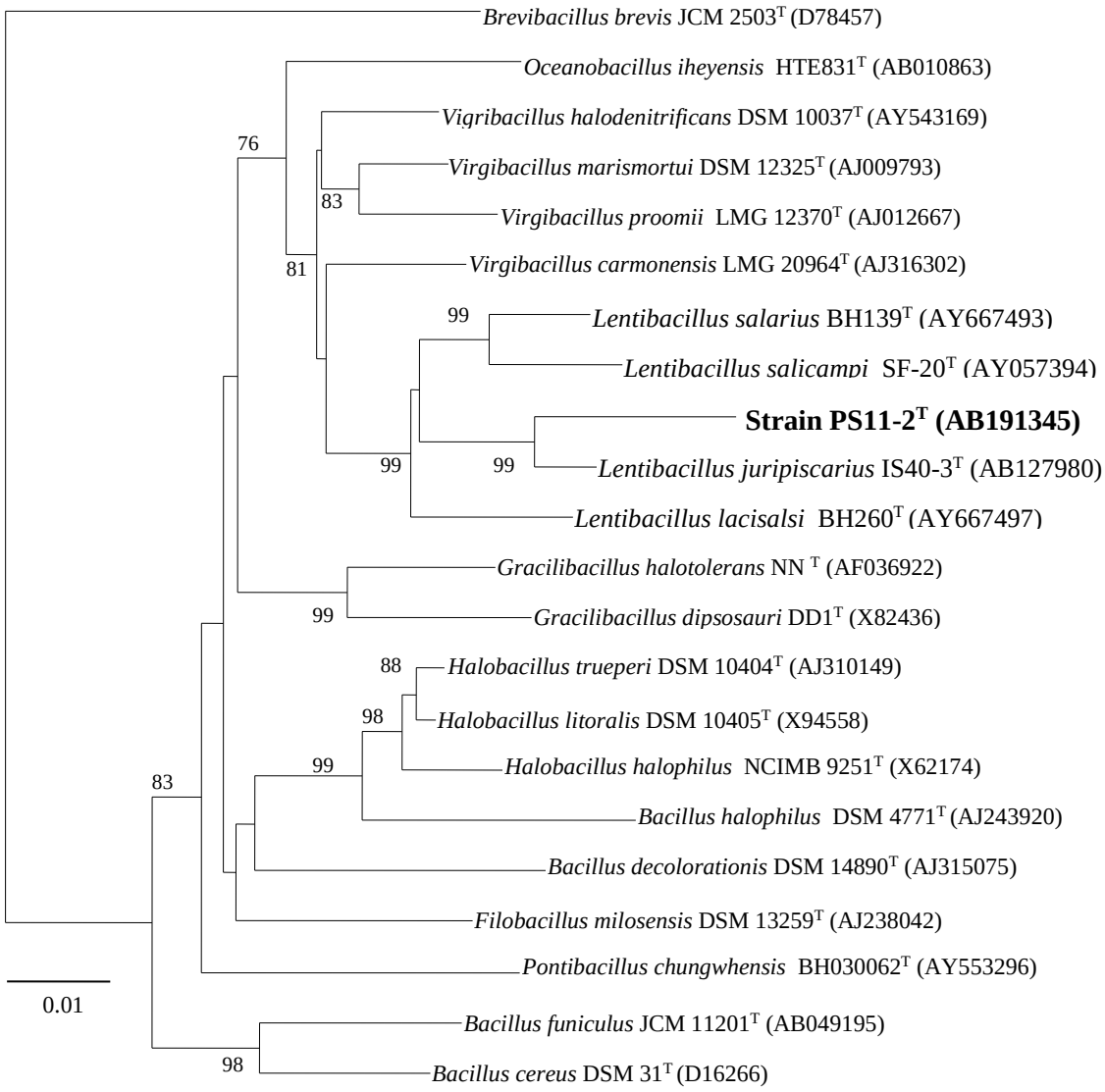


Fig.10. Phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain PS11-2^T and related bacterial species. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages above 75%, based on 1,000 replications, are shown at the nodes. Bar,1 substitution per 100 nucleotide positions.

Table 14. Strain designations, source, fermentation time, DNA base composition and DNA-DNA relatedness of strains

Strains	Factory /Fermentation time (month)	G+C content (mol %)	% Homology with labelled strain		
			11462^T	$PS11\text{-}2^T$	PB7-3
KF1-1	A/1	42.5	14.2	93	103.4
PB7-3 (=JCM 12150 =TISTR 1550)	A/7	42.6	11.9	93.8	100
$PS11\text{-}2^T$ (=$PCU240^T$= JCM 12149^T = TISTR 1549^T)	A/11	42.4	11.3	100	103
DB1-1	B/1	42.1	13.7	95.2	99.4
DS1-3B	B/1	ND	13.9	97.7	95.8
DB4-2	B/4	42.3	11.5	93.4	95.4
DS4-2	B/4	ND	11.5	88.2	97.2
DB5-1B	B/5	42.6	19.4	102.8	112.9
DS6-1	B/6	42.8	12.5	96.4	103.4
DS7-5	B/7	42.5	14	96.2	99.8
DB8-6	B/8	42.6	12.8	95.7	103.4
DB9-1	B/9	42.9	4.9	78.7	88.3
DS10-3B	B/10	42.9	17.5	96.8	90.9
DS26-3	B/26	43.1	13.3	70.8	101.4
CB0-1	C/0	42.7	11.1	84	70.9
L. juripiscarius JCM 12147^T (=$TISTR1536^T$)	Fish sauce	43.4	12.9	17	17.1
L. salicampi JCM 11462^T	Salt field	44	100	13	10.9

Abbreviations; PCU, Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; JCM, Japan Collection of Microorganism, RIKEN BioResource Center, Tsukuba, Japan; TISTR, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand; ND, not determined.

Group II contained two strains. They were strictly aerobic, moderately halophilic Gram-positive rods, slender-rod-shaped, strictly aerobic, red-pigmented, non-motile and approximately 0.2–0.460.8–2.5 mm in size. Spherical endospores are formed terminally in swollen sporangia (Fig. 11). Its were observed after high-temperature treatment (80^{o}C) of a culture for 30 min. Flagella are not observed. Colonies are low convex, smooth and circular (0.2–1.3 mm in diameter). Growth occurs at temperatures between 15 and 45^{o}C (optimum at 37^{o}C), at pH 5–9(optimum at pH 7.0) and with 5–30 % NaCl (optimum at 15 % NaCl). Anaerobic growth is not observed in the presence of 1 % (w/v) nitrate. Positive in tests for catalase, oxidase, and urease activity and nitrate reduction. Hydrolyses arginine and gelatin, but not aesculin, casein, Tween 80, tyrosine, starch, xanthine or hypoxanthine. Produces acid from D-fructose, D-galactose, D-glucose, glycerol, D-mannitol, D-mannose, D-ribose, sorbitol andsucrose, but not from amygdalin, L-arabinose, cellobiose, aesculin, gluconate, myo-inositol, inulin, lactose, maltose, melibiose, melezitose, methyl a-D-glucoside, raffinose, L-rhamnose, salicin, D-trehalose or D-xylose. Contains *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell wall peptidoglycan. The predominant isoprenoid quinone found was MK-7. The DNA G+C content of strain PN7-6^{T} is 41.6 mol% and had the cellular fatty acids included anteiso-$C_{15:0}$ (38.3 %), iso-$C_{16:0}$ (23.4 %) and iso-$C_{14:0}$ (14.8 %), and phosphatidylglycerol, diphosphatidylglycerol and two unidentified glycolipids are predominant in the polar lipid profile, whereas *L. salicampi* JCM 11462^{T} contained phosphatidylglycerol, diphosphatidylglycerol and one unidentified glycolipid (Tables 15 and 16).

On the basis of these characteristics and the data from 16S rRNA gene sequencing, strain PN7-6^{T} was included in a monophyletic cluster consisting of the *Lentibacillus* species, as shown in Fig. 12 (Yoon et al., 2002; Jeon et al., 2005a; Lim et al., 2005b; Namwong et al., 2005). The values for 16S rRNA gene sequence similarity between strain PN7-6^{T} and *L. salarius* KCTC 3911^{T}, *L. salicampi* JCM11462^{T}, *L. juripiscarius* JCM 12147^{T}, *L. lacisalsi* KCTC 3915^{T} and *L. halophilus* JCM12149^{T} were 96.5, 96.3, 96.3, 95.6 and 94.7 %, respectively. In addition, strain PN7-6^{T} showed

94.5–95.1 %16S rRNA gene sequence similarity to members of the genus *Virgibacillus* and 90.8–94.2 % sequence similarity to the other related bacteria (Fig. 12). The DNA–DNA hybridization study revealed that strains PN5-2 and PN7-6^T were closely related, exhibiting 98–118 % similarity, but they showed only low levels of DNA–DNA relatedness to *L. salicampi* JCM 11462^T (2.3–16.5 %) and *L. juripiscarius* JCM 12147^T (5.9–16.2 %) (Table 17), indicating that the two novel strains are unrelated to these *Lentibacillus* species (Wayne et al., 1987). The DNA G+C contents of strains PN5-2 and PN7-6^T were in the range 41.2–41.6 mol%. The two novel strains could be differentiated from *L. salarius* KCTC 3911^T and related species on the basis of pigmentation, motility, maximum growth temperature, NaCl tolerance, oxidase activity, hydrolysis of aesculin, casein and Tween 80, acid production from maltose, D-trehalose and D-xylose and DNA G+C content, as shown in Table 15. Thus, strains PN5-2 and PN7-6^T represent a novel species within the genus *Lentibacillus*, for which we propose the name *Lentibacillus kapialis* sp. nov. (ka.pi.a.lis. N.L. n. *kapium* from Korean n. *ka-pi* shrimp paste; L. suff. -*alis* adjectival suffix meaning pertaining to; N.L. masc. adj. *kapialis* pertaining to shrimp paste, the source of isolation of the strains). The type strain, PN7-6^T (=JCM 12580^T=PCU 259^T=TISTR 1551^T), was isolated from fermented shrimp paste (‘ka-pi’) produced in Thailand.

Table 15. Characteristics that differentiate strain PN7-6^T from related *Lentibacillus* species

Characteristics	PN7-6^T	*L. salarius*	*L. salicampi*	*L. juripiscarius*	*L. lacisalsi*	*L. halophilus*
Spore shape	Spherical	Spherical /oval	Spherical /oval	Oval	Spherical	Spherical
Pigmentation	Red	-	-	-	-	-
Motility	-	+	+	-	+	+
Colony size (mm, diameter)	1.2-3.0	NA	0.2-1.3	1.3-3.2	NA	0.2-.06
Maximum temp. growth (^oC)	45	50	40	45	40	40
NaCl range (%)	5-30	1-20	3-25	3-30	5-25	12-30
Oxidase activity	+	-	+	+	+	+
Nitrate reduction	+	+	+	+	+	-
Hydrolysis of :						
Aesculin	-	+	-	-	-	-
Casein	-	-	+	+	-	-
Tween 80	-	-	+	+	-	-
Acid production from:						
Celloboise	-	NA	w	-	NA	-
D-Fructose	+	+	-	+	+	-
D-Galactose	+	NA	w	-	NA	-
D-Glucose	+	+	+	+	-	-
Maltose	-	+	w	-	-	-
D-Mannitol	+	w	-	-	-	-
D-Mannose	W	+	w	-	-	-
D-Ribose	+	+	-	+	+	-
Salicin	-	-	w	-	-	-
Sucrose	W	NA	-	w	NA	-
D-Trehalose	-	w	-	-	-	-
D-Xylose	-	+	-	+	w	-
Major fatty acids	anteiso-$C_{15:0}$, iso-$C_{16:0}$	anteiso-$C_{15:0}$, iso-$C_{16:0}$	anteiso-$C_{15:0}$, anteiso-$C_{17:0}$	anteiso-$C_{15:0}$, iso-$C_{16:0}$	anteiso-$C_{15:0}$, anteiso-$C_{17:0}$	anteiso-$C_{15:0}$ anteiso-$C_{17:0}$
DNA G+C content (mol%)	41.6	43	42.4	43.4	44	42

Strains: PN7-6^T, *L. salarius* KCTC 3911^T (data from Jeon et al., 2005b*), L. salicampi* JCM 11462^T (Yoon et al., 2002; Namwong et al., 2005*), L. juripi*scarius JCM 12147^T (Namwong et al., 2005), *L . lacisalsi* KCTC3915^T (Lim et al., 2005b), *L. halophilus* JCM 12149^T (Tanasupawat et al., 2006). +, positive; - , negative; w, weak; NA, no data available.

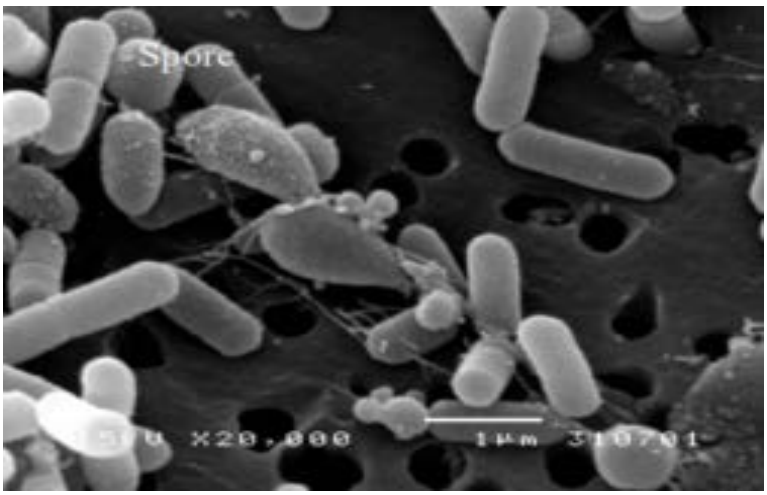


Fig. 11. Scanning electron micrograph of strain PN7-6^T grown on JCM medium no. 168 with 15 % NaCl at 37 °C. Bar, 1μm.

Table 16. Cellular fatty acid compositions of strain PN7-6^T and related taxa

Fatty acid	PN7-6T	[a]*L. salarius* KCTC 3911^T	[b]*L. salicampi* JCM 11462^T	[c]*L. juripiscarius* JCM 12147^T	[d]*L. lacisalsi* KCTC3915^T	[e]*L. halophilus* JCM 12149^T
Saturated fatty acids						
$C_{14:0}$	0.15	0.2	0.2	0.2	NA	1.0
$C_{15:0}$	0.16	0.3	0.1	0.1	NA	0.4
$C_{16:0}$	0.71	1.0	0.9	0.8	1.3	5.4
Unsaturated fatty acids						
$C_{16:1}$ *ω7c* alcohol	NA	NA	NA	0.6	1.5	NA
Branched fatty acids						
iso-$C_{14:0}$	14.8	13.9	7.9	10.2	5.7	1.0
iso-$C_{15:0}$	6.9	16.5	4.1	4.9	8.0	4.7
anteiso-$C_{15:0}$	38.3	25.3	48.6	45.1	50.8	53.9
iso-$C_{16:0}$	23.4	26.5	16.3	20.2	12.0	4.3
iso-$C_{17:0}$	0.9	4.4	0.9	0.5	1.9	0.8
anteiso-$C_{17:0}$	14.1	11.5	20.9	16.7	18.2	25.3

[a]Data from Jeon et al., 2005a; [b]Data from Yoon et al., 2002 and Namwong et al., 2005; [c]Data from Namwong et al., 2005); [d]Data from Lim et al., 2005b; [e]Data from Tanasupawat et al., 2006;
NA, no data available.

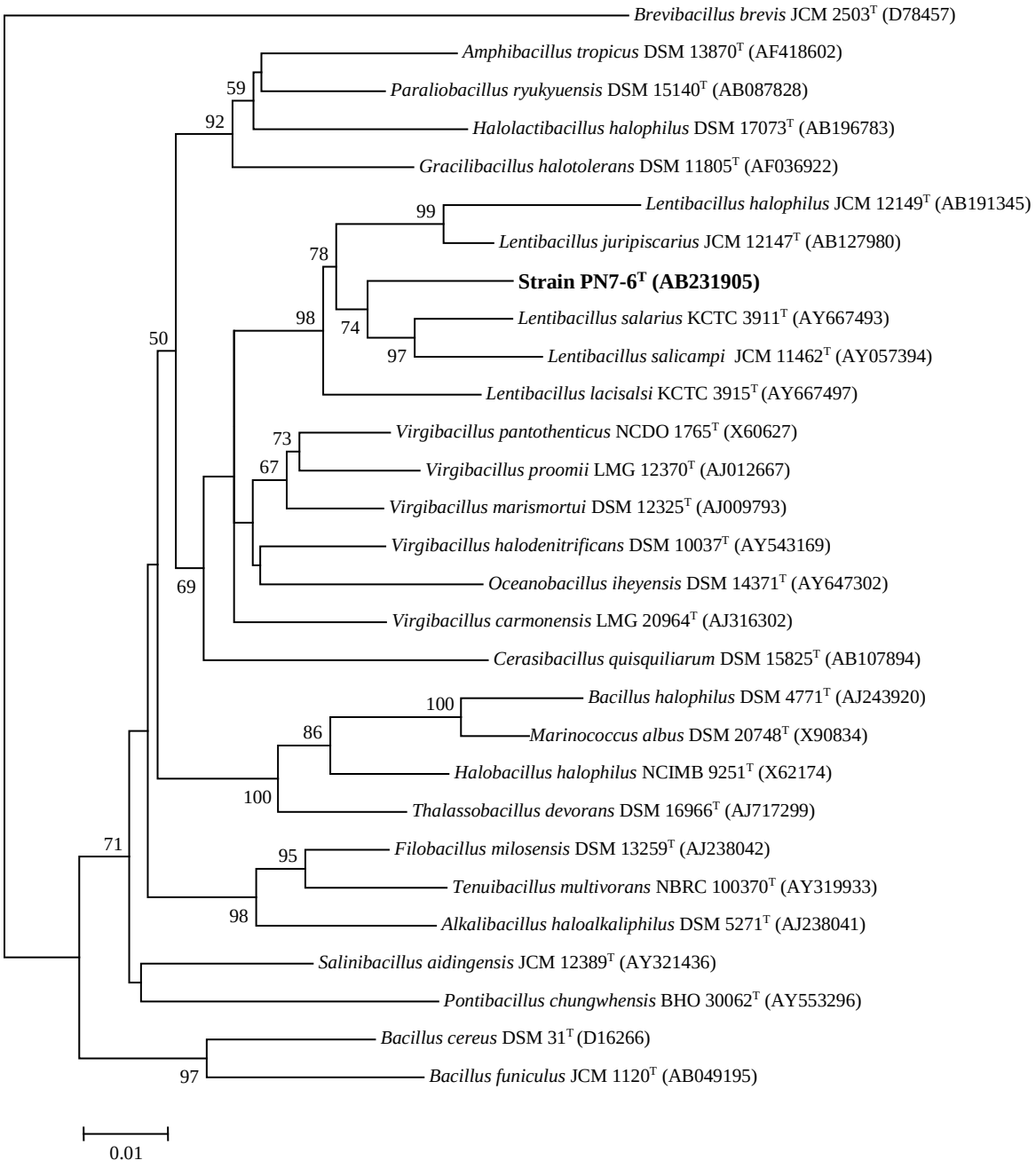


Fig. 12. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain PN7-6^{T}and related bacterial species. Bootstrap percentages above 50 %, based on 1,000 replications, are shown at the nodes. Bar, 1 substitutions per 100 nucleotide positions.

Table 17. Strain designations, source, DNAG+C content and levels of DNA-DNA relatedness for strains PN7-6^T and PN5-2 and related *Lentibacillus* species.

Strains	Culture collection			Sources	G+C	% Homology with labelled strain:			
	JCM	PCU	TISTR		(mol%)	PN5-2	PN7-6^T	11462^T	12149^T
PN5-2	12581		1552	Shrimp paste	41.2	100	98	2.3	3.4
PN7-6	12580^T	259^T	1551^T	Shrimp paste	41.6	118	100	10.2	12.7
L. juripiscarius	12147^T	229^T	1535^T	Fish sauce	43.4	16.2	5.9	18	18.8
L. salicampi	11462^T			Salt field	44	16.5	16.4	100	4.5
L. halophilus	12149^T	240^T	1549^T	Fish sauce	42.4	8	6	6.2	100

Abbreviations; JCM, Japan Collection of Microorganism, RIKEN BioResource Center, Tsukuba, Japan PCU, Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; TISTR, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

Group III contained fifteen strains. They were strictly aerobic moderately halophilic, Gram-positive cocci, approximately0.6–0.8 mm in diameter, occurring singly and in pairs or in tetrads (Fig. 13). Non-motile. Endospores and flagella were not observed. Colonies were smooth, circular, of low convexity and orange in colour (Fig. 14). Anaerobic growth was not observed. Growth occurs at pH 6 and 9 (optimally at pH 8.5), at temperatures of 15–45 ºC (optimally at 37ºC), but no growth was observed at 10 or 50 ºC, and in JCM medium no. 377 with 1.5–25 % (w/v) NaCl (optimally at 10 % NaCl). Catalase- and oxidase positive, but urease-negative. Unable to reduce nitrate. Negative for the indole, methyl red and Voges–Proskauer tests and for utilization of citrate. Aesculin, casein, arginine, gelatin, starch and Tween 80 are not hydrolysed. Acid is produced from D-fructose, D-glucose, glycerol, D-ribose and trehalose, but not from amygdalin, L-arabinose, cellobiose, aesculin, D-galactose, gluconate, *myo*-inositol, inulin, lactose, maltose, D-mannitol, D-mannose, melibiose, melezitose, methyl α-D-glucoside, raffinose, L-rhamnose, salicin, sorbitol, sucrose or D-xylose. On the basis of

phenotypic and chemotaxonomic characteristics of the 15 novel strains are detailed in the species description below and in Tables 18 and 19. Representative strain PN1-2 contained L-Lys in the cell-wall peptidoglycan. This strain had isoprenoid quinone with six isoprene units (MK-6) as a predominant component, and had a polar lipid profile of phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. The dominant cellular fatty acids of strains PN1-2^T, PN1-8, PN2-2 and PN7-1 were anteiso-$C_{15:0}$ (38.7–49.8 %) and iso-$C_{15:0}$ (10.4–24.5 %) (Table 19). The DNA G+C contents of the novel strains ranged from 44.5 to 47.5 mol%.

On the basis of 16S rRNA gene sequence analyses with the neighbour-joining algorithm, strains PN1-2^T and PN7-1 were included in a monophyletic cluster consisting of species of the genus *Salinicoccus*, as shown in Fig. 15 (Ventosa et al., 1990, 1992; Zhang et al., 2002). Levels of 16S rRNA gene sequence similarity between strains PN1-2^T and PN7-1 and *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T, *S. alkaliphilus* T8^{T}and *J. halotolerans* YKJ-101^T were 97.3–97.7, 96.5, 95.9 and 93.5 %, respectively. The novel strains were considered to represent the same species based on levels of DNA–DNA relatedness of over 76.6 % with strain PN1-2^T (Wayne et al., 1987) (Table 20). Strain PN1-2^T showed low DNA–DNA relatedness to *S. roseus* JCM 14630^T (21.7 %) and, reciprocally, *S. roseus* JCM 14630^T showed low DNA–DNA relatedness to strain PN1-2^T and the other 14 related strains (2.0–29.0 %). In addition, all 15 novel strains could be differentiated from *S. roseus* JCM 14630^T and related species based on pigmentation, maximum growth temperature, NaCl tolerance, hydrolysis of casein, gelatin, Tween 80 and starch, acid production from fructose and D-glucose and DNA G+C content, as detailed in Table 18. Furthermore, they could be differentiated from *Salinicoccus salsiraiae* LMG 22840^T based on pigmentation, maximum growth temperature, NaCl tolerance, hydrolysis of casein and gelatin and acid production from maltose and sucrose (Françya et al., 2006). Thus, the 15 novel strains isolated herein are considered to represent a single novel species of the genus *Salinicoccus*, for which we propose the name *Salinicoccus siamensis* sp. nov. (si.am.eń sis. N.L. masc. adj. *siamensis* pertaining to Siam, the old name of Thailand, from where the first strains were

isolated). The type strain, PN1-2^T (=JCM 12822^T =PCU 242^T =TISTR 1562^T), was isolated from fermented shrimp paste ('ka-pi') in Thailand.

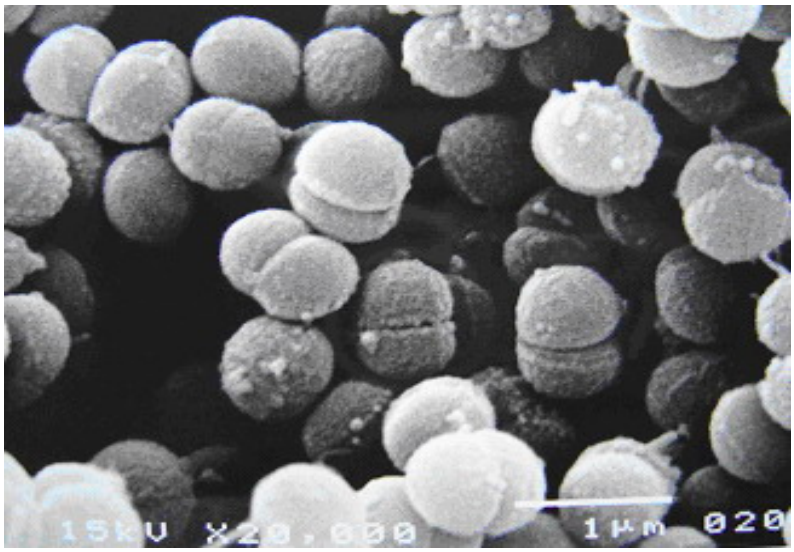


Fig. 13. Scanning electron micrograph of strain PN1-2^T grown on JCM medium no.377 at 37 ºC for 5 days. Bar, 1 μ.

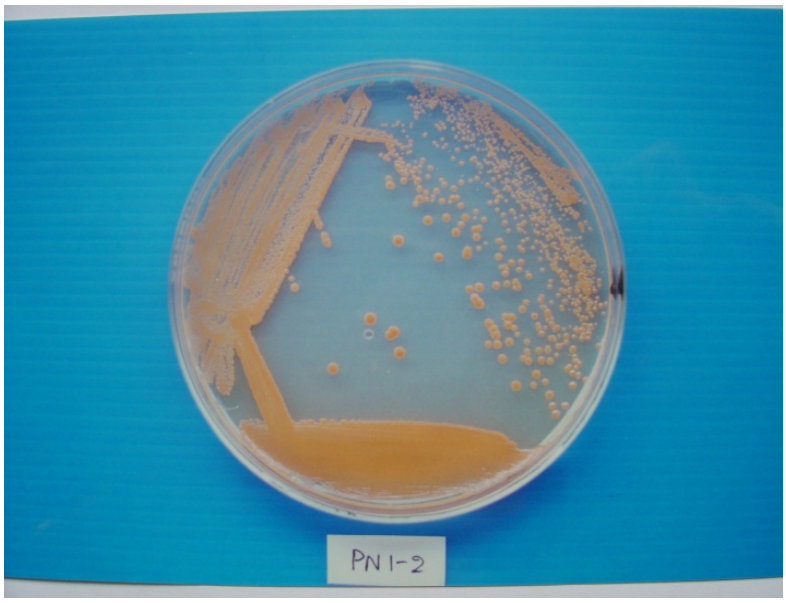


Fig. 14. Colonial appearance of strain PN1-2^T grown on JCM medium no. 377 medium at 37 ºC for 5 days.

Table 18. Differential characteristics of strain PN1-2^T and related taxa

Characteristics	PN1-2^T and 14 related strains	*S. roseus*[a] DSM 5351^T	*S. hispanicus*[b] DSM 5352^T	*S. alkaliphilus*[c] JCM 11311^T	*J. halotolerans*[d] JCM 11198^T
Cell size (μm)	0.6-0.8	1-2.5	1-2.5	0.5-0.8	0.6-1.1
Colony pigmentation	Orange	Pink-red	Reddish orange	Pinkish	Light yellow
pH range for growth	6-9	5-9	6-9	6.5-11.5	7-8
Optimum pH for growth	8.5	7.5	8.0	9.5	NA
NaCl range for growth (%)	1.5-25	0.5-25	0.9-25	0-25	0-20
Temp. range for growth (^{o}C)	15-45	15-37	15-40	10-49	4-42
Optimum temp. for growth	37	37	37	32	30-35
Methyl red test	-	-	+	-	NA
Urease test	-	-	+	+	-
Nitrate reduction	-	-	+	+	+
Hydrolysis of:					
Casein	-	+	-	-	-
Aesculin	-	-	+	+	-
Gelatin	-	+	+	-	NA
Tween 80	-	+	-	-	-
Starch	-	+	-	-	-
Acid production from:					
D-Fructose	+	-	+	-	-
D-Galactose	-	-	+	-	-
D-Glucose	+	-	+	+	-
Maltose	-	-	+	-	-
D-Mannitol	-	-	+	+	+
Major fatty acids	iso-$C_{15:0}$ or anteiso-$C_{15:0}$	anteiso-$C_{15:0}$ or anteiso-$C_{17:0}$	iso-$C_{15:0}$ or anteiso-$C_{15:0}$	iso-$C_{15:0}$ or anteiso-$C_{15:0}$	iso-$C_{15:0}$ or anteiso-$C_{15:0}$
DNA G+C content (mol%)	44.5-47.5 [e]	51.2	45.6-49.3	49.6 [f]	42

[a]Data from Ventosa et al., 1990, [b]Data from Marquez et al.,1990, [c]Data from Zhang et al., 2002, [d]Data from Yoon et al., 2003, [e]The G+C content of the type strain is 46 mol%, [f]Range of values for five strains given by Marquez et al. (1990). The value for the type strain is 45.7 mol%. +, positive; -, negative; NA, no data available.

Table 19. Cellular fatty acid compositions of strain PN1-2^T and related taxa

Fatty acids	PN1-2^T	PN1-8,PN2-2, and PN7-1	*S. roseus*[a] DSM 5351^T	*S. hispanicus*[b] DSM 5352^T	*S. alkaliphilus*[c] JCM 11311^T	*J. halotolerans*[d] JCM 11198^T
Saturated fatty acids:						
$C_{14:0}$	0.5	0.2-0.5	NA	0.5	1.6	0.4
$C_{15:0}$	ND	0.1-0.2	0.7	0.5	NA	1.0
$C_{16:0}$	2.4	0.9-2.4	1.0	2.7	1.5	3.4
Unsaturated fatty acids:						
$C_{16:1}$ $\omega 7c$ alcohol	ND	0.2-1.6	0.6	0.8	5.8	0.8
Branched fatty acids:						
iso-$C_{14:0}$	2.4	1.2-3.2	0.6	0.5	4.4	2.6
iso-$C_{15:0}$	14.2	10.4-24.5	12.5	20.8	22.3	16.7
anteiso-$C_{15:0}$	38.7	49.2-49.8	32.9	35.9	27.6	33.5
iso-$C_{16:0}$	12.1	8.3-17.0	4.3	2.5	10.1	12.6
iso-$C_{17:0}$	4.9	3.3-6.1	6.2	8.8	3.3	5.7
iso-$C_{17:1}$ $\omega 10c$	4.5	3.4-10.8	7.3	3.5	4.0	4.7
anteiso-$C_{17:0}$	12.1	12.1-24.2	13.5	11.9	8.9	8.9
iso-$C_{19:0}$	0.4	0.2-0.4	2.3	2.5	2.8	1.8
Unknown fatty acids	3.4	1.6-3.4	1.4	1.2	0.9	5.0

[a]Data from Ventosa et al., 1990, [b]Data from Marquez et al.,1990, [c]Data from Zhang et al., 2002, [d]Data from Yoon et al., 2003. NA, no data available, ND, not detected.

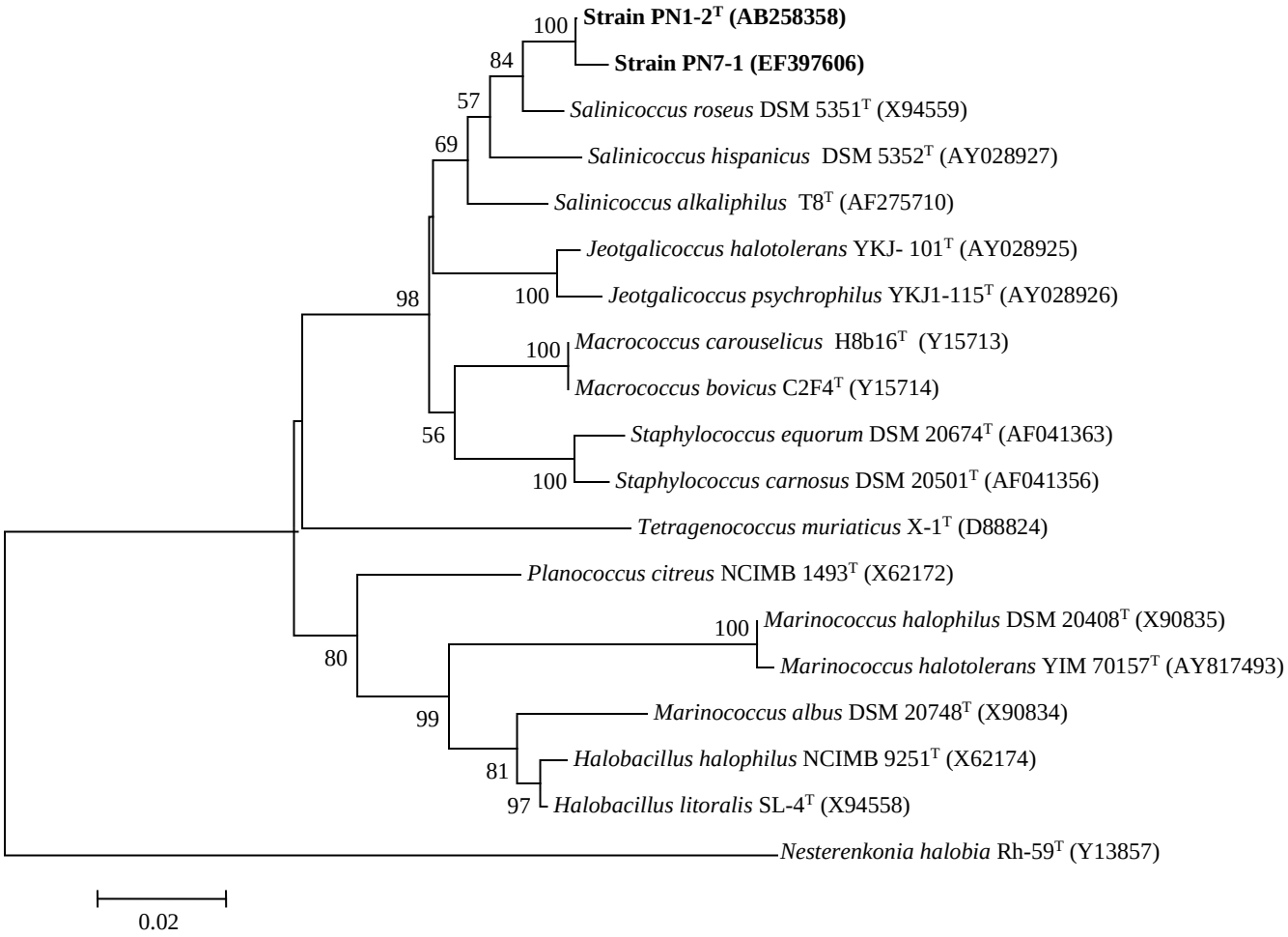


Fig. 15. Phylogenetic tree showing the relationships between strains PN1-2^T and PN7-1, and related bacterial species, based on 16S rRNA gene sequences. The branching pattern was generated by the neighbor-joining method. Bootstrap percentages above 50%, based on 1,000 replications, are shown at nodes. Bar, 2 substitutions per 100 nucleotide positions.

Table 20. DNA G+C content and DNA-DNA relatedness among the novel strains and *Salinicoccus roseus* JCM 14630^T

Strain	G+C content (mol%)	% Homology with with labelled strains	
		PN1-2^T	JCM 14630 ^{T}
PN1-2^T	46.0	100	17.3
PN1-7	ND	97.4	16.8
PN1-8	47.5	89.0	29.0
PN1-9	ND	85.4	10.3
PN1-10	44.5	89.9	3.0
PN2-2	44.9	76.6	5.6
PN2-9	ND	98.8	16.1
PN2-14	ND	92.9	17.2
PN2-15	ND	93.4	9.9
PN2-16	ND	82.9	2.0
PN2-20	ND	96.7	7.3
PN7-1	46.3	91.2	16.8
PN7-7	46.1	96.4	23.0
PN7-8	ND	85.0	14.0
PN7-9	ND	92.5	19.0
S. roseus JCM 14630^T	51.2*	21.7	100

ND, Not determined. *Data from Ventosa et al. (1990).

3.2.3 Lactic acid bacteria isolated from *Miang*

The rod-shaped and sphere-shaped strains were widely distributed in *Miang*. All the strains were placed in a monophyletic cluster consisting of *Lactobacillus* and *Pediococcus* species. They were Gram-positive, catalase negative, and they appeared singly, in pairs, or in chains. Colonies on MRS agar plate were circular, low convex with entire margin. The strains were divided into 7 groups by phenotypic characteristics, isomers of lactic acid, cell wall composition, and DNA base composition and DNA-DNA relatedness (Tables 21, 22, and 23). All the representative strains produced DL-lactic acid, but the strain in Group IV produced L-lactic acid. Strains of Groups I, II and V had *meso*-diaminopimelic acid (DAP) in cell wall (Table 21).

Table 21. General characteristics of strains

Characteristics	Group I (6)[a]	Group II (5)	Group III (5)	Group IV (2)	Group V (4)	Group VI (2)	Group VII (1)
Cell form	Rods	Rods	Rods	Rods	Rods	Rods	Cocci
Cell size (μm)	0.4-1x2-6	0.8-1x2-6	0.5-0.6x2-6	0.6-1x2-5	0.5-0.8x2-5	0.6-1x3-8	0.5-0.7
Gas from glucose	-	-	-	-	+	+	-
Gas from gluconate	-	+	-	-	+	+	-
Arginine hydrolysis	-	-	-	-	-	+	-
Esculin hydrolysis	+	+	+	+	-	-	+
Nitrate reduction	-	-	-	-	-	+	-
Reaction in litmus milk:							
Acidification	+	+(-2)	-	-	-	-	-
Coagulation	-(+3)	+(-2)	-	-	-	-	-
Reduction	-(+2)	+(-2)	-	-	-	-	-
Growth at 15 °C	+	+	w	+	w	-	+
45 °C	-	-	-	-	-	+	+
Growth at pH 3.5	-	+	-	-	-	+	-
pH 8.0	+(-1)	+	+	+	+	+	+
pH 8.5	-(+1)	+	+	-	-	-	+
pH 9.0	-	-(+1)	-	-	-	-	-
Growth in 4 % NaCl	+(-1)	+	+	-	-	+	+
6 % NaCl	+(-1)	+	-(w1)	-	-	-	-
8 % NaCl	-	+(-1)	-	-	-	-	-
meso-DAP in cell wall peptidoglycan	+	+	-	-	+	-	-
Isomer of lactic acid	DL	DL	DL	L	DL	DL	DL

+, positive; w, weak positive;-, negative reaction.
[a]Number of strains. Numbers in parentheses indicate the number of strains showing the reaction.

Table 22. Acid from carbohydrates of strains

Carbohydrates	Group I (6)[a]	Group II (5)	Group III (5)	Group IV (2)	Group V (4)	Group VI (2)	Group VII (1)
D-Amygdalin	+(-1)	+	+(-1)	+	-	-	+
L-Arabinose	-	+(-2)	-(+1)	+	+	+	-
D-Fructose	+	+	+	+	-	+	+
Cellobiose	+	+	+	+	-	-	+
Esculin	+	+	+	+	-	-	+
D-fructose	+	+	+	+	-	+	+
D-Galactose	+	+(-2)	+	+	-	+	+
Gluconate	-	+(w2)	-	-	+(w1)	w	-
Lactose	+	+(-2)	+(-1)	-	-	+	+
Maltose	+(-2)	+	-	+	+	+	-
D-Mannitol	-	+(w2)	-	+	-	-	-
D-Mannose	+	+	+	+	-	+	+
Melibiose	-	+(-2)	-	-	+(w3)	w	-
α-Methyl-D-glucoside	-	+(-2)	-	-	-	-	-
Raffinose	-	+(-2)	-	-	-	+	-
Rhamnose	w(-2)	-(w1)	-	-	-	-	-
Ribose	-	+(-1)	-	-	-	w	-
Salicin	+	+	+	+	-	-	+
Sucrose	-	+	-	-	-	+	-
Sorbitol	-	-(+2)	-	-	-	-	-
Sucrose	-	+	-	-	-	+	-
Trehalose	+(-1)	+	-	-	-	+	+
D-Xylose	-	+(w2)	-	+	+	+	-

+, positive; w, weak positive; -, negative reaction; [a] Number of strains. Numbers in parentheses indicate the number of strains showing the reaction. All are positive for glucose but negative for glycerol, inulin, and melezitose

The rod-shaped strains, FP37-1 (Group I) and MCH5-2 ^{T} (Group III) showed, 99.1 and 98.7% of 16S rDNA sequence similarity to *L. pantheris* LMG 21017^{T} while MCH4-2 (Group VI), MCH3-1^{T} (Group IV), and MCH2-2 (Group V) showed 99.5, 96.1 and 98.6% of 16S rRNA gene sequence similarity to *L. fermentum* CECT 562^{T}, *L. manihotivorans* LMG 18010^{T} and *L. suebicus* CECT 5917^{T}, respectively. They were placed in a monophyletic cluster consisting of *Lactobacillus* species (Fig. 16). The coccal strain, MCH3-2^{T} (Group VII) showed 96.8% of 16S rDNA sequence similarity to *Pediococcus cellicola* Z-8^{T} that was placed in a monophyletic cluster consisting of *Pediococcus* species as shown in Fig. 16.

Group I contained six strains (FP37-1, MCH1-1, MCH1-2, MCH1-3, MCH1-4, MCH1-6). These strains had DAP in the cell wall. All the strains produced acid from cellobiose, esculin, D-fructose, D-galactose, D-glucose, lactose, D-mannose and salicin (Tables 21 and 22). A representative strain, FP37-1 showed the high degree of DNA-DNA relatedness (over 77.9%) to strains in the group, and the strains showed the high degree of DNA-DNA relatedness (over 81.6%) to *Lactobacillus pantheris* NRIC 0613^{T} (=LMG 21017^{T}). The strains in this group showed the low degree of DNA-DNA relatedness (less than 11.2%) to *L. plantarum* NRIC 1067^{T}, *L. pentosus* NRIC 1069^{T} and *L. paraplantarum* CNRZ 1885^{T} (Table 23). Furthermore, the strain FP37-1 showed 99.1% similarity of 16S rDNA sequence to *Lactobacillus pantheris* LMG 21017^{T}, and it was located in a monophyletic cluster (Fig.16). The DNA G + C content of strains tested ranged from 50 to 52 mol%, which was closed to that of *Lactobacillus pantheris* NRIC 0613^{T} (Table 24). Therefore, the isolates in this group were identified as *L. pantheris* (Liu and Dong, 2002; Wayne et al., 1987). *L. pantheris* was originally established based on two strains, and was reported to produce D-lactic acid (Liu and Dong, 2002). In this study, *L. pantheris* NRIC 0613^{T} and our isolates produced DL-lactic acid and had *meso*-diaminopimelic acid (DAP) in the cell wall. In addition, strain FP37-1 required riboflavin, biotin, niacin, calcium-pantothenate, and folic acid for growth but not thiamine, *p*-aminobenzoic acid, and pyridoxal. The strain FP37-1 had the straight chain fatty acids of $C_{18:1}$ and $C_{16:0}$ as major components but had no cyclopropane acids as

reported by Tanasupawat et al. (1992a). The detailed description of this species mentioned here will be useful for their circumscription.

Group II included five strains (MCH1-7, MCH2-5, MCH4-3, MCH5-5, and MCH5-6). They had *meso*-diaminopimelic acid in the cell wall, and the tested strains produced DL-lactic acid. All the strains produced acid from D-amygdalin, cellobiose, esculin, D-fructose, D-glucose, maltose, D-mannose, salicin, sucrose, and trehalose (Tables 21 and 22). All the strains showed the high degree of DNA-DNA relatedness (over 95.2 %) to *L. pentosus* NRIC 1069^T, and they were identified as *L. pentosus* (Hammes et al., 1992; Tanasupawat et al., 1992a; Wayne et al., 1987; Zanoni et al., 1987).

Group III contained five strains (MCH4-1, MCH4-4, MCH5-2^T, MCH5-4, and MCH5-9). These strains did not have *meso*-diaminopimelic acid in the cell wall. All the strains produced acid from esculin, D-fructose, D-galactose, D-glucose, D-mannose and salicin (Tables 21 and 22). Weak growth was observed at 15 but not at 45^{o}C. They were separated from the strains in Groups I, II, IV, V and VI by phenotypic characteristics (Tables 21 and 22). The representative strain in this group, MCH5-2^T showed 98.7% similarity of 16S rRNA gene sequence to *L. pantheris* LMG 21017^T (Liu and Dong, 2002) (Fig.16). The strain MCH5-2^{T}contained L-lysine-aspartate in cell wall. However, they showed the low degree of DNA-DNA relatedness (less than 12.5 %) to *L. pantheris* NRIC 0613^T (Table 23). They were differentiated from *L. pantheris* NRIC 0613^T by the acid production from D-amygdalin, maltose, and trehalose, and *meso*-diaminopimelic acid in the cell wall (Table 24). Therefore, a new species *Lactobacillus thailandensis* sp. nov. is proposed for this group. (thai. lan' den. sis. M. L. fem. adj. *thailandensis*, pertaining to Thailand, where the strains were isolated). The type strain is MCH5-2^T (BCC 21235^T=JCM 13996^T=NRIC 0671^T=PCU 272^T), which has 49 mol% of DNA G+C content.

Group IV contained two strains (MCH3-1^T and MCH3-3). All the strains produced acid from D-amygdalin, L-arabinose, cellobiose, esculin, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, salicin, and D-xylose (Tables

21 and 22). Growth was observed at 15 but not at 45ºC. The representative strain, MCH3-1^T (Group IV), was closely related to *L. manihotivorans* LMG 18010^T, *L. zeae* ATCC 15820^T, and *L. casei* ATCC 334 ^{T} with 96.1, 95.4, and 95.3% of 16S rRNA gene sequence similarity, respectively (Fig.16). The two strains showed the low degree of DNA-DNA relatedness (less than 28.5 %) to *Lactobacillus manihotivorans* JCM 12514^T (= LMG 18010^T) (Table 23). The strains were separated from the strains in Groups I, II, III, V and VI by phenotypic characteristics (Tables 21 and 22). Strain MCH3-1^{T}contained L-lysine-aspartate in the cell wall. Further, they were differentiated from *L. manihotivorans* LMG 18010^T, *L. casei* ATCC 334^T, and *L. zeae* ATCC 15820^T (Dicks et al.,1996; Kandler and Weiss, 1986; Morlon-Guyot et al., 1998) by the growth at 45ºC; acid production from L-arabinose, lactose, mannitol, melibiose, raffinose, sucrose, trehalose and D-xylose; and DNA G + C content (Table 24). Therefore a new species *Lactobacillus camelliae* sp. nov. is proposed for this group. (*ca. mel. li'. ae* N. L. gen. n. *camelliae* of *Camellia,* fermented tea (*Camellia sinensis*) leaves, a source of the strains isolated). The type strain is MCH3-1^T(BCC 21233^T=JCM 13995^T=NRIC 0672^T=PCU 273^T), which has 51.9 mol% of DNA G+C content.

Group V contained four strains (MCH2-1, MCH2-2, MCH2-3, and MCH2-4). All the strains produced gas from glucose heterofermentatively and produced acid from L-arabinose, D-glucose, maltose, and D-xylose. The strains tested had *meso*-diaminopimelic acid in the cell wall. The strains produced DL-lactic acid and had DNA G + C content from 39 to 39.3 mol% (Tables 21 and 22). The representative strain in this group, MCH2-2, was related to *L. suebicus* CECT 5917^T with 98.6% similarity of 16S rDNA sequence (Fig.16). However, MCH2-2 and other strains showed the high degree of DNA-DNA relatedness (over 78.8%) to that of *Lactobacillus suebicus* NRIC 1637^T (=DSM 5007^T) (Table 23). Thus, they were identified as *Lactobacillus suebicus* (Kleynmans et al., 1989).

Table 23. DNA base composition and DNA-DNA relatedness of strains.

Species	Strains	G+C content (mol%)	% DNA relatedness with labelled strains	
Group I			FP37-1	NRIC 0613^T
	FP 37-1	52	100	92.8
	MCH1-1	51	92.6	88.8
	MCH1-2		91.4	89.2
	MCH1-3	50	93.2	81.6
	MCH1-4		90.9	88.6
	MCH1-6		77.9	73.1
L. plantarum	NRIC 1067^T		5.4	6.6
L. pentosus	NRIC 1069^T		5	6.5
L. paraplantarum	CNRZ 1885^T		11.2	6.7
L. pantheris	NRIC 0613^T		99.2	100
Group II			NRIC 1069^T	MCH5-6
	MCH1-7		100.3	93.1
	MCH2-5		101.3	89.5
	MCH4-3		95.2	97.7
	MCH5-5		102.8	102.9
	MCH5-6		100.3	100
L. pentosus	NRIC 1069^T		100	76.2
L. plantarum	NRIC 1067^T		31.3	32.4
L. paraplantarum	CNRZ 1885^T		35.9	49.2
Group III			MCH5-2^T	NRIC 0613^T
	MCH4-1	49.4	89.7	4.7
	MCH4-4		92.4	11.8
	MCH5-2^T	49	100	7
	MCH5-4	50.3	98.3	12.5
	MCH5-9	49.7	95	9.3
L. pantheris	NRIC 0613^T		10.8	100
Group IV			MCH3-1^T	JCM 12514^T
	MCH3-1^T	51.9	100	22.9
	MCH3-3	51.5	101.2	28.5
L. manihotivorans	JCM 12514^T		12.8	100
Group V			MCH2-2	NRIC 1637^T
	MCH2-1		93.4	78.8
	MCH2-2	39.3	100	82.7
	MCH2-3		109.6	102.2
	MCH2-4	39	86	85.2
L. suebicus	NRIC 1637^T		94.7	100
Group VI			MCH4-2	
	MCH4-2	50.2	100	
	MCH4-5		96.7	

Table 24. Differential characteristics of *L. thailandensis*, *L. camelliae* and closely related species.

Characteristics	*L. thailandensis*	*L. camelliae*	[a]*L. pantheris*	[b] *L. manihotivorans*	[c]*L. casei*	[d] *L. zeae*
	MCH 5-2^T	MCH3-1^T	NRIC 0163^T	JCM 12514^T	ATCC 334^T	ATCC 15820^T
D-Amygdalin	+	+	-	+	-	+
L-Arabinose	-	+	-	-	+	-
Gluconate	-	-	-	-	+	+
Lactose	+	-	+	+	+	+
Maltose	-	+	+	+	+	+
Mannitol	-	+	-	-	+	+
Melezitose	-	-	-	-	+	+
Melibiose	-	-	-	+	-	-
Raffinose	-	-	-	+	-	-
Rhamnose	-	-	-	-	-	+
Ribose	-	-	-	-	+	+
Sorbitol	-	-	-	-	+	-
Sucrose	-	-	-	+	+	+
Trehalose	-	-	+	+	+	+
D-Xylose	-	+	-	-	-	-
NH_3 from arginine	-	-	-	ND	-	-
Lactic acid isomer	DL	L	DL	L	L	-
Growth at 45 ^oC	-	-	-	+	ND	+
meso-DAP in the cell wall	-	-	+	+	-	-
DNA G+C (mol %)	49	51.9	52.7	48.4	45-47	48-49

+, positive; -, negative reaction; ND, No data. [a]Data from Liu and Dong (2002); [b]Data from Morlon-Guyot et al. (1998); [c]Data from Kandler and Weiss (1986); [d]Data from Dicks et al. (1996).

Group VI contained two strains (MCH4-2 and MCH4-5). The strains produced gas from D-glucose heterofermentatively (Table 21). They hydrolyzed arginine, reduced nitrate, grew at 45^{o}C and in 4% NaCl, and produced acid from L-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, raffinose, sucrose, trehalose and D-xylose (Tables 21 and 22). Their phenotypic characteristics and DNA G + C content (50.2 mol %) were the same as those described by Tanasupawat et al. (1993). In addition, a representative strain, MCH4-2 was closely related to *L. fermentum* CECT 562^T with 99.5% similarity of 16S rDNA gene sequence (Fig. 16). MCH4-2 and

MCH4-5 showed a high DNA-DNA relatedness (96.7%) to each other (Table 23). Therefore, they were identified as *L. fermentum* (Tanasupawat et al., 1993).

Group VII contained one strain, MCH3-2^T. It did not have *meso*-diaminopimelic acid but contained L-lysine-aspartate in the cell wall. Growth was observed at 15 and 45ºC. Strain MCH3-2^T was closely related to *Pediococcus cellicola* Z-8^T, *P. damnosus* DSM 20331 ^{T} and *P. inopinatus* LMG 11409 ^{T} with 96.8, 96.4, and 96.2% of 16S rRNA gene sequence similarity, respectively (Fig.16). This strain was differentiated from *P. cellicola* Z-8^T (Zhang et al., 2005) by acid production from L-arabinose, maltose, rhamnose, D-ribose, sucrose, and D-xylose; DNA G + C content (42 mol%), and 16S rRNA gene sequence similarity (Table 25, Fig. 16). Therefore a new species *Pediococcus siamensis* sp. nov. is proposed for this group.

In the previously, the isolation of strains of *Lactobacillus plantarum, L. pentosus, L. vaccinostercus, Lactobacillus* sp., *Enterococcus casseliflavus*, and *Enterococcus* sp. from *miang* were reported by Okada *et al*. (1979; 1986); Tanasupawat *et al*. (1992a; 1992b); Tanasupawat and Komagata. (1995). Further, I found the strains of *L. pantheri*s, *L. pentosus*, *L. suebicus*, and *L. fermentum*, and the three novel species, *L. thailandensis*, *L. camelliae*, and *P. siamensis* in fermented tea leaves (*miang*). Consequently, lactic acid bacteria would play roles in the acid production and preservation of *miang*. In addition, the new *Lactobacillus* species are close to *L. pantheri*s from faeces of jaguar and *L. manihotivorans* strains from sour fermented cassava starch (Liu and Dong, 2002; Morlon-Guyot et al., 1998) including the new *Pediococcus* species closed to *P. cellicola* strains from a distilled-spirit-fermenting cellar (Zhang et al., 2005) showed high DNA base compositions.

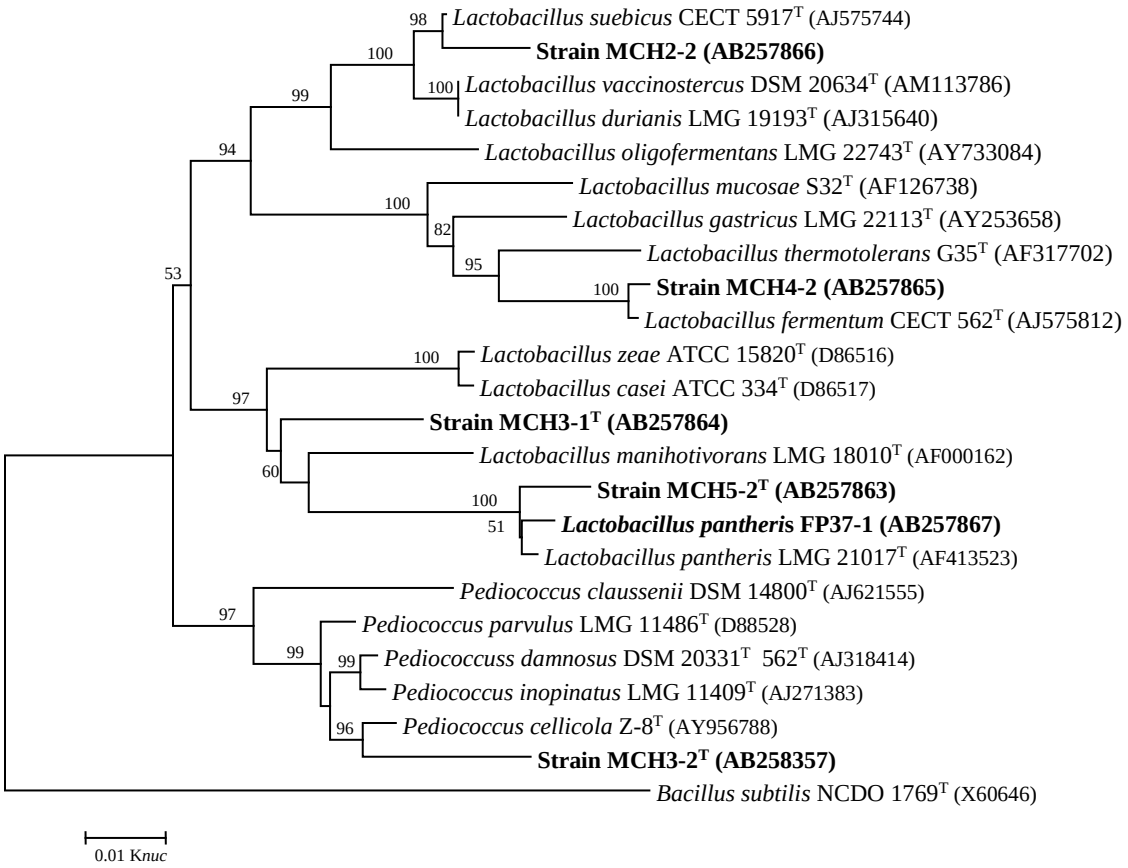
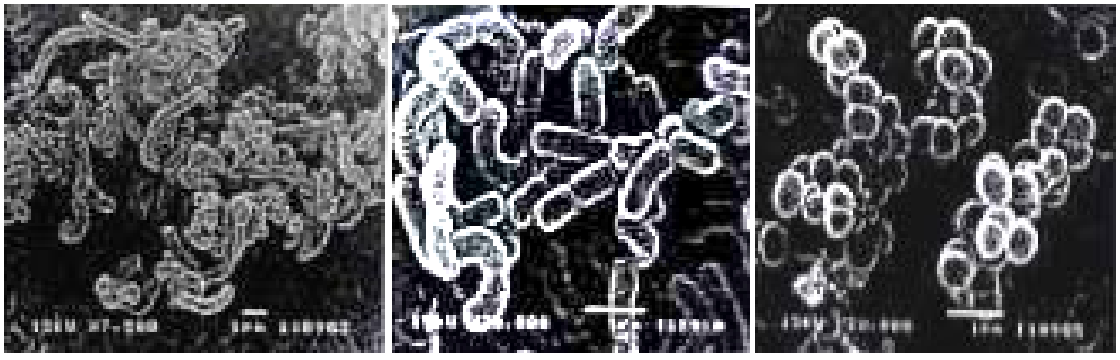


Fig. 16. Phylogenetic tree, based on 16S rRNA gene sequences showing the relationships among strains FP37-1, MCH2-2, MCH3-1^T, MCH3-2^T, MCH4-2, MCH5-2^T, *Lactobacillus* species and *Pediococcus* species. The branching pattern was generated by the neighbor-joining method. Bootstrap percentages above 51%, based on 1,000 replications, are shown at the nodes. Bar, 1 substitution per 100 nucleotide positions

Table 25. Differential characteristics of *P. siamensis* and related *Pediococcus* species.

Characteristics	*P. siamensis* MCH3-2^T	*P. cellicola*[a] Z-8^T	*P. damnosus*[b]	*P. inopinatus*[b]
L-Arabinose	-	+	-	-
Lactose	+	+	-	+
Maltose	-	+	D+	+
Melezitose	-	-	D+	-
Rhamnose	-	+	-	-
Ribose	-	+	-	-
Sucrose	-	+	D+	D+
D-Xylose	-	+	-	-
α-Methyl-D-glucoside	-	-	D+	D+
Growth at pH 7.0	+	+	-	+
Growth at 30 ^oC	+	+	-	+
DNA G+C (mol %)	42	37	40.0[c]	ND

+, positive; D+, some strains positive; -, negative; ND, no data. [a]Data from Zhang et al.(2005); [b]Data from Back (1978); [c]Data from Tanasupawat and Komagata (1988).



(A) (B) (C)

Fig. 17. Scanning electron micrograph of *Lactobacillus camelliae* MCH3-1^T (A), *L. thailandensis* MCH5-2 ^{T} (B) and *Pediococus siamensis* MCH3-1^T (C) grown on MRS agar at 30 ^oC for 3 days. Bar, 1 μ. (Tanasupawat, 2014)

3.2.4 High diacetyl-producing LAB isolated from milk and fermented foods

All seven selected strains were gram positive, nonmotile, and nonsporing. Colonies on MRS agar plates were circular, low convex with entire margin, and nonpigmented. They did not produce catalase or reduce nitrate. On the basis of phenotypic characteristics of isomer of lactic acid and peptidoglycan type of cell wall in Tables 26 and 27 the selected strains AP17-1, SR4-2 and SR8-1 were induced in the genus *Lactobacillus* (Hammes et al., 1992; Kandler and Weiss, 1986). A strain AP2-1 was in *Weissella* (Collin et al., 1993; Schillinger et al., 1989) which was different from genus *Leuconostoc* (Collins et al., 1993) and the strains PM3-13, PM3-14 and PM4-9 were in *Enterococcus* (Devriese et al., 1991; Tanasupawat et al., 1992b). From the DNA-DNA relatedness results as shown in Table 28, the strains AP17-1, SR4-2 and SR8-1 showed high DNA relatedness (93.3-111.6%) with *Lactobacillus pentosus* NRIC 1069^T but showed low DNA similarity with *Lactobacillus plantarum* NRIC 1067^T. They were identified as *Lactobacillus pentosus* (Tanasupawat et al., 1992a, 1998, 2000; Wayne et al., 1987). The strain AP2-1 showed high DNA similarity (100.5%) with *Weisella confusa* NRIC 0207^T. It was identified as *Weissella confusa* (Collin et al., 1993; Hammes et al., 1992; Kandler and Weiss, 1986; Schillinger et al., 1989; Tanasupawat et al., 1993, 2000; Wayne et al., 1987). The strains PM3-13, PM3-14 and PM4-9 showed high DNA similarity (72.1-99.6%) with *Enterococcus faecium* NRIC 1145^T but showed low DNA similarity (2.7-17.1%) with *Enterococcus faecalis* TISTR 379^T. They were identified as *Enterococcus faecium* (Facklam and Collins, 1989; Tanasupawat et al, 1992b; Wayne et al., 1987).

Table 26. Characteristics of rods strains.

Characteristics	AP17-1	SR4-2	SR8-1	[a]*L. pentosus* NRCT 1069[T]	[b]*L. plantarum* NRCT 1067[T]
Cell form	……..……..Rods……				
Cell size (μm)	….....……0.8-1.0 x 1.5-5.0……				
Cell arrangement	……..…..…….. singly, in pairs or chains……				
Gas from glucose	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-
Slime formation	-	-	-	-	-
Growth at: 45 °C	-	-	-	-	-
pH 4.0	+	+	+	+	+
pH 9.6	w	+	+	+	-
Isomer of lactic acid	DL	DL	DL	DL	DL
Peptidoglycan type: *meso*-DAP	+	+	+	+	+
Acid from:					
L-Arabinose	+	+	+	+	+
Gluconate	+	+	+	+	+
Glycerol	+	+	+	+	-
Lactose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Melibiose	+	+	+	+	+
D-Melezitose	w	w	w	-	+
Raffinose	w	w	w	-	+
L-Rhamnose	w	+	w	+	-
D-Ribose	+	+	+	+	+
Salicin	+	+	+	+	+
D-Sorbitol	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Xylose	+	+	+	+	-

+, positive; w, weak; -, negative; ND, no data. All produced acid from D-cellobiose, esculin, D-fructose, D-galactose and D-glucose. NRIC, NODAI Research Institute Culture Collection, Tokyo, Japan.
[a]Data from Tanasupawat et al.(2002).
[b]Data from Tanasupawat et al. (1992a, 1993, 2000).

Table 27. Characteristics of cocci strains.

Characteristics	AP2-1	[a] *W. confusa* NRIC 0207^T	[a] *L. mesenteroides* NRIC 1541^T	PM3-13	PM3-14	PM4-9	[b] *E. faecium* NRIC 1145^T	[b] *E. faecalis* NRIC 1142^T
Cell form	……...……..…...Cocci……......							
Cell size (μm)	…… ……..……..…….....…0.5 - 1.0……...							
Cell arrangement	……..……..……. singly, in pairs or chains…….							
Gas from glucose	+	+	+	-	-	-	-	-
Arginine hydrolysis	+	+	-	+	+	+	+	+
Slime formation	+	+	+	-	-	-	-	-
Growth at: 45 ºC	-	-	-	+	+	+	+	+
pH 4.0	+	+	+	-	-	-	ND	ND
pH 9.6	-	-	-	+	+	+	ND	ND
Isomer of lactic acid	DL	DL	D	L	L	L	L	L
Peptidoglycan type: *meso*-DAP	-	-	-	-	-	-	-	-
Acid from:								
L- Arabinose	-	-	+	+	+	+	+	-
Gluconate	+	+	-	+	+	+	+	-
Glycerol	w	ND	ND	w	w	-	-	+
Lactose	-	-	+	+	+	+	+	+
D-Mannitol	-	-	+	w	w	+	+	+
D-Melibiose	-	-	+	w	w	w	-	-
D-Melezitose	-	-	ND	-	-	-	-	+
Raffinose	-	-	+	-	-	w	-	-
L-Rhamnose	-	-	-	w	w	w	-	-
D-Ribose	+	+	-	+	+	+	ND	ND
Salicin	-	-	+	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-	-	+
Sucrose	+	+	+	w	w	+	+	-
D-Trehalose	-	-	+	w	w	w	-	+
D-Xylose	+	+	+	w	-	-	-	-

+, positive; w, weak; -, negative; ND, no data. All produced acid from D-cellobiose, esculin, D-fructose, D-galactose and D-glucose. NRIC, NODAI Research Institute Culture Collection, Tokyo, Japan.
[a]Data from Collin et al. (1993); Hammes et al. (1992); Kandler and Weiss (1986); Schillinger et al. (1989); Tanasupawat et al. (1993, 2000).

[b]Data from Facklam and Collins (1989); Tanasupawat et al. (1992b)

Table 28. DNA-DNA relatedness of strains

Strains	% Similarity with labeled strains				
	NRIC 1069 T	NRIC 1067 T	NRIC 0207 T	NRIC 1145 T	TISTR 379T
AP17-1	111.6	19.6			
SR4-2[a]	93.3	30.9			
SR8-1[a]	100.9	53.6			
L. pentosus NRIC 1069T	100	53.3			
L. plantarum NRIC 1067T	38.9	100			
AP2-1			100.5		
W. confusa NRIC 0207 T			100		
PM3-13				83.8	5.5
PM3-14				72.1	2.7
PM4-9				99.6	17.1
E. faecium NRIC 1145 T				100	3.4
E. faecalis TISTR 379T				19.7	100

[a]Data from Tanasupawat et al. (2002).
TISTR,Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

3.3 Study on diacetyl production of lactic acid bacteria

3.3.1 Screening of diacetyl and acetoin-producing LAB

The results showed that 115 isolates which could produce diacetyl/acetoin ranged from 0.01 to 6.49 mM. Homofermenttative rod-shaped strains produced 0.01 to 6.49 mM and the homofermentative coccal strains 0.04 to 5.09 mM as reported in *Lacticoccus lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 125 (Phalip et al., 1994). Furthermore, the heterofermentative rod-shaped and coccal strains produced

diacetyl/cacetoin 0.12 to 3.62 mM and 2.62 to 4.41 mM, respectively. The selected strains SR8-1, SR4-2, PM3-14, AP17-1, PM4-9, AP2-1, PM3-13 were cultivated in 400 ml MMRS broth for 24 hours. As determined by gas-liquid chromatography, their diacetyl productions were 3.25, 3.14, 2.88, 2.79, 2.55, 2.53 and 2.19 mM under static conditions, and were 2.21, 2.23, 2.30, 2.76, 2.03, 2.07 and 2.57 mM under shaken conditions, respectively (Fig. 18A). On other hand, their acetoin productions were 3.26, o.48, 1.05, 0.48, 1.69, 2.34 and 1.25 mM under static conditions, and were 0.45, 0.38, 4.27, 0.56, 2.36, 0.14 and 4.20 mM under shaken conditions, respectively (Fig. 18B).

3.3.2 Study on diacetyl production of lactic acid bacteria

The determination of growth, pH, duacetyl and acetoin of 3 selected strains in 400 ml MMRS broth revealed that diacetyl production was found at the exponential phase (Figs. 19, A and B, 20 A and B, 21, A and B). All selected strains could produce the maximum of diacetyl concentration at 18 to 36 hours of fermentation time under stationary phase as reported by Ray (1992). *L. pentosus* SR4-2 could produce 2.97 mM diacetyl under shaken condition and 3.35 mM under static condition while the pH decreased to 5.81 and 5.74 mM after 30 hours incubation, respectively (Fig. 19, A and B). In addition, *L. pentosus* SR8-1 produced 2.35 mM diacetyl under shaken conditions and 3.25 mM under static conditions while the pH decreased to 5.84 and 6.48 after 36 and 24 hours incubation, respectively and diacetyl production of *L. pentosus* AP17-1 did not differ between conditions. *W. confusa* AP2-1 produced 2.84 mM diacetyl under shaken condition and 3.16 mM under static conditions while the pH increased to 7.27 and 7.51 after 36 and 30 hours incubation, respectively (Fig. 20, A and B). *E. faecium* PM3-14 produced 2.30 mM diacetyl under shaken conditions while the pH increased to 7.96 after 24 hours incubation and 3.27 mM under static conditions while the pH decreased to 6.41 after 18 hours incubation, respectively (Fig. 21 A and B). *E. faecium* PM3-13 produced 2.57 mM diacetyl under shaken conditions while the pH increased to 7.94 after 24 hours incubation and 2.98 mM under static conditions while the pH increased to 7.0 after 36 hours incubation. *E. faecium* PM4-9 produced 2.46 mM diacetyl under shaken conditions

while the pH increased to 7.55 after 18 hours incubation and 3.04 mM under static conditions while the pH increased to 6.96 after 30 hours incubation, respectively

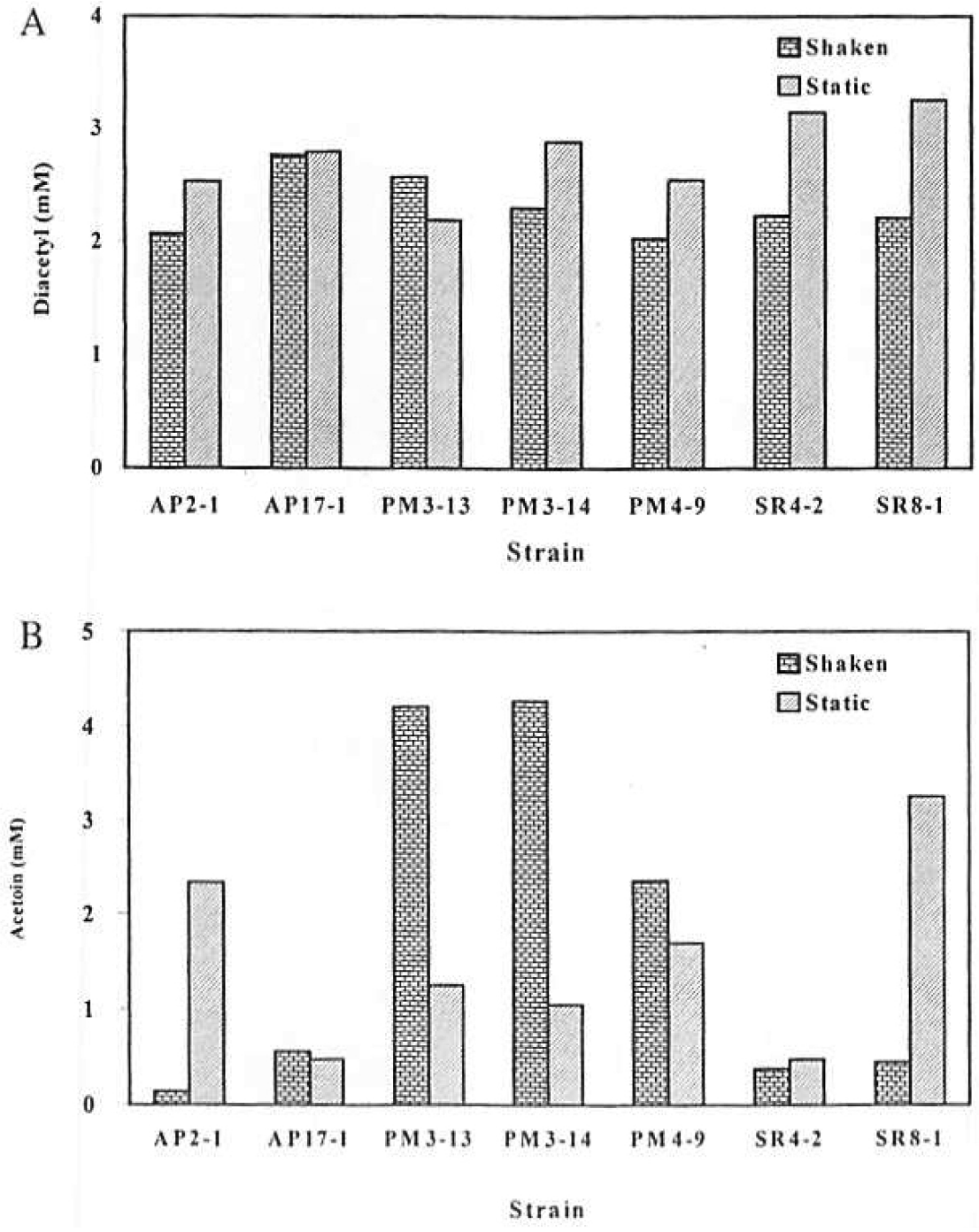
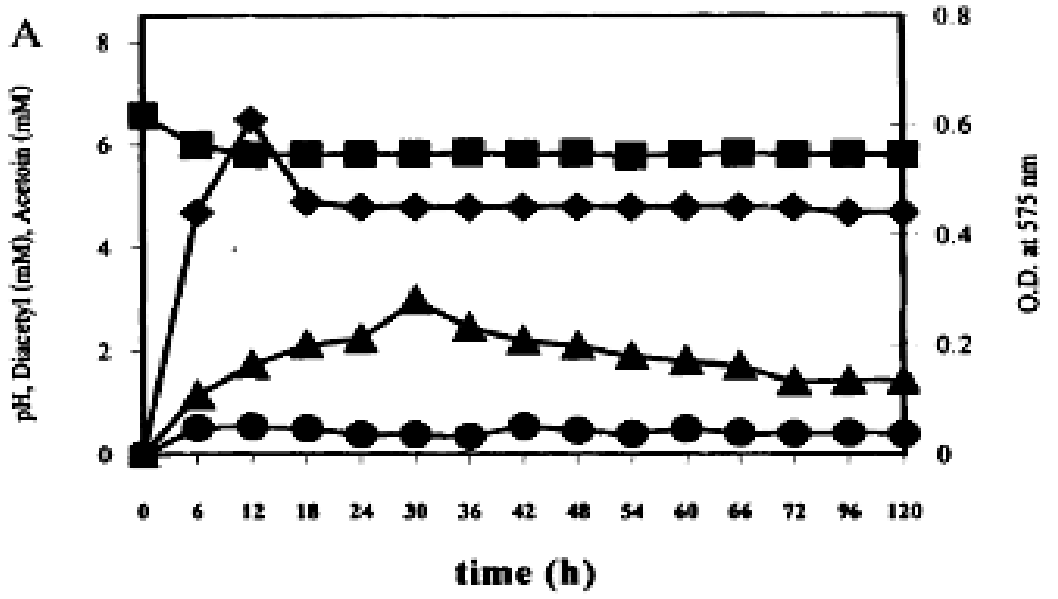


Fig. 18. Diacetyl production (A) and acetoin production (B) of selected strains under shaken and static conditions at 30 ºC for 24 hours.

On the other hand, SR4-2, AP2-1 and PM3-14 strains could produce 0.54, 0.49 and 4.59 mM acetoin under shaken conditions after 12, 36 and 120 hours incubation while their productions were 0.89, 3.82 and 1.88 mM under static conditions after 66, 48 and 60 hous incubation, respectively (Figs. 19, A and B, 20, A and B, 21, A and B). In addition, the strains SR8-1, AP17-1, PM3-13 and PM4-9 could produce 0.67, 0.75, 4.20 and 2.36 mM acetoin under shaken conditions after 48, 30, 24 and 24 hours incubation, while their productions were 6.36, 1.18, 2.03 and 1.84 mM under static conditions after 96, 42, 60 and 36 hours incubation, respectively. As mentioned above, most of homofermentative strains could produce more diacetyl than the heterofermentative strains did as reported previously (Christensen and Pederson, 1958). Moreover, all selected strains could produce high diacetyl concentration in the medium containing citrate under static conditions, while was different from previous reports (Boumerdassi et al., 1996; Kaneko et al., 1990, 1991). However, most lactic acid bacteria produced a high amount of diacetyl in the medium without citrate under aerobic conditions (Kaneko et al., 1990, 1991). Therefore, the diacetyl metabolism and enzyme activities cultivated in the medium with citrate of these strains should be further studied.

In fermented milk, the aroma and flavor are basically due to the production of nonvolatile and volatile acids and carbonyl compounds by starter cultures. Diketones, 2,3-butanedione and 2,3-pentanedione belong to the key aroma compounds and 2,3-butanedione can be reduced to 2,3-butanediol through acetoin (Beshkova et al., 2003). In Thailand, *L. pentosus* SR4-2 and SR8-1 were isolated from soy sauce mash (Tanasupawat et al., 2002), *L. pentosus* AP17-1 from fermented fish (*pla-ra*), *W. confuse* AP2-1 from pork sausage (*mu-yor*), and *E. faecium* PM3-13, PM3-14 and PM4-9 were isolated from pasteurized milk. Their diacetyl production as flavor will be a role other than that of lactic acid fermentation in foods.


A
pH, Diacetyl (mM), Acetoin (mM)
O.D. at 575 nm
0
2
4
6
8
0
0.2
0.4
0.6
0.8
0 6 12 18 24 30 36 42 48 54 60 66 72 96 120
time (h)

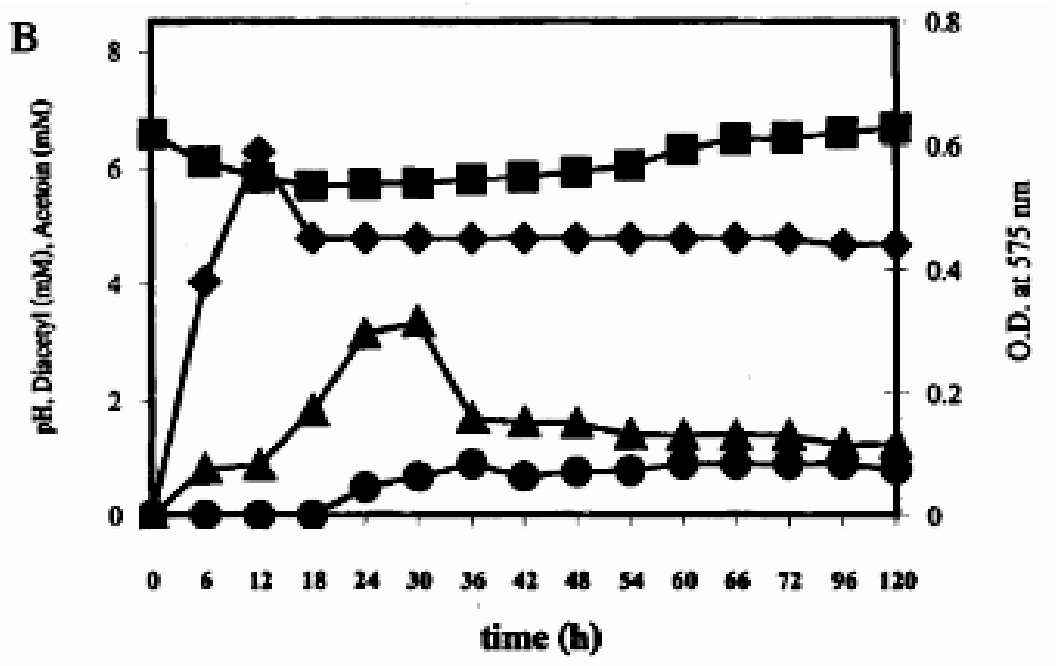

B
pH, Diacetyl (mM), Acetoin (mM)
O.D. at 575 nm
0
2
4
6
8
0
0.2
0.4
0.6
0.8
0 6 12 18 24 30 36 42 48 54 60 66 72 96 120
time (h)

Fig. 19. Relation among ♦growth, ■pH, ▲diacetyl (mM) and ●acetoin (mM) production of *Lactobacillus pentosus* SR4-2 under shaken condition (A) and static condition (B) at 30 °C for 120 hours.

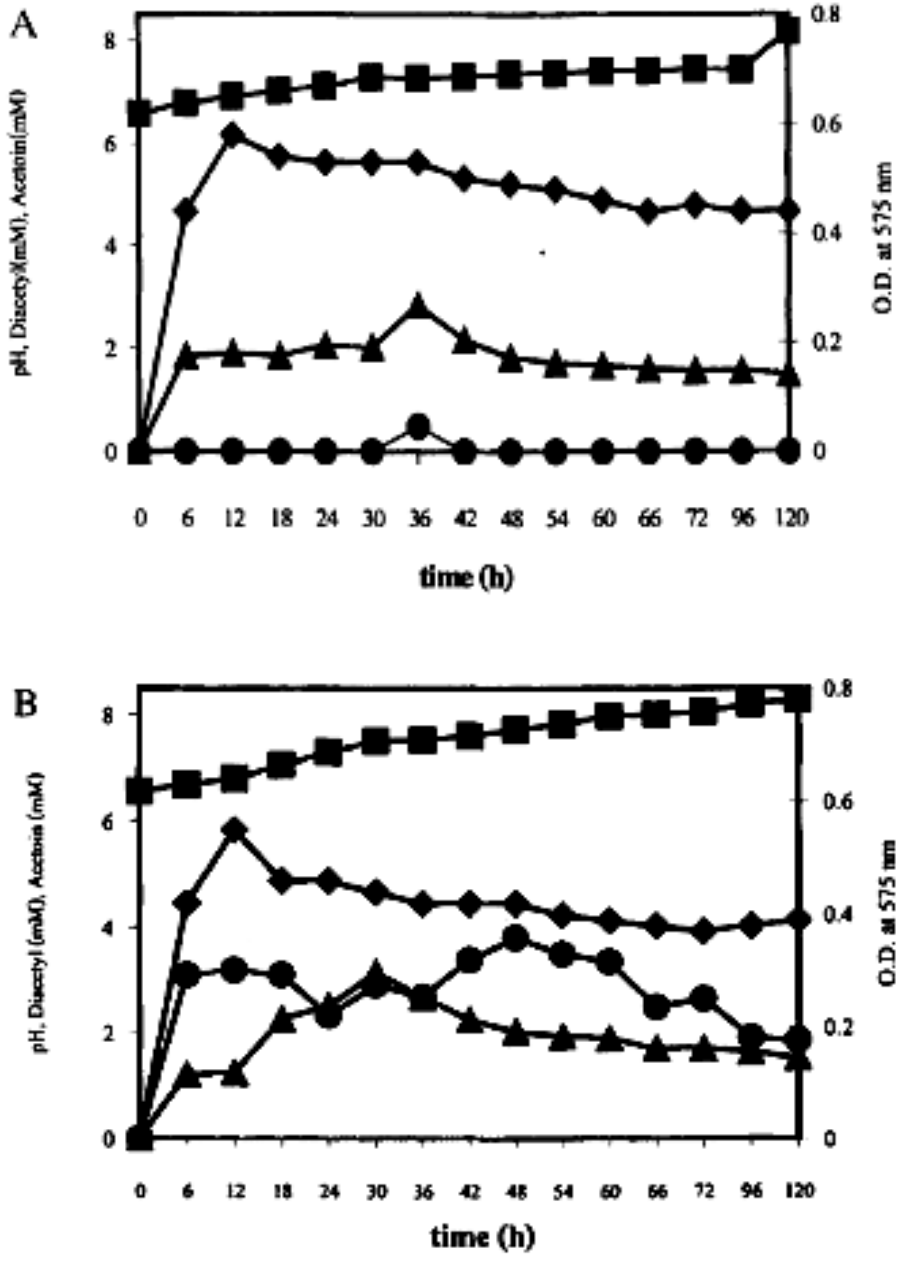


Fig. 20. Relation among ♦growth, ■pH, ▲diacetyl (mM) and ●acetoin (mM) production

of *Weissella confusa* AP2-1 under shaken condition (A) and static condition (B) at 30 ºC for 120 hours.

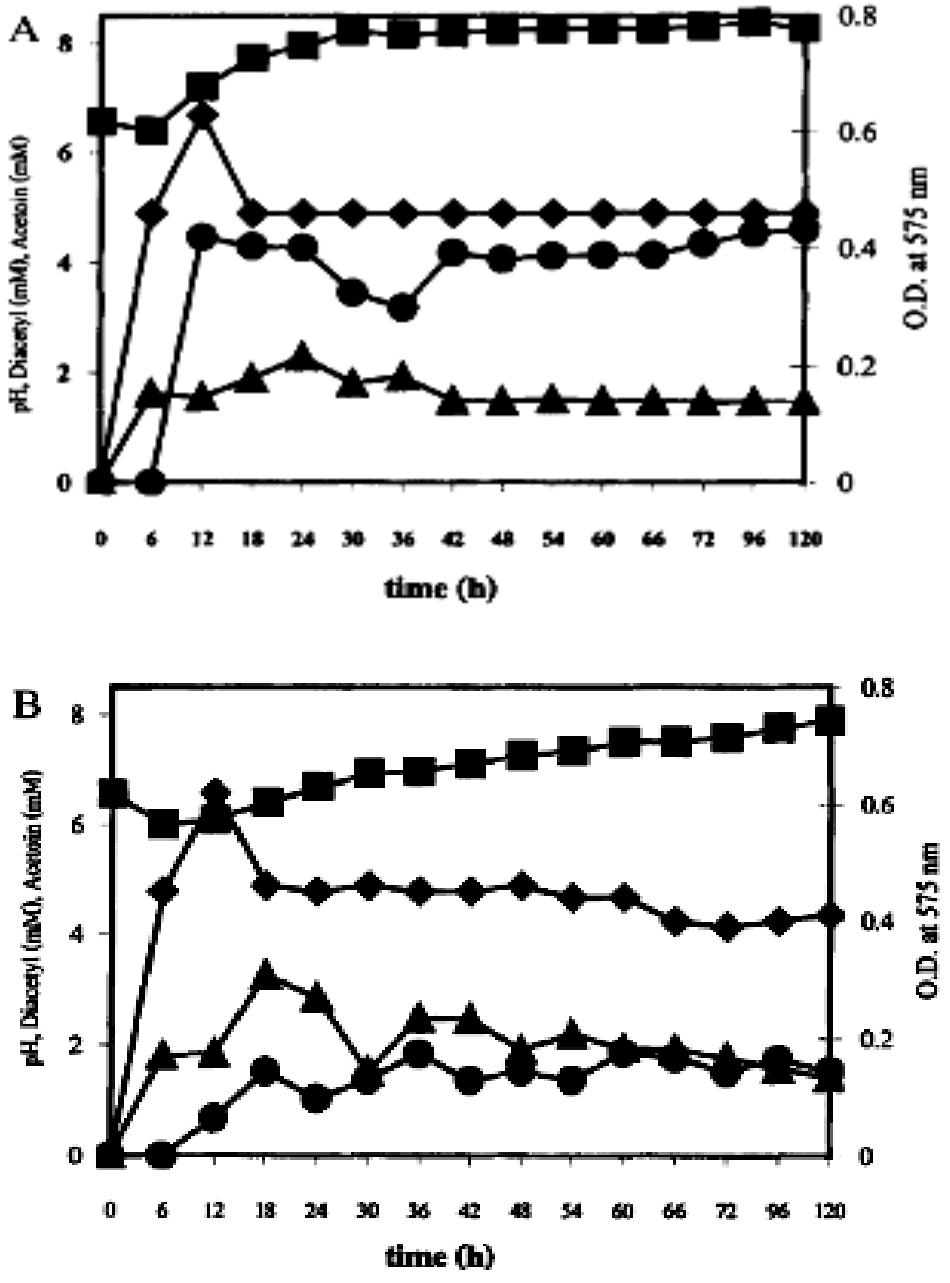


Fig. 21. Relation among ♦growth, ■pH, ▲diacetyl (mM) and ●acetoin (mM) production of *Enterococcus faecium* PM3-14 under shaken condition (A) and static condition (B) at 30 ^{o}C for 120 hours.

CHAPTER IV

CONCLUSION

1. Seventeen strains of halophilic lactic acid bacteria isolated from fermented shrimp paste (*ka-pi*) produced in the southern part of Thailand were belong to the genus *Tetragenococcus* base on the morphological, culture, physiological and biological characteristics. On the basis of DNA-DNA similarity and growth on 5% and 10% NaCl were divided into 2 groups, the representative strain in Group I showed high degree of similarity over 83.6 % and high growth at 5% NaCl with *Tetragenococcus halophilus* ATCC 33315^{T} and were identified as *T. halophilus*. The representative strain in Group II showed high degree of similarity over 94.7 % and high growth at 10% NaCl with *Tetragenococcus muriaticus* JCM 10006^{T} and were identified as *T. muriaticus.* Therefore, the characteristics of phenotypic, DNA-DNA similarity and growth on 5% and 10% NaCl were useful method for identified of *Tetragenococcus* species.
2. Thirty-two strains of halophilic bacteria isolated from *Ka-pi* produced in the southern part of Thailand and fish sauce (*nam-pla*) collected in Thailand at various stages of the fieh-fermentation process were belong to the genus *Lentibacillus* and *Salinicoccus*. These strains were divided into 3 groups, on the basis of phenotypic, chemotaxonomic characteristics, DNA-DNA relatedness and 16S rDNA sequencing.The representative rods strain isolated from *Nam-pla* in Group I (15 strains) were included in the same species on the basis that the levels of DNA-DNA relatedness with type strain PS11-2^{T} were greater than 70%. They could be distinguished from *Lentibacillus juripiscarius* JCM 12147^{T}and other *Lentibacillus* species on the basis of several phenotypic characteristics and low level of DNA-DNA relatedness (≤19.4 %). Therefore, the 15 strains represent a novel species of the genus *Lentibacillus,* for which the name *Lentibacillus halophilus* sp. nov. is proposed. The representative rods strain isolated from *Ka-pi* in Group II (2 strains) could produced a red pigment. They were included in the same species on the basis that the levels of DNA-DNA relatedness with type strain PN7-6^{T} were greater than 70%. Comparative 16S rRNA gene

sequence analyses showed that type strain PN7-6^T was most closely related to *Lentibacillus salarius* KCTC 3911^T with 96.5% sequence similarity. On the basis of phenotypic and molecular properties, the two isolates represent a novel species of the genus *Lentibacillus,* for which the name *Lentibacillus kapialis* sp. nov. is proposed. In addition, the representative cocci strain isolated from *Ka-pi* in Group III (15 strains) could produced orange pigment. They were included in the same species on the basis that the levels of DNA-DNA relatedness with type strain PN1-2^T were greater than 70%. Comparative 16S rRNA gene sequence analyses showed that type strain PN1-2^T was most closely related to *Salinicoccus roseus* JCM 14630^T with 97.3% sequence similarity. On the basis of phenotypic and molecular properties, the 15 strains represent a novel species of the genus *Salinicoccus,* for which the name *Salinicoccus siamensis* sp. nov. is proposed.

3. Twenty-five strains of lactic acid bacteria were isolated from fermented tea leaves (*miang*) produced in the northern part of Thailand were belonged to 7 groups, on the basis of phenotypic, chemotaxonomic characteristics, DNA-DNA relatedness and 16S rDNA sequencing. The isolates were placed in a monophyletic cluster consisting of *Lactobacillus* and *Pediococcus* species. Group I to Group VI belonged to *Lactobacillus.* Group I (6 strains) were identified as *Lactobacillus pantheris*, Group II (5 strains) as *Lactobacillus pentosus,* Group V (4 strains) as *Lactobacillus suebicus* and Group VI (2 trains) as *Lactobacillus fermentum*, while, three new species such as Group IV (2 strains) were most closely related to *Lactobacillus manihotivorans* LMG 18010^T with 96.1% sequence similarity and showed a low degree of DNA-DNA similarity (less than 28.5%) to *L. manihotivorans* JCM 12514^T (=LMG 18010^T). Therefore a new species, *Lactobacillus camelliae* sp. nov., is proposed. Group III (5 strains) were most closely related to *Lactobacillus pantheris* LMG 21017^T with 98.7% sequence similarity and showed a low degree of DNA-DNA similarity (less than 12.5%) to *L. pantheris* NRIC 0613^T. Therefore a new species, *Lactobacillus thailandensis* sp. nov., is proposed. and Group VII (1 strains) was most closely related to *Pediococcus cellicola* Z-8^T with 96.8% sequence similarity and showed

differentiated from *P. cellicola* Z-8^{T} by acid production from L-arabinose, maltose, D-ribose, sucrose, and D-xylose including DNA G+C content (42 mol%). Therefore a new species, *Peddiococcus siamensis* sp. nov., is proposed.

4. The distribution of lactic acid bacteria in milk and fermented foods in Thailand, the most of lactic acid bacteria were found cocci shape in pasteurized milk and raw cow's milk, on the other hand, rods shape usually found in fermented foods. In addition, homofermentative lactic acid bacteria could produce more diacetyl than the heterofermentative lactic acid bacteria. The seven selected strains of high diacetyl-producing lactic acid bacteria such as *Enterococcus faecium* (PM3-14, PM3-12 and PM4-9) were found distribution and role in pasteurized milk, some fermented foods such as *Lactobacillus pentosus* (SR4-2 and SR8-1) were found in soy sauce mash and *L. pentosus* (AP17-1) found in fermented fish (*pla-ra*), and *Weissella confusa* AP2-1 found in pork sausage (*mu-yor*).
5. Diacetyl production of *Lactobacillus pentosus* SR4-2, *Weissella confusa* AP2-1 and *Enterococcus faecium* PM3-14 were found at the exponential phase within 6 hours. All strains could produce the maximum of diacetyl concentration at 18 to 36 hours of fermentation time under stationary phase and showed the maximum growth at 12 hours.
6. The selected strains could produce high diacetyl concentration in the medium containing citrate under static conditions, which was different from previous reports by under static condition, *L. pentosus* SR4-2, *E. faecium* PM3-14 and *W. confusa* AP2-1 could produce high diacetyl equal to 3.35, 3.27 and 3.16 mM at pH 5.74, 6.41 and 7.51 after incubation 30, 18 and 30 hours respectively, while under shaken condition, *L. pentosus* SR4-2, *E. faecium* PM3-14 and *W. confusa* AP2-1 could produce high acetoin equal to 0.54, 4.59 and 0.49 mM after incubation 12, 120 and 36 hours respectively.

REFERENCES

1. Albert, R. A., Archambault, J., Rosselló-Mora, R., Tindall, B. J. and Matheny, M. 2005. *Bacillus acidicola* sp. nov., a novel mesophilic, acidophilic species isolated from acidic *Sphagnum* peat bogs in Wisconsin. Int. J. Syst. Evol. Microbiol.55: 2125-2130.
2. Anonymous. 1994. Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List no. 49. Int. J. Syst. Bacteriol. 44: 370-371.
3. Appleton, J. M., Mc. Gowan, V., and Skerman, V. B. D. 1978–1980. Microorganisms and Man, World Data Center (MIRCEN), Department of Microbiology, University of Queensland, Brisbane, Australia.
4. Arahal, D. R., Marquez, M. C., Volcani, B. E., Schleifer, K. H. and Ventosa, A. 2000. Reclassification of *Bacillus marismortui* as *Salibacillus marismortui* comb. nov. Int. J. Syst. Evol. Microbiol. 50: 1501-1503.
5. Aslam, Z., Lim, J. H., Im, W.-T., Yasir, M., Chung, Y. R. and Lee, S.-T.2007. *Salinicoccus jeotgali* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. Int. J. Syst. Evol. Microbiol. 57: 633–638.
6. Axelsson, L. 1993. Lactic acid bacteria : classification and physiology, pp. 1-63. *In* S. Salminen and A. von Wright (eds.). Lactic Acid Bacteria . Marcel Dekker, Inc., New York.
7. Axelsson, L. 1998. Lactic acid bacteria : classification and physiology, pp. 1-72. *In* S. Salminen and A. von Wright (eds.). Lactic Acid Bacteria : Microbiology and Function Aspects. Marcel Dekker, Inc., New York.
8. Back, W. 1978. Zur Taxonomie der Gattung *Pediococcus*. Phänotypische und genotypische Abgrenzung der bisher bekannten Arten sowie Beschreibung einer neuen bierchä dlichen Art: *Pediococcus inopinatus*. Brauwissenschaft. 31: 237–250, 312–320 (in German).

9. Barrow, G. I. and Feltham, R. K. A. 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria, 3rd edn.Cambridge: Cambridge University Press.
10. Bassit, N., Boguien, C.Y. Picque, D., and Corrieu, G. 1993. Effect of initial oxygen concentration on diacetyl and acetoin production by *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. Appl. Environ. Microbiol. 59: 1893-1897.
11. Beshkova, D. M., Simova, E. D., Frengova, G. I., Simov, Z. I., and Dimitrov, Zh. P. 2003. Production of volatile aroma compounds by kefir starter cultures. Int. Dairy J. 13: 529-535.
12. Boumerdassi, H., Desmadzeau, M., Monnet, C., Boquien, C.Y., and Corrieu, G. 1996. Improvement of diacetyl production by *Lactococcus lactis* subsp.*lactis* CNRZ483 through oxygen control. J. Dairy Sci.79: 775-781.
13. Boumerdassi, H., Monnet, C., Desmazeau, M.,and Corrieu, G. 1997. Isolation and properties of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* CNRZ483 mutants producing diacetyl and acetoin from glucose. Appl. Environ. Microbiol. 63: 2293-2299.
14. Cathy, J. 1994. Lactic acid bacteria. *Danone world newsletter*. Vol. 5.
15. Chen, Y.-G., Cui, X.-L., Pukall, R., Li, H.-M., Yang, Y.-L., Xu, L.-H., Wen, M.-L., Peng, Q. and Jiang, C.-L. 2007. *Salinicoccus kunmingensis* sp. nov., a moderately halophilic bacterium isolated from a salt mine in Yunnan, south-west China. Int. J. Syst. Evol. Microbiol. 57: 2327–2332.
16. Christensen, M. D., and Pederson, C. S. 1958. Factors affecting diacetyl production by lactic acid bacteria. Appl. Microbiol**.** 6: 319-322.
17. Collins , M. D., Wiliam, A. M., and Wallbanks, S. 1990. The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis : description of *Tetragenococcus* gen. nov. FEMS Microbiol Lett**.** 70: 255- 262.
18. Collins, M. D., Samelis, J., Metaxopoulos, J., and Wallbanks, S. 1993. Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteriodes* group of species. J. Appl. Bacteriol. 75: 595-603.

19. Cogan, T. M. 1981. Constitutive nature of the enzymes of citrate metabolism in *Streptococcus lactis* subsp. *diacetylactis*. J. Dairy Sci. 48: 489-495.
20. Condon, S. 1987. Responses of lactic acid bacteria to oxygen. Microbiol. Rev. 46: 269-280.
21. Coster, E. and White, H. R. 1964. Further studies of the genus *Pediococcus*. J. Gen. Microbiol. 37: 15-31.
22. De Man, J. C., Rogosa, M., and Sharpe, M. E. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23: 130–135.
23. Dellaglio, F., Trovatelli, L. D. and Sarra, P. G. 1981. DNA-DNA homology among representative strains of the genus *Pediococcus* . Zbl. Bakt. Hyg. , I. Abt. Orig. 2: 140-150.
24. Devriese, L. A., Collins, M. D., and Wirth, R.1991. The genus *Enterococcus. In* The Prokaryotes, Vol. I. ed. by Balows, A., Trüper, H. G., Dworkin, M., Harder, W., and Schleifer, K. H., Springer-Verlag, New York, pp. 1465-1481.
25. Dicks, L. M. T., F. Dellaglio and M. D. Collins. 1995.Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [Corrig.] gen. nov. Int. J. Syst. Bacteriol. 45: 395-397.
26. Dicks, L. M. T., Du. Plessis, E. M., Dellaglio, F., and Lauer, E. 1996. Reclassification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15280 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*. Int. J. Syst. Bacteriol. 46: 337–340.
27. Ennahar, S., and Cai, Y. 2005. Biochemical and genetic evidence for the transfer of *Enterococcus* solitaries Collins et al. 1989 to the genus *Tetragenococcus* as *Tetragenococcus solitaries* comb. nov.Int. J. Syst. Evol. Microbiol. 55: 589-592.
28. Ezaki, T., Hashimoto, Y. and Yabuuchi, E. 1989. Fluorometric deoxyribonucleic acid- deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol. 39: 224-229.

29. Facklam, R. R. and Collins, M. D. 1989. Identification of *Enterococcus* species isolated from human infection by a convention test scheme. J. Clin. Microbiol. 27: 731-734.
30. Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39: 783-791.
31. Fisher, K. and Phillips, C. 2009. The ecology, epidemiology and virulence of *Enterococcus.* Microbiology 155 (Pt 6): 1749–57
32. Forbes, L. 1981. Rapid flagella stain. *J Clin Microbiol.* 13: 807-809.
33. Franca, L., Rainey, F. A., Nobre, M. F. and da Costa, M. S. 2006. *Salinicoccus salsiraiae* sp. nov.:a new moderately halophilic gram-positive bacterium isolated from salted skate. Extremophiles. 10: 531-536.
34. Fungsin, B. 2014. An overview of traditional fermented foods of Thailand and their microflora promoting to health benefits. Seminar in Asian fermented foods: The sources of novel functional foods. 17-18 July, Thailand Institute of Scientific and Technological Research (TISTR), Pathumtani, Thailand.
35. Grant, W. D. and Larsen, H. 1989. Extremely halophilic archaeobacteria, order Halobacteriales ord. nov. In *Bergey's Manual of Systematic Bacteriology*, vol. 3. pp.2216-2233. Edited by J. T. Staley, M. P. Bryant, N. Pfenning and J. G. Holt. Baltimore: Williams and Wilkins.
36. García, M. T., Gallego, V., Ventosa, V. and Mellado, E. 2005. *Thalassobacillus devorans* gen. nov.,sp. nov.,a moderately halophilic, phenol-degrading, Gram-positive bacterium. *Int J Syst Evol Microbiol.* 55: 1789-1795.
37. Garvie, E. I. 1986. Genus *Pediococcus*, In *Bergey' s Manual of Systematic Bacteriology*. Vol. 2, ed. by Sneath, P. H. A., Mair, N. S., Sharpe, M. E. and Holt., J. G., The Williams & Wilkins, Baltimore, pp. 1075-1079.
38. Gasson, M. J. 1983. Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. 154, 1-9.
39. Gilmore M. S. 2002. *The Enterococci:* Pathogenesis, Molecular Biology, and Antibiotic Resistance. Washington, D.C.: ASM Press

40. Gould, G. W. 1995. New methods of food preservation. Britain: Chapman and Hall.
41. Haakensen, M; Dobson, C. M; Hill, J. E; Ziola, B. 2009. Reclassification of Pediococcus dextrinicus (Coster and White 1964) back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus *Lactobacillus*. Int. J. Syst. Evol. Microbiol. **59** (3): 615–621.
42. Hammes, W. P., Weiss, N., and Holzapfel, W. 1992. The genus *Lactobacillus* and *Carnobacterium*. *In* The Prokaryotes, Vol. II, ed. by Ballows, A., Trüper, H. G., Dworkin, M., Harder, W., and Schleifer, K. H., Springer-Verlag, New York, pp. 1536–1594.
43. Hao, M. V., Kocur, M. and Komagata, K. 1984. *Marinococcus* gen. nov., a new genus for motile cocci with *meso*-diaminopimelic acid in the cell wall; and *Marinococcus albus* sp. nov. and *Marinococcus halophilus* (Novitsky and Kushner) comb. nov. J. Gen. Appl. Microbiol. 30: 449-459.
44. Herbin, S., Mathieu, F., Brule, F., Branlant, C., Lefebvre, G., and Lebrihi, A. 1997. Characteristics and genetic determinants of bacteriocin activities produced by Carnobacterium pisciola CP5 isolated from cheese. Current Microbiology 35: 319-326.
45. Heydrickx, M., Lebbe, L., Kersters, K., De Vos., P., Forsyth, G. and Logan , N. A. 1998. *Virgibacillus*: a new genus to accommodate *Bacillus panthothenticus* (Proom and Knight 1995). Emended description of *Virgibacillus panthothenticus*. Int. J. Syst. Bacteriol. 48: 99-106.
46. Heyrman, J., Logan, N. A., Busse, H-J., Balcaen, A., Lebbe, L., Rodriguez-Diaz, M., Swings, J., and De Vos, P. 2003. *Virgibacillus carmonensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus picturae* sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of genus *Salibacillus* to *Virgibacillus*, as *Virgibacillus marismortui* comb. nov. and *Virgibacillus salexigens* comb. nov., and emended description of genus *Virgibacillus*. Int. J. Syst. Evol. Microbiol. 53: 501-511.

47. Hucker, G. J. and Conn, H. J. 1923. Method of Gram staining. N Y Sate Agric Exp Stn Tech Bull. 93: 3-37.
48. Hugenholtz, J. 1993. Citrate metebolism in lactic acid bacteria. FEMS Microbiol. Rev.12: 165-178.
49. Hugenholtz, J., and M. J. C. Starrenburg. 1992. Diacetyl production by difference strains of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc spp*. Appl. Microbiol. Biotechnol**.** 38: 17-22.
50. Ishikawa, M., Ishizaki, S., Yamamoto, Y. and Yamasato, K. 2002. *Paraliobacillus ryukyuensis* gen. nov., sp. nov., a new Gram-positive, slightly halophilic, extremely halotolerant, facultative anaerobe isolated from a decomposing marine alga. J. Gen. Appl. Microbiol. 48: 269-279.
51. Ishikawa, M., Nakajima, K., Itamiya, Y., Furukawa, S., Yamamoto, Y. and Yamasato, K. 2005. *Halolactibacillus halophilus* gen. nov., sp. nov. and *Halolactibacillus miurensis* sp. nov., halophilic and alkaliphilic marine lactic acid bacteria constituting a phylogenetic lineage in *Bacillus* rRNA group 1. Int. J. Syst. Evol. Microbiol. 55: 2427-2439.
52. Jay, J. M. 1982. Antimicrobial properties of diacetyl. Appl. Environ. Microbiol. 44: 525-532.
53. Jeon, C. O., Lim, J.–M., Lee, G. S., Lee, J.–M., Xu, L.–H., Jiang, C.-L. And Kim, C.-J. 2005a. *Lentibacillus salarius* sp. nov., isolated from saline sediment in China and emended description of genus *Lentibacillus*. Int. J. Syst. Evol. Microbiol. 55: 1339-1343.
54. Jeon, C. O., Lim, J.-M., Lee, J.-M., Xu, L.-H., Jiang, C.-L. and Kim, C.-J. 2005b. Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. Int. J. Syst. Evol. Microbiol. 55: 1891 - 1896.

55. Justé, A., Van Trappen, S., Verreth, C., Cleenwerck, I., De Vos, P., Lievens, B., et al. 2012. Characterization of *Tetragenococcus* strains from sugar thick juice reveals a novel species, *Tetragenococcus osmophilus* sp. nov., and divides *Tetragenococcus halophilus* into two subspecies, *T. halophilus* subsp. *halophilus* subsp. nov. and *T. halophilus* subsp. *flandriensis* subsp. nov. Int. J. Syst. Evol. Microbiol. 62: 129-137.
56. Haakensen, M., Dobson, C. M., Hill, J. E., Ziola, B. 2009. Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. Nov., and emended description of the genus Lactobacillus. Int. J. Syst. Evol. Microbiol. 59 (Pt 3): 615–21.
57. Kämpfer, P. and Kroppenstedt, R. M. 1996. Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. Can. J. Microbiol. 42: 989-1005.
58. Kandler, O. and Weiss, N. 1986. Genus *Lactobacillus* Beijerinck 1901, 212 ^{AL}. *In* Bergey's Manual of Systematic Bacteriology, Vol.2, ed. by Sneath, H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G., Williams & Wilkins, Baltimore, pp. 1209–1234.
59. Kaneko, T., Takashi, M., and Suzuki, H. 1990. Acetoin fermentation by citrate-positive *Lactococcus lactis* subsp. *lactis* 3022 grown aerobically in the presence of hemin or Cu^{+}. Appl. Environ. Microbiol. 56: 2644-2649.
60. Kaneko, T., Watanabe, Y., and Suzuki, H. 1991. Differences between *Lactobacillus casei* subsp. *casei* 2206 and citrate positive *Lactococcus lactis* subsp. *lactis* 3022 in the characteristics of diacetyl production. Appl. Environ. Microbiol. 57: 3040-3042.
61. Karnop, V. G. 1988. Histaminn in salzsardellen. Arch. Lebensmittelhyg. 39: 57-84.
62. Kimura, M. 1980. Simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.
63. Kimura, B., Konagaya, Y., and Fujii, T. 2001. Histamine formation by *Tetragenococcus muriaticus,* a halophilic lactic acid bacterium isolated from fish sauce. Int. J. Food Microbial. **70** (1-2): 71–77.

64. Kleynmans, U., Heinzi, H., and Hammes, W. P. 1989. *Lactobacillus suebicus* sp. nov., an obligately heterofermentative *Lactobacillus* species isolated from fruit mashes. Syst. Appl. Microbiol. 11: 267–271.
65. Kobayashi, T., Kajiwara, M., Wahyuni, M., Kitakado, T., Hamada-Sato, N., Imada, C., and Watanabe, E. 2003. Isolation and characterization of halophilic lactic acid bacteria isolated from "terasi" shrimp paste: A traditional fermented seafood product in Indonesia. J. Gen. Appl. Microbiol. 49: 279-286.
66. Komagata, K. and Suzuki, K. 1987. Lipids and cell wall analysis in bacterial systematics. Methods Microbiol. 19: 161-207.
67. Kumar, S., Tamura, K., Jakobson, I. -B. and Nei, M. 2001. MEGA 2: molecular evolutionary genetics analysis software, Bioinformatics 17, 1244-1245.
68. Lee, N. K., and Paik, H. D. 2001. Partial characterization of lacticin NK24, a newly identified bacteriocin of *Lactococcus lactis* NK24 isolated from Jeot-gal. Food Microbiology 18: 17-24.
69. Lee, M., Kim, M. K., Vancanneyt, M., Swings, J., Kim, S.-H., Kang, M. S., and Lee, S. –T. 2005. *Tetragenococcus koreensis* sp. nov., a novel rhammolipid-producing bacterium. Int. J. Syst. Evol. Microbiol. 55: 1409-1413.
70. Lee, S.-Y., Choi, W.-Y., Oh, T.-K. and Yoon, J.-H. 2008a. *Lentibacillus salinarum* sp. nov., isolated from a marine solar saltern in Korea. Int. J. Syst. Evol. Microbiol. 58: 45–49.
71. Lee, J.-C., Li, W.-J, Xu, Li.-H , Jiang, C.-L, and Kim, C.-J. 2008b. *Lentibacillus salis* sp. nov., a moderately halophilic bacterium isolated from a salt lake. Int. J. Syst. Evol. Microbiol. 58: 1838–1843.
72. Leifson, E. 1963. Determination of carbohydrate metabolism of marine bacteria. J. Bacteriol. 85: 1183-1184.
73. Li, W. –J., Schumann, P., Zhang, Y. –Q., Chen, G. Z., Tian, X. –P., Xu, L. –H., Stackebrandt, E. and Jiang, C. –L. 2005. *Marinococcus halotolerans* sp. nov., isolated from Qinghai, north-west China. Int. J. Syst. Evol. Microbiol. 55: 1801-1804.

74. Lim, J. -M., Jeon, C. O., Song, S. M. and Kim, C. –J. 2005a. *Pontibacillus chungwhensis* gen. nov., sp. nov., a moderately halophilic Gram-positive bacterium from a solar saltern in Korea. Int. J. Syst. Evol. Microbiol. 55: 161-170.
75. Lim, J. -M., Jeon, C. O., Song, S. M., Lee, J-C., Ju, Y. J., Xu, L. H., Jiang, C. –L. and Kim, C. –J. 2005b. *Lentibacillus lacisalsi* sp. nov., a moderately halophilic bacterium isolated from a saline lake in China. Int. J. Syst. Evol. Microbiol. 55: 1805-1809.
76. Liu, B. and Dong, X. 2002. *Lactobacillus pantheris* sp. nov., isolated from faeces of a jaguar. Int. J. Syst. Evol. Microbiol. 52: 1745–1748.
77. Lu, J., Nogi, Y. and Takami, H. 2001. *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. FEM Microbiol. Lett. 205: 291-297.
78. Marquez, M. C., Ventosa, A. and Ruiz-Berraquero, F. 1990. *Marinococcus hispanicus*, a new species of moderately halophilic gram-positive cocci. Int. J. Syst. Bacteriol. 40: 165-169.
79. Mattessich, J., and Cooper, J. R. 1989. The spectrophotometric determination of diacetyl. Anal. Biochem. 180: 349 –350.
80. Minnikin, D. E., O' Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. and Parlett, J. H. 1984. An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipid. J. Microbiol. Methods. 2: 233-241.
81. Mishara, C., and Lambert, J. 1996. Production of anti-microbiology substances by probiotics. Asia Pacific J. Clin. Nutrition 5: 20-24.
82. Monnet, C., Phalip, V., Schmitt, P., and Divies, C. 1994. Comparison of α-acetolactate synthase and α-acetolactate decarboxylase in *Lactococcus* spp. and *Leuconostoc* spp. Biotechnol. Lett. 16: 257-262.
83. Morlon-Guyot, J., Guyot, J. P., Pot, B., Jacobe de Haut, I., and Raimbault, M. 1998. *Lactobacillus manihotivorans* sp. nov., a new starch-hydrolysing lactic acid bacterium isolated during cassava sour starch fermentation. Int. J. Syst. Bacteriol. 48: 1101–1109.

84. Nakagawa, A. and Kitahara, K. 1959. Taxonomic studies on the genus *Pediococcus*. J. Gen. Appl. Microbiol. 5: 95-126.
85. Nakamura, K., Haruta, S., Ueno, S., Ishii, M., Yokota, A. and Igarashi, Y. 2004. *Cerasibacillus quisquiliarum* gen. nov., sp. nov. isolated from a semi-continuous decomposing system of kitchen refuse. Int. J. Syst. Evol. Microbiol. 54: 1063-1069.
86. Namwong, S., Tanasupawat, S., Smitinont, T., Visessanguan., W., Kudo, T. and Itoh, T. 2005. Isolation of *Lentibacillus salicampi* strains and *Lentibacillus juripiscarius* sp. nov. from fish sauce in Thailand. Int. J. Syst. Evol. Microbiol. 55: 315-320.
87. Niimura, Y., Koh, E., Yanagida, F., Suzuki, K.-I., Komagata, K. and Kozaki, M. 1990. *Amphibacillus xylanus* gen. nov., sp. nov., a facultatively anaerobic sporeforming xylan-digesting bacterium with lacks cytochrome, quinone, and catalase. Int. J. Syst. Bacteriol. 40*:* 297-301.
88. Okada, S., Daengsubha, W., Uchimura, T., Ohara, N., and Kozaki, M. 1986. Flora of lactic acid bacteria in *miang* produced in northern Thailand. J. Gen. Appl. Microbiol. 32: 57–65.
89. Okada, S., Suzuki, Y., and Kozaki, M. 1979. A new heterofermentative *Lactobacillus* species with *meso*-diaminopimelic acid in peptidoglycan,*Lactobacillus vaccinostercus* Kozaki and Okada sp. nov. J. Gen. Appl. Microbiol. 25: 215–221.
90. Okada, S., Toyoda, T., and Kozaki, M. 1978. An easy method for the determination of the optical types of lactic acid produced by lactic acid bacteria. Agric. Biol. Chem. 42: 1781–1783.
91. Pavia, D. L., Gary M. L., George S. K. and Randall G. E. 2006. Introduction to Organic Laboratory Techniques: A Microscale Approach, 4th ed. Belmont, CA: Cengage Learning.
92. Phalip, V., Schemitt, P., and Dives, C. 1994. A method for screening diacetyl and acetoin – producing bacteria on agar plates. J. Basic Microbiol. 34: 277 – 280.
93. Phithakpol, B., Varanyanond, W., Reungmaneepaitoon, S. and Wood, H. 1995. *The Traditional Fermented Foods of Thailand.* Kuala Lumpur: ASEAN Food Handling Bureau Level 3.

94. Phithakpol, B., Varanyanond, W., Reungmaneepaitoon, S., and Wood, H. 1995. *The Traditional Fermented Foods of Thailand,* Institute of Food Research and Product Development, Kasetsart University, Bangkok, 157 pp.
95. Ray, B. 1992. Diacetyl of lactic acid bacteria as a food biopreservative. *In* Food Biopreservatives of Microbial Origin, ed. by Ray, B. and Daeschel, M., CRC Press, Boca Raton, pp. 137-151.
96. Ren, P. -G. and Zhou, P. -J. 2005a. *Tenuibacillus multivorans* gen. nov., sp. nov., a moderately halophilic bacterium isolated from saline soil in Xin-Jiang, China. Int. J. Syst. Evol. Microbiol. 55: 95-99.
97. Ren, P. -G. and Zhou, P. -J. 2005b. *Salinibacillus aidingensis* gen. nov., sp. nov., and *Salinibacillus kushneri* sp. nov., moderately halophilic bacteria isolated from a neutral saline lake in Xin-Jiang, Chaina. Int. J. Syst. Evol. Microbiol. 55: 949-953.
98. Ryan, K. J., Ray, C. G. 2004. Sherris Medical Microbiology (4th ed.). McGraw Hill. pp. 294–5.
99. Saisithi, P.1994. Traditional fermented fish: fish sauce production. In: Martin, A.M. fisheries processing: biotechnological application. pp. 111-131. (ed). Chapman & Hall, London.
100. Saito, H. and Miura, K. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta. 72: 619-629.
101. Saitou, N. and Nei, M. 1987. The neighboring-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.
102. Sakaguchi, K. and Mori, H. 1969. Comparative study on *Pediococcus halophilus, P. soyae, P. homari, P. urinae-equi* and related species. J. Gen. Appl. Microbiol. 15: 159 -167.
103. Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical Note 101. Newark, DE: MIDI. Inc.
104. Satomi, M., Kimura, B., Mizoi, M., Sato, T. and Fujii, T. 1997. *Tetragenococcus muriaticus* sp. nov., a new moderately halophilic lactic acid bacterium isolated from fermented squid liver sauce. Int. J. Syst. Bacteriol. 47: 832-836.

105. Schillinger, U., and Holzapfel, W., and Kandle, O. 1989. Nucleic acid hybridization studies on *Leuconostoc* and heterofermentative lactobacilli and description of *Leuconostoc amelibiosum* sp. nov . Syst. Appl. Microbiol. 12: 48-55.

106. Schlesner, H., Lawson, P. A., Collins, M. D., Weiss, N., Wehmeyer, U., Volker, H. and Thomm, M. 2001. *Filobacillus milensis* gen. nov.sp. nov., a new halophilic spore-forming bacterium with Orn-D-Glu-type peptidoglycan. Int. J. Syst. Evol. Microbiol. 51: 425-431.

107. Seearunruangchai, A. Tanasupawat, S., Keeratipibul, S., Thawai, C., Itoh, T. and Yamada,Y. 2004. Identification of acetic acid bacteria isolated from fruits and related materials collected in Thailand. J. Gen. Appl. Microbiol. 50: 47-53.

108. Sharpe, M. E. 1981. The genus *Lactobacillus,* In *The Procaryotes,* A Hand book on Habitates, Isolation and Identification of Bacteria M.P. Starr, H.Stolp, H.G. Truper, A. Balows, and H.G. Schlegel, eds., Spring-Verlag, Berlin .pp. 1653-1674.

109. Spring, S., Ludwig, W., Marquez, M. C., Ventosa, A. and Schleifer, K.-H. 1996. *Halobacillus* gen. nov., with description of *Halobacillus litoralis* sp. nov. and *Halobacillus trueperi* sp. nov., and transfer of *Sporosarcina halophila* to *Halobacillus halophilus* comb. nov. Int. J. Syst. Bacteriol. 46: 492-496.

110. Stackebrandt, E., Koch, C., Gvozdiak, O. and Schumann, P. 1995. Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. Int. J. Syst. Bacteriol. 45: 682-692.

111. Tamaoka, J. , S. and Komagata, K. 1984. Determination of DNA base composition by reverse-phase high-performance liquid chromatography. FEMS Microbiol. Lett. 25: 125-128.

112. Tanasupawat, S.. 2014. Selection, identification and preservation of microorganisms, probiotic from fermented foods. Seminar in Asian fermented foods: The sources of novel functional foods. 17-18 July, Thailand Institute of Scientific and Technological Research (TISTR), Pathumtani, Thailand.

113. Tanasupawat, S. 2009. Thai lactic acid bacteria; diversity and applications. SWU Sci. J. 25 (1): 1-13.

114. Tanasupawat, S. and Komagata, K. 1995. Lactic acid bacteria in fermented foods in Thailand. World J. Microbiol. Biotechnol., 11: 253-256.

115. Tanasupawat , S. and Daengsubha, W. 1983. *Pediococcus* species and related bacteria found in fermented foods and related materials in Thailand**.** J. Gen. Appl. Microbiol. 29: 487-506.

116. Tanasupawat, S. and Komagata, K. 1988. DNA base composition, quinone systems, and cellular fatty acid composition of strains in *Pediococcus* species. Bull. JFCC. 4: 63–67.

117. Tanasupawat, S., Okada, S., Suzuki, K., Kozaki, M., and Komagata, K. 1993. Lactic acid bacteria, particularly heterofermentative lactobacilli found in fermented foods in Thailand. Bull. JFCC. 9: 65-78.

118. Tanasupawat, S., and Komagata, K. 2001. Lactic acid bacteria in fermented foods in Southeast Asia In *Microbial Diversity in Asia*. *Technology and Prospects*. pp.43-59. Edited by B. H. Nga, H. M. Tan and K. Suzuki. Singapore: World Scientific Publishing.

119. Tanasupawat, S., Ezaki., T., Suzuki., K., Okada., S., Komagata., K., and Kozaki, M. 1992a. Characterization and identification of *Lactobacillus pentosus* and *Lactobacillus plantarum* strains from fermented foods in Thailand. J. Gen. Appl. Microbiol**.** 38: 121-134.

120. Tanasupawat, S., Okada, S., Suzuki, K., Kozaki, M., and Komagata, K. 1992b. Identification of *Enterococcus hirae, E. faecalis, E. faecium* and *E. casseliflavus* strains from fermented foods. Bull. JFCC. 8: 86-94.

121. Tanasupawat, S., Okada, S., and Komagata, K. 1998. Lactic acid bacteria found in fermented fish in Thailand. J. Gen. Appl. Microbiol. 44: 193–200.

122. Tanasupawat, S., Pakdeeto, A., Namwong, S., Thawai, C., Kudo, T. and Itoh, T. 2006. *Lentibacillus halophilus* sp. nov., from fish sauce in Thailand. Int. J. Syst. Evol. Microbiol. 56: 1859-1863.

123. Tanasupawat, S., Shida, O., Okada, S. and Komagata, K. 2000. *Lactobacillus acidipiscis* sp. nov., and *Weissella thailandensis* sp. nov. isolated from fermented fish in Thailand. Int. J. Syst. Evol. Microbiol. 50: 1479-1485.

124. Tanasupawat, S., Thawai, C., Yukphan, P., Moonmangmee, D., Itoh, T., Adachi, O., and Yamada, Y. 2004. *Gluconobacter thailandicus* sp. nov., an acetic acid bacterium in the a-proteobacteria. J. Gen. Appl. Microbiol. 50: 159–167.

125. Tanasupawat, S., Thongsanit, J., Okada, S., and Komagata, K. 2002. Lactic acid bacteria isolated from soy sauce mash in Thailand. J. Gen. Appl. Microbiol. 48: 201-209.

126. Tanasupawat**,** S. and W. Vissessanguan. 2008. Thai Fermented Foods: Microorganisms and Their Health Benefits In: *Handbook of Fermented Functional Foods* by E. R. Farnworth, 2nd ed., Boca Raton, FL.: CRC Press Taylor & Francis Group, pp.495-511.

127. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. -G. 1997. CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876–4882.

128. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.

129. Thongsanit, J., Tanasupawat, S., Keeratipibul, S., and Jatikavanich, S. 2002. Characterization and identification of *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* strains from fish sauce (nam-pla). Jpn. J. Lactic Acid Bacteria. 13: 46-52.

130. Thornhill, P. J. and Cogan, T. M. 1984. Use of gas-liquid chromatography to determine the end products of growth of lactic acid bacteria. Appl. Environ. Microbiol. 47: 1250-1254.

131. Thornley, M. J. 1960. The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. 23: 37-52.

132. Uchida, K. 1982. Multiplicity in soy pediococci carbohydrate fermentation and its application for analysis of their flora. J. Gen. Appl. Microbiol. 28: 215-223.

133. Ventosa, A., Garcia, M. T., Kamekura, M., Onishi, H. and Ruiz-Berraquero, M. 1989. *Bacillus halophilus* sp. nov., a moderately halophilic *Bacillus* species. Syst. Appl. Microbiol. 12: 162-165.

134. Ventosa, A., Marquez, M. C., Ruiz-Berraquero, F. and Kocur, M. 1990. *Salinicoccus roseus* gen. nov., sp. nov., a new moderately halophilic Gram-positive coccus. Syst. Appl. Microbiol. 13: 29-33.

135. Ventosa, A., Marquez, M. C., Weiss, N. and Tindall, B. J. 1992. Transfer of *Marinococcus hispanicus* to the genus *Salinicoccus* as *Salinicoccus hispanicus* comb. nov. Syst. Appl. Microbiol. 15: 530-534.

136. Ventosa, A., Nieto, J. J. and Oren, A. 1998. Biology of moderately aerobic bacteria. Microbiol. Mol. Biol. Reveiws. 504-544.

137. Villar, M., A. P. de R. Holgado, J. J. Sanchez, R. E., Trucco and Oliver, G. 1985. Isolation and characterization of *Pediococcus halophilus* from salted anchovies (*Engraulis anchoita*). Appl. Environ. Microbiol. 49: 664-666.

138. WainØ, M., Tindall, B. J., Schumann, P. and Ingvorsen, K. 1999. *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. Int. J. Syst. Bacteriol. 49: 821-831.

139. Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., and Trüper, H. G. 1987. International Committee on Systematic Bacteriology. Report of the *Ad Hoc* committee on the reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37: 463–464.

140. Weiss, N. 1992.The genera *Pediococcus* and *Aerococcus*. In *The Prokaryotes.*Vol.1 ed. By Balows , A., Trüper, H. G., Dworkin, M., Harder, W., and Schleifer, K. H., Springer-Verlag. New York, pp. 1502-1504.

141. Whittenbury, R. 1963. The use of soft agar in the study of conditions affecting the utilization of fermentable substrates by lactic acid bacteria. J. Gen Microbiol. 32: 375-384.

142. Whittenbury, R. 1965. A study of some pediococci and their relationship to *Aerococcus viridans* and the enterococci. J. Gen. Microbiol. 40: 97-106.

143. Yamada, K. and Komagata, K. 1970. Taxonomic studies on coryneform bacteria. III. DNA base composition of coryneform bacteria. J. Gen. Appl. Microbiol. 16: 215–224.

144. Yoon, J. –H., Kang, K. H. and Park, Y. –H. 2002. *Lentibacillus salicampi* gen, nov., sp, nov., a moderlately halophilic bacterium isolated from a salt field in Korea. Int. J. Syst. Evol. Microbiol. 52: 2043-2048.

145. Yoon, J. –H., Lee, K. –C., Weiss, N., Kang, K. H., and Park, Y. –H. 2003. *Jeotgalicoccus halotolerans* gen. nov., sp. nov. and *Jeotgalicoccus psychrophilus* sp. nov., isolated from the traditional Korean fermented seafood jeotgal. Int. J. Syst. Evol. Microbiol. 53: 595-602.

146. Yoon, J. –H., Oh, T. K. and Park, Y. –H. 2004. Transfer of *Bacillus halodenitrificans* Denariaz *et al.* 1989 to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov. Int. J. Syst. Evol. Microbiol. 54: 2163-2167.

147. Yuan, S., Ren, P., Liu, J., Xue, Y., Ma, Y., and Zhou, P. 2007. *Lentibacillus halodurans* sp. nov., a moderatelyhalophilic bacterium isolated from a salt lake in Xin-Jiang, China. Int. J. Syst. Evol. Microbiol. 57: 485-488.

148. Zanoni, P., Farrow, J. A. E., Phillips, B. A., and Collins, M. D. 1987. *Lactobacillus pentosus* (Fred, Peterson, and Anderson) sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* 37: 339–341.

149. Zhang, B., Tong, H., and Dong, X. 2005. *Pediococcus cellicola* sp. nov., a novel lactic acid coccus isolated from a distilledspirit-fermenting cellar. Int. J. Syst. Evol. Microbiol. 55: 2167–2170.

150. Zhang, W., Xue, Y., Ma, Y., Zhou, P., Ventosa, A. and Grant, W. D. 2002. *Salinicoccus alkaliphilus* sp. nov., a novel alkaliphile and moderate halophile from

Baer Soda Lake in Inner Mongolia Autonomous Region, China. Int. J. Syst. Evol. Microbiol. 52: 789-793.

151. Zhang, Y.-Q., Yu, L.-Y., Liu, H.-Y., Zhang, Y.-Q., Xu, L.-H. and Li, W.-J. 2007. *Salinicoccus luteus* sp. nov., isolated from a desert soil. Int. J. Syst. Evol. Microbiol. 57: 1901–1905.

152. Zhilina, T. N., Garnova, E. S., Tourova, T. P., Kostrikina, N. A. and Zavarzin, G. A. 2001. *Amphibacillus fermentum* sp. nov., *Amphibacillus tropicus* sp. nov., new alkaliphilic, facultatively anaerobic, saccharolytic bacilli from Lake Magadi. Microbiology (English translation of *Mikrobiologia*) 70: 711-722.

LIST OF PUBLICATIONS

1. Pakdeeto, A., Naranong, N., and Tanasupawat, S. 2003. Diacetyl of lactic acid bacteria from milk and fermented foods in Thailand. J. Gen. Appl. Microbiol. 49: 301-307.
2. Utarapichat, B., Pakdeeto, A., and Tanasupawat, S. 2006. Identification of halophilic lactic acid bacteria from shrimp paste (*ka-pi*). Thaksin Sci. J. 3(1): 71-84.
3. Tanasupawat, S., Pakdeeto, A., Namwong, S., Thawai, T., Kudo, T., and Itoh, T. 2006. *Lentibacillus halophilus* sp. nov., from fish sauce in Thailand. Int. J. Syst. Evol. Microbiol. 56: 1859-1863.
4. Tanasupawat, S., Pakdeeto, A., Thawai, C., Yukphan, P., and Okada, S. 2007. Identification of lactic acid bacteria from fermented tea leaves (*miang*) in Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov., and *Pediococcus siamensis* sp. nov. J. Gen. Appl. Microbiol. 53: 7-15.
5. Pakdeeto, A., Tanasupawat, S., Thawai, C., Moonmangmee, S., Kudo, T., and Itoh, T. 2007. *Lentibacillus kapialis* sp. nov., from fermented shrimp paste in Thailand. Int. J. Syst. Evol. Microbiol. 57: 364-369.
6. Pakdeeto, A., Tanasupawat, S., Thawai, C., Moonmangmee, S., Kudo, T., and Itoh, T. 2007. *Salinicoccus siamensis* sp. nov., isolated from fermented shrimp paste in Thailand. Int. J. Syst. Evol. Microbiol. 57: 2004-2008.