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Characterization and lipolytic activity of lactic acid bacteria isolated from Thai fermented meat

Somboon Tanasupawat[1*], Mukkharin Phoottosavako[2], Suwimon Keeratipibul[2]

[1]Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand.
[2]Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

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ABSTRACT

Twenty six strains of lactic acid bacteria (LAB) were isolated from Thai fermented meat products, including 7 samples of *Nham* (fermented pork), 7 *Sai-krog-prieo* (fermented sausage) and one *Mum* (fermented beef). The isolates were identified as *Lactobacillus pentosus* (5 isolates), *Lactobacillus* sp. (11 isolates), *Pediococcus pentosaceus* (5 isolates) and each isolate of *Pediococcus lolii, Leuconostoc fallax, Weissella thailandensis, W. cibaria,* and *W. paramesenteroides* based on their phenotypic characteristics and 16S rRNA gene sequence similarities (99.8-100 %). The isolates were primary screened for their lipolytic activity on agar plates. The rod-shaped isolates showed 0.031±0.030-0.938±0.127 U/ml of lipase activity in broth supplemented with Tween 20, Tween 40, Tween 60 or Tween 80. The isolate SS50-1 identified as *Lactobacillus pentosus* showed the highest activity in Tween 80 (0.938±0.127 U/ml). The coccal isolates showed lipase activity ranged from 0.029±0.006-1.090±0.033 U/ml when Tween 20, Tween 40, Tween 60 or Tween 80 was used as a substrate. The isolate SS48-4 identified as *Pediococcus lolii* showed the highest activity in Tween 80 (1.090±0.033 U/ml).

INTRODUCTION

Lipase, the hydrolase of glycerol esters EC 3.1.1.3, has high catalytical potential to catalyse the hydrolysis and trans-esterification of triacylglycerols, enantioselective synthesis, and hydrolysis of a variety of esters. Lipase is extensively distributed in plants, animals and microorganisms. The strains of *Bacillus*, *Pseudomonas*, *Burkholderia*, *Acinetobacter* and *Staphylococcus*, and fungi, *Aspergillus terreus* and *Fusarium heterosporum* are reported to produce lipase (Walavalkar and Bapat, 2001; Mrozik *et al.*, 2006; Gayathri *et al.*, 2013). Lipase and esterase of lactic acid bacteria distributed in cheese and milk products and involved milk fat hydrolysis, alcoholysis and esterification including the control of bioflavor and safety in fermented sausages (Meyers *et al.*, 1996; de Fátima Silva Lopes *et al*., 1999; 2002; Demeyer *et al*., 2000; Hollanda *et al.*, 2005). Microbial lipases are commercially significant in food industry because of low production cost, greater stability and wider availability than plant and animal lipases. Lipases are commonly used in the production of a variety of products, ranging from fruit juices, baked foods, vegetable fermentation and in flavour development (Hasan *et al*., 2005). Bacterial lipase is linked to their role as biocatalysts in many biochemical processes such (Joshi and Vinay, 2007). In Thailand, there are many kinds of fermented meat such as *Nham* (fermented pork), *Sai-krog-prieo* or *Mum (*fermented pork or beef sausage), *Pla-ra* (fermented fish) and *Kung-chom* (fermented shrimp) that have been consumed daily by Thai people (Tanasupawat and Komagata, 2001). In *Nham* (fermented pork), the changes in lipid composition and fatty acid profile during fermentation have been reported (Visessanguan *et al.*, 2006). However, a lipolysis of pork fat by bacterial cultures has not been studied in Thailand compared to the meat starter culture, *Staphylococcus xylosus* that was applied as starter in meat fermentation (Sørensen, 1997). The aim of the research was to isolate, identify, and screen the lipase activity of lactic acid bacteria (LAB) isolated from Thai fermented meat.

* *Corresponding Author*
Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, 254 Phayathai Road, Wangmai, Bangkok 10330, Thailand.
E-mail: Somboon.T@chula.ac.th, tanalab@yahoo.com

MATERIALS AND METHODS

Sources and Isolation methods

Fifteen fermented meat including 7 *Nham* (fermented pork) samples collected in Utaradit and Bangkok provinces, 7 *Sai-krog-prieo* (fermented sausage) samples collected in Mahasarakham and one *Mum* (fermented beef) collected in Phetchabun province, Thailand (Table 1) were used for the isolation. Bacterial strains were isolated by spread plate technique using one gram of the fermented food samples diluted in 99 ml of 0.1% peptone solution and then mixed by stomacher for 2 min. It was then 10-fold serially diluted with peptone solution, and 0.1 ml of each proper diluted sample was transferred to Tryptic soy agar (TSA) plate before being spread with a glass spreader and incubated at 37°C for 48 h. The bacterial cells were counted and the colonies which showed different appearance were picked up for purification and then were transferred to TSA slant.

Identification methods

Phenotypic characterization

The morphological, cultural, physiological and biochemical characteristics including Gram staining, cell morphology, colonial appearance, catalase, nitrate reduction, MR-VP, hydrolysis of gelatin, starch and arginine of the isolates were determined (Barrow and Feltham, 1993; Tanasupawat *et al.*, 1998). Growth in 2, 4 and 6% NaCl (w/v), at pH 4, 5, 6, 8 and 9, at 20, 25, 30, 40 and 45 °C were performed. Acid production from carbohydrates was determined as described by Tanasupawat *et al.* (1998).

Genotypic characterization

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers 20F (5'-AGTTTGATCCTGGCTC-3'), 1530R (5'-AAGGAGGTGATCCAGCC-3'), 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The amplified 16S rRNA gene sequence was analyzed by Macrogen Inc., Korea. Sequence alignment was employed using the BLAST software from the Gen Bank. Multiple alignments of the sequences determined were performed with a program CLUSTAL_X (version 1.83; Thompson *et al*., 1997). Gaps and ambiguous bases were eliminated prior to construction of a phylogenetic tree. A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) with the program MEGA version 6 (Tamura *et al.*, 2013). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 replications.

Screening for lipolytic activity

All of the isolates were screened for lipolytic activity on agar plate. The medium consisted of peptone 1%, $CaCl_2$. $2H_2O$ 0.01%, agar 2% and 1% of Tween 20, Tween 40, Tween 60 or Tween 80 (Barrow and Feltham, 1993) and were incubated at 30 °C for 48 h. The lipolytic activity of the isolates was detected by the appearance of an opaque zone around the colonies.

The selected isolates that showed their lipolytic activity to different substrates on agar medium were cultivated and 0.1% (v/v) seed cultures (0.5 McFarland standard) were inoculated in 50 ml Nutrient broth (NB) (250 ml) with 1% Tween as substrates and then incubated at 30 °C on a rotary shaker (200 rpm) for 24 h. The fermentation broth was collected and centrifuged at 10,000 rpm, 4 °C for 10 min and 50 µl of the supernatant was used as crude enzyme for the assay.

Lipase activity was determined by a spectrophotometric assay with ρ-nitrophenyl palmitate (*ρ*-NPP) as a substrate. The reaction mixture consisted of 135 µl of 0.4% Triton X, 0.1% gum arabic in 50 mM Tris-HCl buffer (pH 7) and 15 µl of 30 mg, *p*-NPP in 10 ml of isopropyl alcohol. The mixture was added with 50 µl of crude enzyme incubated at 37˚C for 1 h, the colour change of activity was measured at 405 nm (Arora, 2013). The enzyme activity was calculated as described by Lee *et al.*, 2003.

RESULTS AND DISCUSSION

Isolation of isolates

Eight rod-shaped lactic acid bacteria were isolated from *Nham* collected in Utaradit and Bangkok provinces and 8 isolates from *Sai-krog-prieo* collected in Mahasarakham province. One coccal isolate was isolated from *Mum* collected in Phetchabun province, 3 isolates were isolated from *Nham* collected in Utaradit and Bangkok provinces and 6 isolates were from *Sai-krog-prieo* collected in Mahasarakham province (Table 1). The total bacterial cell count in *Nham*, *Sai-krog-prieo* and *Mum* ranged from 2.63×10^8-5.75×10^9; 2.05×10^8-1.71×10^{10} and 1.39×10^9 CFU/g, respectively.

Identification of isolates

Twenty-six isolates were Gram-positive none spore forming, catalase and oxidase negative lactic acid producing bacteria. They fermented glucose homofermentatively and some isolates that produced gas from glucose were heterofermentative. They did not grow at 50 °C. They showed negative reaction to nitrate reduction, hydrolysis of arginine and starch. All isolates were divided into seven groups based on the cell form, growth at different temperatures, pH and NaCl concentrations including the acid production from carbohydrates (Table 2) as described here.

Lactobacillus group (Group 1 and 2) consisted of 16 isolates NM8-2, NM39-2, NM43-1, NM43-2, NM43-5, NM44-2, NM47-1, NM47-2, SS48-2, SS49-2, SS49-3, SS50-1, SS50-2, SS52-1, SS52-3 and SS56-1. Cells were rod-shaped. Colonies were smooth, circular, convex and white in colour. They fermented glucose homofermentatively and produced no gas from glucose. Most of isolates grew at 20-40 °C, pH 4-8.0 and in 2% NaCl. Acid was produced from D-cellobiose, D-galactose, D-glucose, D-ribose, salicin and sucrose Variable characteristics are found in L-arabinose, glycerol, inulin, lactose, D-mannitol, D-

melezitose, D-melibiose, methyl-α D-glucoside, raffinose, L-rhamnose, D-sorbitol, D-trehalose and D-xylose (Table 2). The 16S rRNA gene sequence of the representative isolates, NM8-2 (1,274 bp), NM44-2 (1,388 bp), SS49-2 (1,214 bp), SS50-1 (1,398 bp) and SS50-2 (1,386 bp) were closely related to *Lactobacillus pentosus* JCM 1558^T (99.9-100% similarity) (Figure 1). Therefore, these isolates were identified as *L. pentosus* (Zanoni *et al*., 1987). The remained isolated were identified as *Lactobacillus* species.

Pediococcus group (Group 3 and 4) consisted of 6 isolates, MA34-3, NM46-2, NM46-3, SS48-4, SS51-2 and SS52-4. Cells were tetrad forming cocci. Colonies were smooth, circular, convex and white in colour. They fermented glucose homofermentatively and produced no gas from glucose. Most of them grew at 20-40 °C, pH 5-8.0 and in 2% NaCl. Group 3 isolates produced acid from D-cellobiose, D-galactose, D-glucose, D-ribose, salicin, sucrose and D-trehalose but did not produce acid from glycerol, inulin, D-mannitol, D-melezitose and D-sorbitol. Group 4 isolates produced acid from many substrates. Variable characteristics are shown in Table 2. The representative isolate SS48-4 (1,397 bp) showed 99.8% 16S rRNA gene sequence similarity to *Pediococcus pentosaceus* DSM 20336^T (Figure 1). In this study, isolates MA34-3, NM46-2, NM46-3 and SS52-4 showed almost the same phenotypic characteristics with SS48-4. Their characteristics were similar to the isolates as described by Tanasupawat *et al*. (1993). Therefore, all of them were identified as *P. pentosaceus* (Tanasupawat *et al*., 1993).

The isolate SS51-2 (1,380 bp) showed 100% 16S rRNA gene sequence similarity to *Pediococcus lolii* NGRI 0510Q^T. This strain was differentiated from Group 3 isolates in the growth at 45 °C. Therefore, this isolate was identified as *Pediococcus lolii (*Doi *et al*., 2009*).*

Leuconostoc group (Group 5) contained an isolate SS49-1. Cells were cocci in chains and non spore forming. Colonies were smooth, circular, convex and white in colour. They fermented glucose heterofermentatively and produced gas from glucose. They grew at 20-40 °C, pH 4-7 and in 6% NaCl. Acid was produced from glucose, D-mannitol, methyl-α D-glucoside, and D-xylose but it did not produce acid from any carbohydrates as in Table 2. The isolate SS49-1 (1,327 bp) showed 99.9% 16S rRNA gene sequence similarity to *Leuconostoc fallax* DSM 20189^T. Therefore, this isolate was identified as *Leu. fallax* (Martinez-Murcia and Collins, 1991).

Weissella group (Group 6, 7 and 8) contained 3 isolates, NM45-2, SS55-3 and SS56-3. Cells were cocci in chains and non spore forming. Colonies were smooth, circular, convex and white in colour. They fermented glucose heterofermentatively and produced gas from glucose.

They grew at 20-40°C, pH 5-7 and in 6% NaCl. Acid was produced from L-arabinose, D-cellobiose, D-galactose, D-glucose, D-ribose, salicin and sucrose as in Table 2.

The isolate NM45-2 (1,339 bp) showed 99.8% 16S rRNA gene sequence similarity to *Weissella thailandensis* FS61-1^T. Therefore, this isolate was identified as *W. thailandensis* (Tanasupawat *et al*., 2000).

Isolate SS55-3 (1,369 bp) showed 99.9% 16S rRNA gene sequence similarity to *Weissella cibaria* LMG 17699^T. Therefore, this isolate was identified as *W. cibaria* (Björkroth *et al.,* 2002). The isolate SS56-3 (1,400 bp) showed 99.9% 16S rRNA gene sequence similarity to *Weissella paramesenteroides* ATCC 33313^T. Therefore, this isolate was identified as *Weissella paramesenteroides* (Collins *et al.,* 1993).

Screening for lipolytic activity

The lipase production in nutrient broth with Tween 20, Tween 40, Tween 60 or Tween 80 of the rod-shaped isolates ranged from 0.032±0.014-0.83±03.083, 0.043±0.017-0.528±0.048, 0.031±0.030-0.672±0.109 and 0.035±0.015-0.938±0.127 U/ml, respectively. The isolates NM8-2, NM43-2 and NM44-2 from *Nham* showed high lipase activity, while the isolate SS50-1 identified as *Lactobacillus pentosus* from *Sai-krog-prieo* showed the highest activity in Tween 80 (0.938±0.127 U/ml). The lipase production in nutrient broth with Tween 20, Tween 40, Tween 60 or Tween 80 of the coccal isolates ranged from 0.073±0.008-0.827±0.048, 0.029±0.006-0.670±0.069, 0.055±0.028-0.956±0.044 and 0.040±0.009-1.090±0.033 U/ml, respectively. The isolate SS48-4 identified as *Pediococcus lolii* showed the highest activity in Tween 80 (1.090±0.033 U/ml). This strain was also isolated from *Sai-krog-prieo*.

The enzyme potential of lactic acid bacteria is an important factor in the formation of characteristic taste of meat products. The ripening of the product are complex and are resulted from the interaction of the remaining enzymes of muscle and fat tissue and the action of bacterial enzymes. This study, LAB isolates showed low lipase activity the same as *Lactobacillus plantarum*, *Lactobacillus sakei* and *Lactobacillus brevis* strains isolated during different stages of fermented traditional Bulgarian meat product that reported by Stoyanovski *et al.* (2013). Production of naturally fermented sausages is a tradition in Southern Europe, Scandinavia and Latin America.

Specific flavor, odor, color and structure of the sausages are due to the characteristics of the raw meat and spices used, natural microflora including lactic acid bacteria. During the ripening of dry sausage biochemical and physicochemical processes occur, which alter the composition and the structure of meat to form end products that give specific flavor, taste, color and other features of the product. Enzymatic activity of microflora, including the lactic acid bacteria plays an essential role in these processes (Demeyer *et al.,* 2000; Vestergaard *et al.,* 2000; Papamanoli *et al.,* 2003). Our results showed that LAB in fermented meat may play the formation of characteristic taste of the meat products.

Table 1: Sources, location, isolate number, identification and 16S rRNA gene sequence similarity (%) of the representative LAB isolates.

Fermented food	Province	Isolate no.	Identification and Nearest Relative (%)
Nham	Utaradit	NM8-2	*Lactobacillus pentosus* JCM 1558^T (100%)
Mum	Phetchabun	MA34-3	*Pediococcus pentosaceus*
Nham	Bangkok	NM39-2	*Lactobacillus* sp.
Nham	Utaradit	NM43-1	*Lactobacillus* sp.
		NM43-2	*Lactobacillus* sp.
		NM43-5	*Lactobacillus* sp.
Nham	Utaradit	NM44-2	*L. pentosus* JCM 1558^T (99.9%)
Nham	Utaradit	NM45-2	*Weissella thailandensis* FS61-1^T (99.8%)
Nham	Bangkok	NM46-2	*P. pentosaceus*
		NM46-3	*P. pentosaceus*
Nham	Bangkok	NM47-1	*Lactobacillus* sp.
		NM47-2	*Lactobacillus* sp
Sai-krog-prieo	Mahasarakham	SS48-2	*Lactobacillus* sp.
		SS48-4	*P. pentosaceus* DSM 20336^T (99.8%)
Sai-krog-prieo	Mahasarakham	SS49-1	*Leuconostoc fallax* DSM 20189^T (99.9%)
		SS49-2	*L. pentosus* JCM 1558^T (100%)
		SS49-3	*Lactobacillus* sp
Sai-krog-prieo	Mahasarakham	SS50-1	*L. pentosus* JCM 1558^T (100%)
		SS50-2	*L. pentosus* JCM 1558^T (99.9%)
Sai-krog-prieo	Mahasarakham	SS51-2	*P. lolii* NGRI 0510Q^T (100%)
Sai-krog-prieo	Mahasarakham	SS52-4	*P. pentosaceus*
		SS52-1	*Lactobacillus* sp.
		SS52-3	*Lactobacillus* sp.
Sai-krog-prieo	Mahasarakham	SS55-3	*W. cibaria* LMG 17699^T (99.9%)
Sai-krog-prieo	Mahasarakham	SS56-3	*W. paramesenteroides* ATCC 33313^T (99.9%)
		SS56-1	*Lactobacillus* sp.

Table 2: Phenotypic characteristics of LAB isolates.

Characteristics	1	2	3	4	5	6	7	8
No. isolate	**5**	**11**	**5**	**1**	**1**	**1**	**1**	**1**
Cell shape	Rods	Rods	Tetrads	Tetrads	Cocci	Cocci	Cocci	Cocci
Arginine hdrolysis	-	-(+4)	- (+1)	-	+	-	-	-
Gas from glucose	-	-	-	-	+	+	+	+
Growth at 40°C	+	-(+4)	- (+1)	+	+	+	+	+
Growth at 45°C	-	-	-	+	-	-	-	-
Growth at pH 4	-(+2)	-(+2)	-	+	-	-	-	-
Growth at pH 5	+(-1)	-(+5)	+(-1)	+	+	+	+	+
Growth at pH 6	+	+	+	+	+	+	+	+
Growth at pH 8	+(-1)	-(+5)	- (+1)	+	-	-	+	-
Growth at pH 9	-(+1)	-(+3)	-	-	-	-	+	-
4% NaCl	-(+1)	-(+1)	-	+	+	+	+	+
6% NaCl	-(+1)	-	-	-	+	+	+	+
Acid from :								
L- Arabinose	+	-	+(-1)	+	-	+	+	+
D-Cellobiose	+	+	+	+	-	+	+	+
D-Galactose	+	+	+	+	-	+	+	+
Glycerol	+(-2)	-(+4)	-	-	-	-	-	-
Inulin	-	-(+6)	-	-	-	-	+	+
Lactose	+	-	+(-2)	+	-	-	+	+
D-Mannitol	+	-(+5)	-	-	+	-	+	+
D-Melezitose	+	+(-6)	-	-	-	-	-	-
D-Melibiose	+	-	+(-1)	+	-	+	-	+
Methyl-α D-glucoside	+	-(+5)	- (+1)	+	+	-	-	-
Raffinose	+	-	+(-1)	+	-	-	-	-
L-Rhamnose	-	-(+5)	- (+1)	+	-	-	-	-
D-Ribose	+	+	+	+	-	+	+	+
Salicin	+	+	+	+	-	+	+	+
D-Sorbitol	+	-(+3)	-	-	-	-	-	-
Sucrose	+	+	+	+	-	+	+	+
D-Trehalose	+	-(+5)	+	+	-	-	-	-
D-Xylose	-(+2)	-	-	-	+	-	-	-

1, *L. pentosus*; 2, *Lactobacillus* spp.; 3, *P. pentosaceus*; 4, *P. lolii*; 5, *Leuconostoc fallax*; 6, *W. thailandensis*; 7, *W. cibaria;* 8, *W. paramesenteroides*. +, positive reaction; -, negative reaction. Numbers in parentheses indicate the number of strains showing a positive or negative reaction.

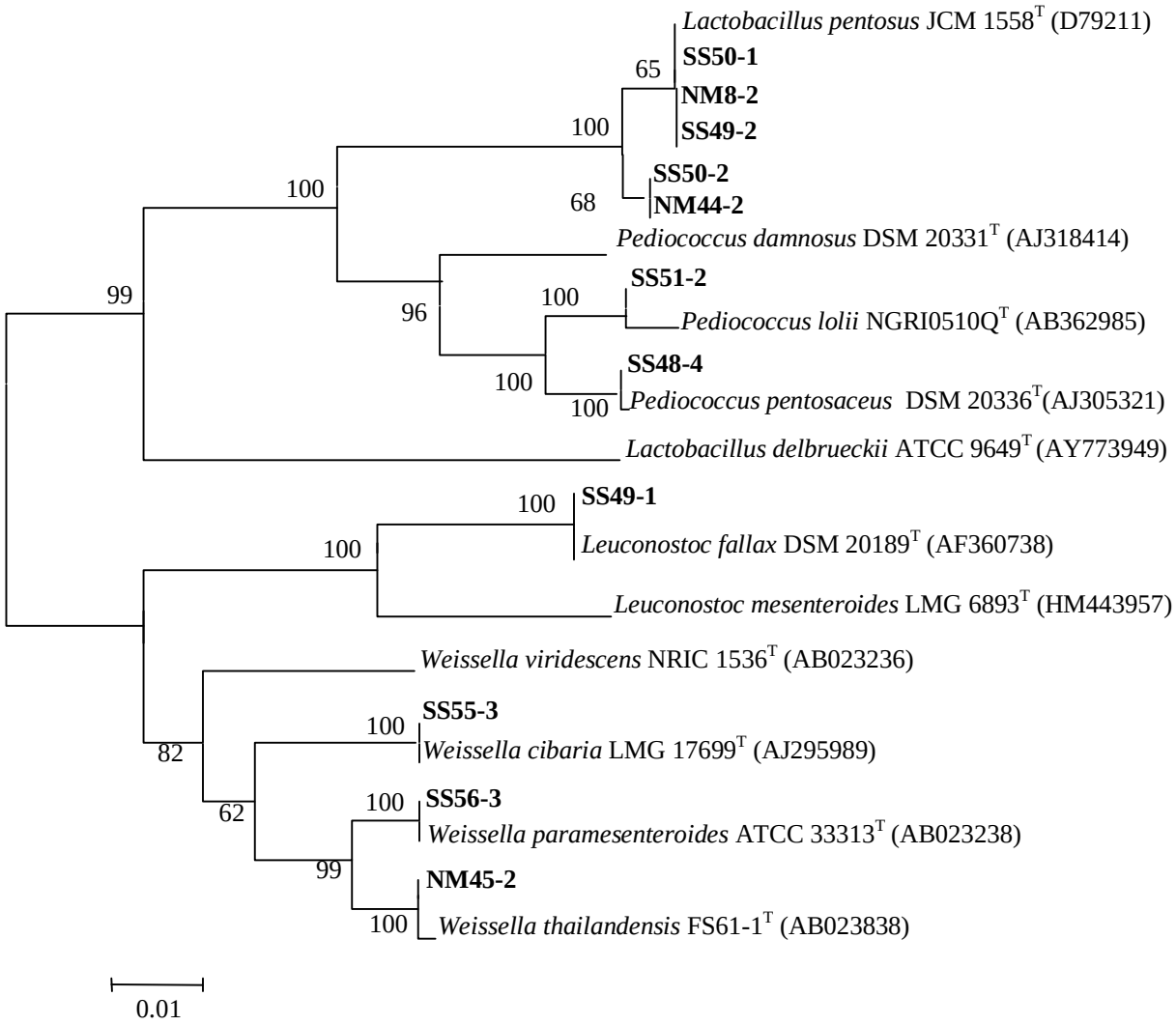

Fig. 1: Neighbour-joining tree based on 16S rRNA gene sequences showing relationships among LAB isolates and related species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.01 substitutions per nucleotide position.

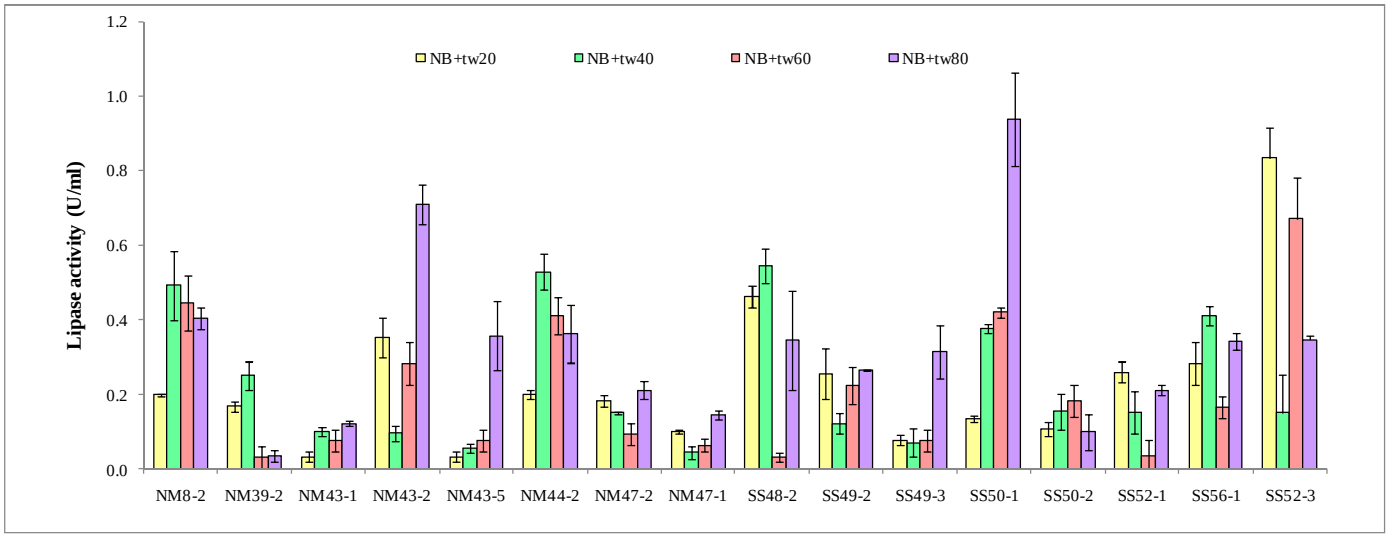

Fig. 2: Lipase activity (U/ml) of rod-shaped isolates in nutrient broth (NB) with Tween 20, Tween 40, Tween 60 or Tween 80.

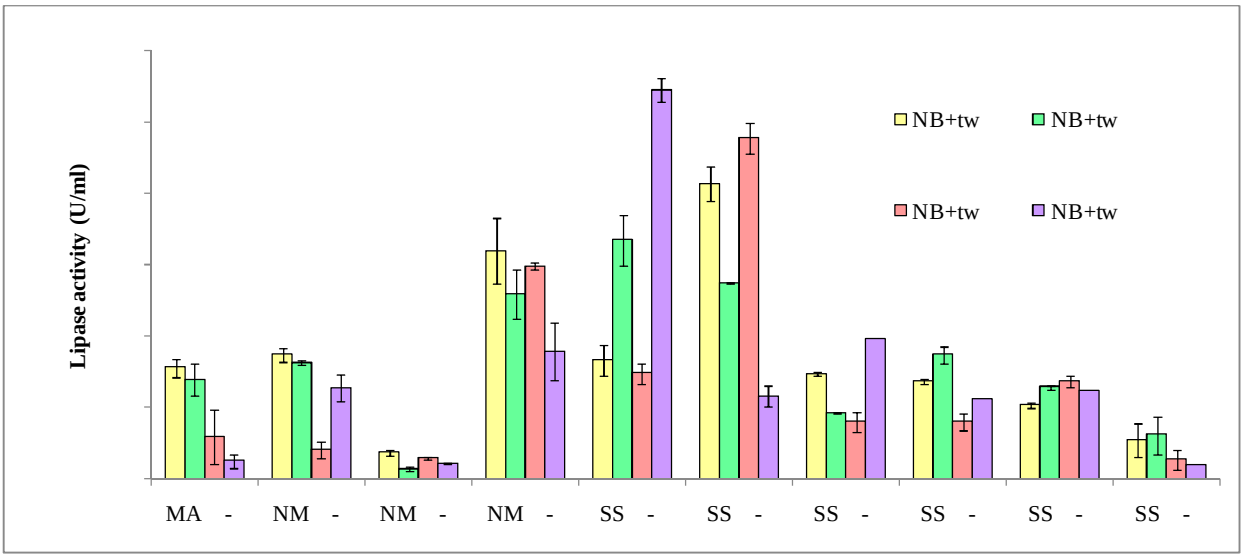

Fig. 3: Lipase activity (U/ml) of coccal isolates in nutrient broth (NB) with Tween 20, Tween 40, Tween 60 or Tween 80.

CONCLUSION

In this study, lactic acid bacteria including 5 isolates of *Lactobacillus pentosus*, and 11 isolates of *Lactobacillus* sp. were isolated from *Nham* (fermented pork) and *Sai-krog-prieo* (fermented sausage). Five *Pediococcus pentosaceus* isolates were distributed in *Nham*, *Sai-krog-prieo* and *Mum* (fermented beef). Each isolate of *Pediococcus lolii, Leuconostoc fallax, Weissella thailandensis*, *W. cibaria*, and *W. paramesenteroides* was found in *Nham* and *Sai-krog-prieo*. The isolates showed 0.029±0.006-1.090±0.033 U/ml when Tween 20, Tween 40, Tween 60 or Tween 80 was used as a substrate. The isolate SS50-1 identified as *Lactobacillus pentosus* showed the highest activity in Tween 80 (0.938±0.127 U/ml) while isolate SS48-4 identified as *Pediococcus lolii* showed the highest activity in Tween 80 (1.090±0.033 U/ml).

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