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Identification and characterization of lipolytic bacteria from Thai fermented foods

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ABSTRACT

Aims: Gram-positive spore forming rod-shaped bacteria from Thai fermented foods were isolated, identified and screened for lipolytic activity.
Methodology and results: Bacterial strains were isolated from Thai fermented foods by the standard dilution technique using Tryptic soy agar. Seven isolates which belong to the genus *Bacillus* and one isolate which belongs to *Paenibacillus* were characterized based on their phenotypic characteristics and 16S rRNA gene sequence analyses. Isolates NM8-1 (Group 1), PR9-2 (Group 2), PR11-1 (Group 3), KM15-1 and SS49-4 (Group 4), SS48-5 (Group 5) and SS503 (Group 6), were closely related to *Bacillus methylotropicus*, *B. pumilus*, *B. flexus*, *B. cereus*, *B. subtilis* and *B. anthracis*, based on 99.90-100% similarities, respectively. Isolate NM45-3 (Group 7) was closely related to *Paenibacillus pasadenensis* based on 99.55% similarity. All the isolates were susceptible to amikacin, chloramphenicol, clindamycin, erythromycin, gentamicin, imipenem, kanamycin, levofoxacin, novobiocin, streptomycin, tetracycline and vancomycin. Their lipase production in nutrient broth with Tween 20, Tween 40, Tween 60 or Tween 80 ranged from 0.014±0.129 - 3.231±0.087 U/mL. *Bacillus subtilis* SS48-5 exhibited highest lipase production when cultured with Tween 80 at pH 7.5 for 24 h. The optimum lipase production of this strain was at 40 °C after incubated for 30 h.
Conclusion, significance and impact of study: The characterization and evaluation of the lipolytic activity of *Bacillus* and *Paenibacillus* strains isolated from Thai fermented foods will be useful for the species diversity, food fermentation and the lipase production.

Keywords: Antibiotic susceptibility, fermented foods, lipolytic bacteria, spore-forming bacteria

INTRODUCTION

Lipolytic enzyme, lipase (the hydrolases of glycerol esters EC 3.1.1.3) has high catalytical potential and is extensively distributed in plants, animals and microorganisms. It helps to catalyse hydrolysis and trans-esterification of triacylglycerols, enantioselective synthesis and hydrolysis of a variety of esters. Many microorganisms in genera *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).

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 as biodiesel synthesis (Joshi and Vinay, 2007; Korman *et al*., 2013). However, bacterial strains are varied in their enzymatic activity, which depend on the species and the cultivating conditions (Sharma *et al.*, 2001; Mrozik *et al.*, 2006). Many researchers have been extensively studied on the screening of lipases and development of the immobilization methods (Yoon *et al*., 2011).

In Thailand, there are many kinds of fermented foods that have been consumed daily by Thai people such as *nham* (fermented pork), *sai-krog-prieo (*fermented sausage), *pla-ra* (fermented fish) and *kung-chom* (fermented shrimp) (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 reported the roles in the fermentation (Sørensen, 1997). The aim of the research was to isolate, identify, and screen the lipase activity of bacterial strains from Thai fermented foods.

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MATERIALS AND METHODS

Sources and isolation methods

Eight fermented foods which included two *nham* (fermented pork) samples collected from Utaradit province, two *pla-ra* (fermented fish) and one *kung-chom* (fermented shrimp), samples collected from Buriram, three *sai-krog-prieo* (fermented sausage) samples collected from Mahasarakram, 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 10-fold serially diluted with peptone solution and 0.1 mL each of proper diluted samples was transferred to Tryptic soy agar (TSA) plate and then spread with 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.

Screening for lipolytic activity

All of the isolates were screened for lipolytic activity on agar plate. The medium consisted of peptone 1%, $CaCl_2 \cdot 2H_2O$ 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 flask) 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, *ρ*-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).

Identification methods

Phenotypic characterization

The morphological, cultural, physiological and biochemical characteristics including Gram staining, cell morphology, colonial appearance, catalase, oxidase, nitrate reduction, MR-VP, indole production, citrate utilization; hydrolysis of gelatin, starch and arginine; and blood haemolysis of the isolates were determined (Barrow and Feltham, 1993). The cell morphology of selected isolate was observed by using a JEOL JSM-5410LV scanning electron microscope at Scientific and Technological Research Equipment Centre, Chulalongkorn University. Growth in 4, 6, 8% 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 (Tanasupawat *et al.*, 1998). Diaminopimelic acid in the cell wall was determined as described by Komagata and Suzuki (1987).

The isolates were examined for antibiotic susceptibility by disc diffusion assay (Lorian, 1991) on Mueller-Hinton agar (Difco, Detroit, MI). The common antibiotics such as amikacin (30 ug), chloramphenicol (30 ug),clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), imipenem (10 µg), kanamycin (30 µg), levofoxacin (5 µg), novobiocin (5 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg), bacitracin (10 U) and penicillin G (20 U), sulphonamide (300 µg) and sulfamethoxazole/trimethoprim (Bactrim, 25 µg) were used.

Genotypic characterization

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers 20F (5'-AGTTTGATC CTGGCTC-3'), 1530R (5'- AAGGAGGTGATCCAGCC–3'), 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'-TACGGYTACCTTGTTACGACTT-3'). The amplified 16S rRNA gene sequence was analyzed by Macrogen®, 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 5.05 (Tamura *et al.*, 2011). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 replications.

Determination of the effects on pH, temperature and incubation time on lipase production

The effect of different substrates including 1% of Tween 20 (Tw20), Tween 40 (Tw40), Tween 60 (Tw60), and Tween 80 (Tw80) in NB (pH 7.4) of the isolates on lipase production were determined by incubating the strains at 30 °C on a rotary shaker (200 rpm) for 24 h. The temperature effect (20, 30, 37 and 40 °C) on lipase activity of the selected isolate, highest lipase production was determined when cultivated in NB with 1% Tween 80 and incubated on rotary shaker (200 rpm) for 24 h. Effect of the initial pH at 5, 6, 7, 8 and 9 and the incubation time (0, 6, 12, 18, 24, 30, 36, 42, and 48 h) on lipase production was determined in NB broth with 1% Tween 80 incubated at 30 °C on rotary shaker (200 rpm) for 24 h. Lipase activity was determined by a spectropho-

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Table 1: Source of isolation, province, isolate number, bacterial cell count, group, 16S rRNA gene sequence similarity (%) and closest species.

Fermented food	Province	Isolate no.	Bacterial cell count (CFU/mL)	Group	Similarity (%)	Closest species
Nham	Utaradit	NM8-1	2.63×10^8	1	99.93	*B. methylotropicus* CBMB 205^T
Pla-ra	Buriram	PR9-2	3.75×10^8	2	100	*B. pumilus* ATCC 7061^T
Pla-ra	Buriram	PR11-1	1.13×10^8	3	100	*B. flexus* IFO 15715^T
Kung-chom	Buriram	KM15-1	2.5×10^6	4	100	*B. cereus* ATCC 14579^T
Sai-krog-prieo	Mahasarakram	SS49-4	5.85×10^8	4	100	*B. cereus* ATCC 14579^T
Sai-krog-prieo	Mahasarakram	SS48-5	2.10×10^9	5	99.93	*B. subtilis* DSM 15029^T
Sai-krog-prieo	Mahasarakram	SS503	2.09×10^9	6	99.90	*B. anthracis* ATCC 14578^T
Nham	Utaradit	NM45-3	2.35×10^9	7	99.55	*P. pasadenensis* SAFN-007^T

tometric assay with *ρ*-nitrophenyl palmitate (*ρ*-NPP) and was calculated as mentioned above (Lee *et al.*, 2003; Arora, 2013)**.**

RESULTS AND DISCUSSION

Eight bacterial strains were isolated from eight samples of fermented food products including *nham* (NM), *sai-krog-prieo* (SS), *pla-ra* (PR) and *kung-chom* (KM), collected from various markets in Thailand. The total bacterial cell count ranged from 2.5×10^6-2.35×10^9 CFU/g (Table 1).

The isolates NM8-1, PR9-2, PR11-1, KM15-1, NM45-3, SS49-4, SS48-5, SS50-3 showed the opaque halos of calcium oleate on agar medium when Tween 80 was used (García-Lepe *et al.*, 1997). They were Gram-positive, aerobic or facultative anaerobic, spore forming rod-shaped bacteria. Colonies were round, regular or irregular, opaque, flat/smooth, or raised and white or creamy-white after incubation at 37 °C on TSA agar plate for 2 days. They were belonged to the genus *Bacillus* and *Paenibacillus* and were divided into seven groups based on their phenotypic characteristics and the 16S rRNA gene sequence analyses. All isolates showed positive reaction for catalase, hydrolysis of gelatin and starch, nitrate reduction, Voges-Proskauer (VP) reaction, and Tween 80, growth at 20-40 °C, and at pH 6-8, but showed negative results for hydrolysis of arginine, indole, methyl red, urea and hydrogen sulfide formation. All produced acid from D-fructose, D-glucose, but did not produce acid from inulin, D-melezitose, D-melibiose, α-methyl-D-glucoside, raffinose, rhamnose, and D-sorbitol. Their specific phenotypic characteristics are described below and in Table 2.

All isolates showed susceptibility to amikacin (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), imipenem (10 µg), kanamycin (30 µg), levofoxacin (5 µg), novobiocin (5 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). Isolate KM15-1 was resistant to bacitracin (10 U) and penicillin G (20 U) while isolate SS49-4 was resistant to penicillin G (20 U), sulphonamide (300 µg) and bactrim (25 µg). Isolate SS503 was resistant to sulphonamide (300 µg) and bactrim (25 µg) (Table 3).

Group 1 contained isolate NM8-1. This isolate grew in 6% NaCl, at 20-45 °C, and pH 5-9. This strain showed the positive for catalase, hydrolysis of gelatin and starch, nitrate reduction and growth at 20-45 °C, and pH 5-9 the same as *B. methylotropicus* CBMB 205^T. However, it was different from *B. methylotropicus* CBMB 205^T in oxidase reaction and growth in 6% NaCl (Madhaiyan *et al.*, 2010). Based on the 16S rRNA gene sequence, isolate NM8-1 (1,352 bp) was closely related to *B. methylotropicus* CBMB 205^T (Figure 1) with 99.93% sequence similarity. This study, we identified it as *B. methylotropicus*.

Group 2 contained isolate PR9-2. It grew in 6% NaCl at 20-40 °C and at pH 5-9. This strain showed the positive for hydrolysis of Tween 80 but negative for acid production from D-maltose and α-methyl-D-glucoside the same as *B. pumilus* ATCC 7061^T (Satomi *et al*., 2006). Based on the 16S rRNA gene sequence, isolate PR9-2 (1,382 bp) was closely related to *B. pumilus* ATCC 7061^T (Figure 1) with 100% sequence similarity. Therefore, it was identified as *B. pumilus* (Satomi *et al*., 2006).

Group 3 contained isolate PR11-1. It grew in 3% and 5% NaCl, at pH 5-9 and at 20-40 °C. This strain showed the positive for hydrolysis of gelatin, starch, Tween 80, Tween 20, acid production from D-fructose, D-glucose, D-galactose, D-maltose but negative for acid production from glycerol, D-sorbitol and D-xylose the same as *B. flexus* strains (Fergus *et al*., 1988). Based on the 16S rRNA gene sequence, isolate PR11-1 (1,386 bp) was closely related to *B. flexus* IFO 15715^T (Figure 1) with 100% sequence similarity. Therefore, it was identified as *B. flexus* (Fergus *et al*., 1988).

Group 4 contained isolates KM15-1 and SS49-4. These two strains were different in growth in 4-6% NaCl and some acid production from carbohydrates for each other as in Table 2. Only isolate SS49-4 produced hemolysis. Based on the 16S rRNA gene sequence, isolates KM15-1 (889 bp) and SS49-4 (1,502 bp) was

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closely related to *B. cereus* ATCC 14579^T (Figure 1) with 100% sequence similarity. However, they showed the positive for Voges-Proskauer test, hydrolysis of gelatin, starch and Tween 80, acid production from D-fructose and D-glucose but negative for acid production from D-galactose, D-mannitol and D-xylose the same as *B. cereus* strains (Logan *et al*., 2002; Yoon and Oh, 2005). In this study, they were identified as *B. cereus*. The isolate KM15-1 was resistant to bacitracin (10 U) and penicillin G (20 U) while isolate SS49-4 was resistant to penicillin G (20 U), sulphonamide (300 µg) and bactrim (25 µg) (Table 3). Comparison to the clinical *B. cereus* strains that were resistant to beta-lactam antibiotics whereas many non-*B. cereus* strains were susceptible to penicillins, semisynthetic penicillins, and cephalosporins (Weber *et al*., 1988; Drobniewski, 1993).

Table 2: Phenotypic characteristics of isolates.

Characteristics	NM8-1	PR9-2	PR11-1	KM15-1	SS49-4	SS48-5	SS503	NM45-3
Group	1	2	3	4	4	5	6	7
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Colony pigment	CW	W	W	W	W	CW	W	CW
Citrate utilization	+	-	-	+	+	-	+	-
Oxidase	-	-	-	-	-	+	+	+
Haemolytic activity	-	-	-	-	+	-	+	-
Esculin	+	+	+	-	+	+	+	+
Growth at 45 °C	+	-	-	-	+	+	+	+
Growth at pH 5	+	+	+	+	+	+	+	+
Growth at pH 9	+	+	+	+	+	+	+	+
4% NaCl (w/v)	+	+	+	+	-	+	+	-
6% NaCl (w/v)	+	+	+	+	-	+	+	-
8% NaCl (w/v)	-	-	-	-	-	-	-	-
Acid from:								
L-Arabinose	-	+	-	-	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-	+
D-Galactose	-	-	+	-	-	-	-	+
Glycerol	-	-	-	-	+	-	-	-
Lactose	-	-	-	-	-	-	-	+
D-Maltose	-	-	+	-	+	-	+	+
D-Mannitol	-	-	-	-	-	-	-	+
D-Mannose	-	+	-	-	+	+	+	-
D-Ribose	-	+	-	-	+	-	+	-
Salicin	-	-	-	-	-	-	-	-
Sucrose	-	+	-	-	-	-	-	-
D-Trehalose	-	-	-	-	+	-	+	-
D-Xylose	-	-	-	-	-	-	+	-

CW, creamy white; W, white; +, positive reaction; -, negative reaction.

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Table 3: Zone diameter (mm)[a] of antimicrobial disc susceptibility test of isolates.

Antibiotics	Strain							
	NM8-1	PR9-2	PR11-1	KM15-1	SS49-4	SS48-5	SS503	NM45-3
Amikacin (30 ug)	39.3±0.4	39.8±0.4	41.2±0.6	37.7±0.2	33.6±0.1	36.6±0.8	36.2±0.6	21.2±0.3
Bacitracin (10 U)	15.1±0.7	14.1±0.1	33.2±0.8	0.0±0.0	22.4±0.1	9.9±0.1	21.8±0.6	33.7±0.4
Chloramphenicol (30 ug)	37.8±0.3	39.6±0.2	40.8±0.3	32.2±0.4	35.5±0.4	34.0±1.0	42.2±0.4	42.1±0.1
Clindamycin (2 µg)	31.9±0.1	37.9±0.6	31.0±0.2	37.3±0.3	31.5±0.3	21.3±0.4	30.5±0.0	33.7±0.2
Erythromycin (15 µg)	44.6±0.4	49.6±0.4	37.2±0.2	40.9±0.6	41.8±0.1	39.4±0.6	38.0±1.0	38.2±0.1
Gentamicin (10 µg)	39.4±0.5	42.1±0.4	39.0±0.4	29.8±0.1	31.2±0.1	38.3±0.4	34.8±0.4	30.5±0.3
Imipenem (10 µg)	61.9±0.1	60.3±0.4	50.4±0.6	50.2±0.4	45.8±0.0	57.2±0.3	56.9±0.2	49.0±0.4
Kanamycin (30 µg)	40.5±0.5	41.4±0.2	42.2±0.3	26.9±0.3	26.6±0.4	40.6±0.6	36.7±0.1	20.3±0.4
Levofoxacin (5 µg)	43.6±0.3	53.9±0.2	37.9±0.1	37.7±0.0	38.3±0.2	40.1±0.6	43.2±0.5	31.4±0.2
Novobiocin (5 µg)	25.5±0.4	34.6±0.2	24.8±0.4	20.2±0.1	22.7±0.1	27.6±0.6	30.5±0.6	34.2±0.2
Penicillin G (20 U)	49.1±0.1	55.7±0.1	49.7±0.4	0.0±0.0	0.0±0.0	31.6±0.6	20.4±0.4	57.3±0.4
Streptomycin (10 µg)	29.0±0.4	33.2±0.5	30.2±0.5	31.2±0.2	32.0±0.7	19.7±0.5	28.9±0.1	25.8±0.4
Sulphonamide (300 µg)	47.9±0.8	59.7±0.2	42.1±0.1	37.0±0.4	0.0±0.0	34.8±0.1	0.0±0.0	53.4±0.3
Sulfa/trimeth (25 µg)	47.8±0.6	64.8±0.3	41.5±0.4	11.8±0.4	0.0±0.0	43.2±0.5	0.0±0.0	45.6±0.6
Tetracycline (30 µg)	21.8±0.7	52.2±0.5	39.7±0.1	9.3±0.4	30.4±0.5	36.1±0.1	31.5±0.2	48.9±0.5
Vancomycin (30 µg)	31.9±0.6	34.9±0.1	31.3±0.4	21.4±0.6	26.8±0.4	28.6±0.8	29.8±0.4	37.0±0.6

[a]Values are expressed as the means of two determinations.

Group 5 contained isolate SS48-5. The strain produced endospore (Figure 2). It grew in 8% NaCl, at pH 5-9 and at 20-45 °C. It showed the positive for catalase, oxidase, nitrate reduction, haemolytic activity, hydrolysis of gelatin and starch, acid production from D-glucose and D-mannose but negative for acid production from D-galactose and lactose the same as *B. subtilis* strains (Gatson *et al.*, 2006; Lim *et al.*, 2006). This strain contained *meso*-diaminopimelic acid in the cell wall peptidoglycan. Based on the 16S rRNA gene sequence, isolate SS48-5 (1,481 bp) was closely related to *B. subtilis* DSM 15029^T (Figure 1) with 99.93% sequence similarity. Therefore, it was identified as *B. subtilis*.

Group 6 contained isolate SS503. The strain grew in 6% NaCl, at pH 5-9, and at 20-45 °C. It produced hemolysin that was different from other strains (Table 2). Based on the 16S rRNA gene sequence, isolate SS503 (1,010 bp) was closely related to *B. anthracis* ATCC 14578^T with 99.9% sequence similarity (Figure 1). The isolate was different from *B. anthracis* strain on growth in 10% NaCl and acid production from D-cellobiose, D-mannose, salicin, and D-xylose as previously reported (Barrow and Feltham, 1993). *B. anthracis* and *B. cereus* strains are closely related species, therefore isolate SS503 is required to differentiate by molecular identification (Henderson *et al.*, 1994).

Group 7 contained isolate NM45-3. The strain grew at pH 5-9 and 20-45 °C but did not grow in 4% NaCl. It showed positive reaction for catalase, oxidase, VP reaction and hydrolysis of gelatin and esculin the same as *P. pasadenensis* SAFN-007^T but was different on growth in 4% NaCl and acid production from D-mannitol (Osman *et al.*, 2006). Based on the16S rRNA gene sequence, isolate NM45-3 (1,337 bp) was closely related to *P. pasadenensis* SAFN-007^T (Figure 1) with 99.55% sequence similarity. It is required for DNA-DNA hybridization experiment to clarify its taxonomic position at a species level.

The isolates showed lipolytic activity for Tween 20 (0.014 ± 0.129 - 0.758 ± 0.009 U/mL), Tween 40 (0.053 ± 0.013 - 1.322 ± 0.022 U/mL), Tween 60 (0.087 ± 0.005 - 1.358 ± 0.046 U/mL) and Tween 80 (0.050 ± 0.020 - 3.231 ± 0.087 U/mL) when they were cultivated in nutrient broth supplemented with Tween 20, Tween 40, Tween 60 or Tween 80, respectively (Figure 3). Isolate SS48-5 identified as *B. subtilis* produced the highest lipase activity (3.231±0.087 U/mL) when Tween 80 was used. The high lipase activity is based on Tween 80 that contained esters of oleic acid and produced rarely esterases compared to Tween 20 that was easily hydrolyzed by esterase as it contained esters of lower chain fatty acids viz. lauric acid (Kumar *et al.*, 2012). The effects of pH, temperature and incubation time on lipase activity of *B. subtilis* SS48-5 were carried out. The results revealed that it showed the highest lipase activity (0.82±0.01 U/mL) on Tween 80 at pH 7.5 after incubated for 24 h (Figure 4). In addition, the optimum temperature was at 40 °C and the incubation time for lipase production of this strain was between 30 h (3.15±0.03 U/mL) and 36 h (3.09±0.03 U/mL) (Figure 5).

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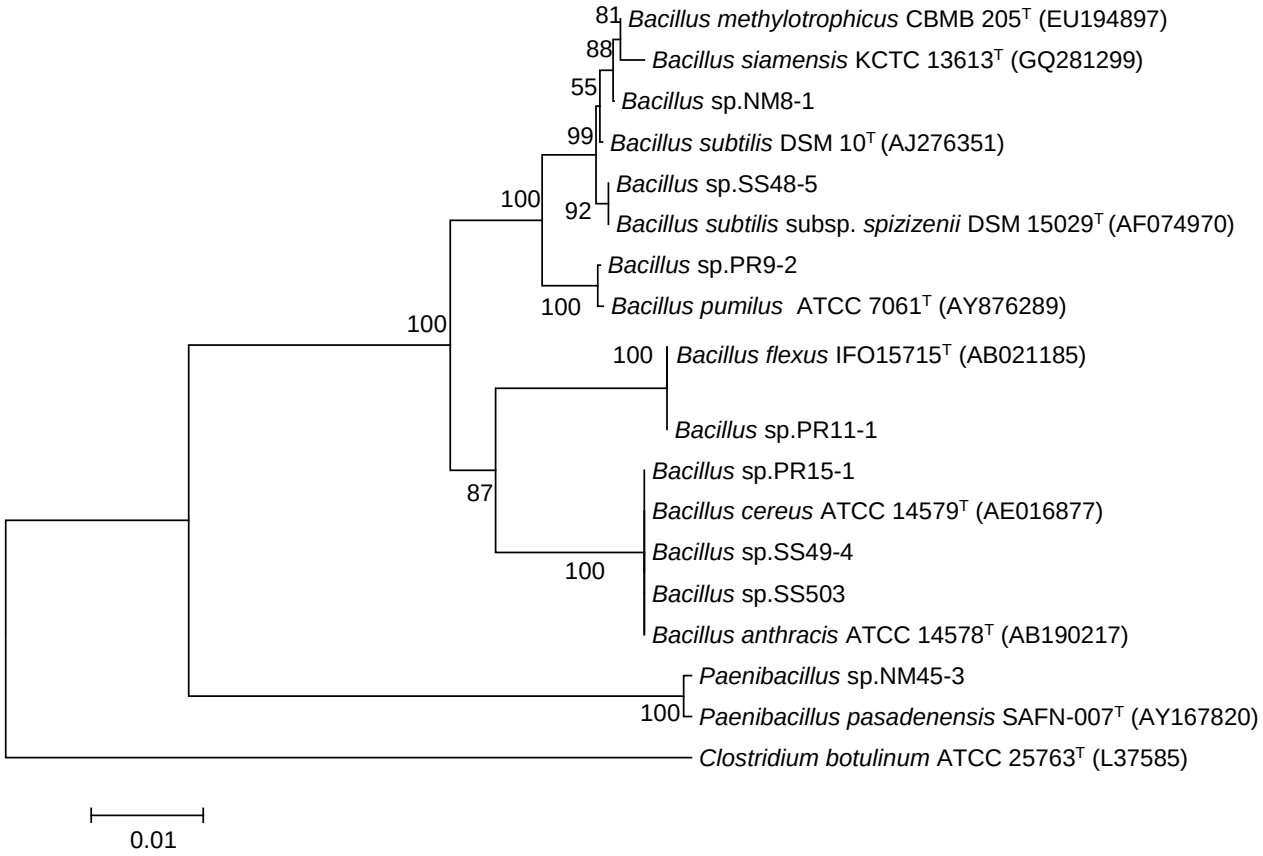

Figure 1: Neighbour-joining tree based on 16S rRNA gene sequences showing relationships among *Bacillus* and *Paenibacillus* isolates and related species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.01 substitutions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

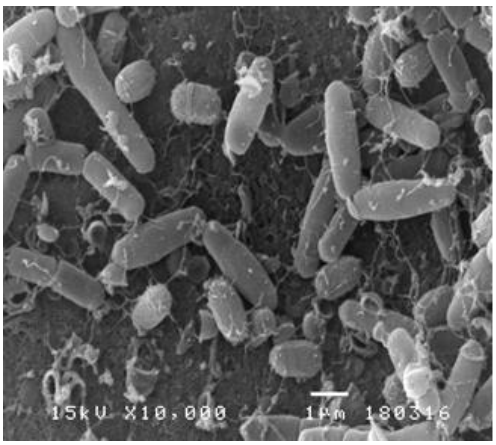

Figure 2: Scanning electron micrograph of isolate SS48-5 grew on TSA. (Bar, 1μm; 10,000× magnification).

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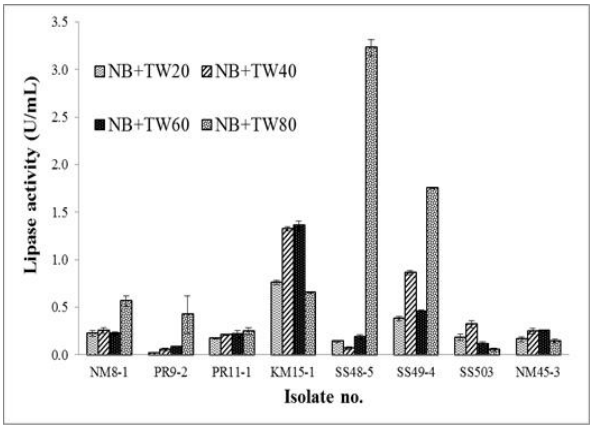

Figure 3: Lipase activity (U/mL) of isolates in nutrient broth (NB) with Tween 20, Tween 40, Tween 60 or Tween 80.

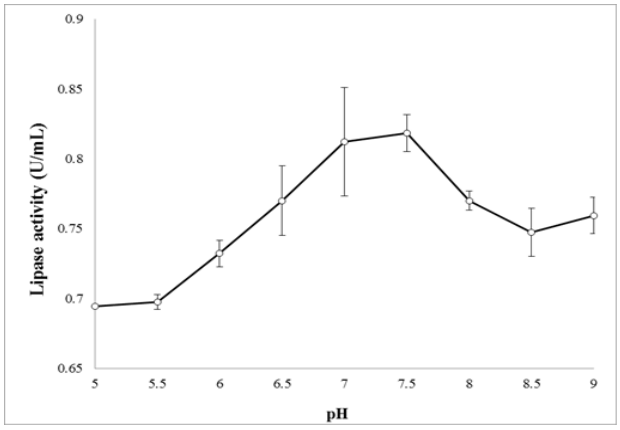

Figure 4: Effect of initial pH on lipase activity of isolate SS48-5 cultivated in NB with 1% Tween 80, at 30 °C for 24 h.

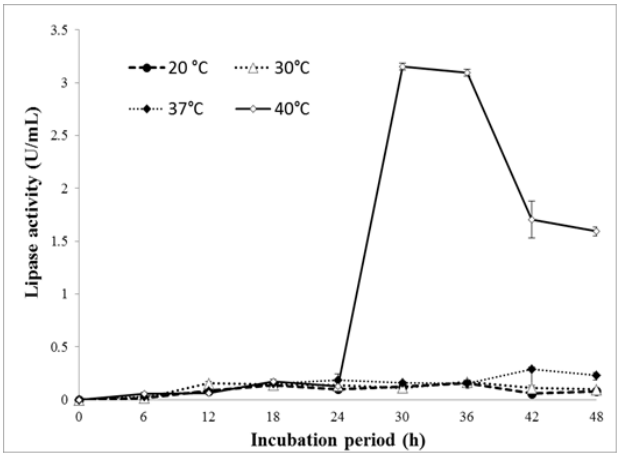

Figure 5: Effect of temperature and incubation time on lipase activity of isolate SS48-5 cultivated in NB with 1% Tween 80, pH 7.5).

As mentioned above, in Thai fermented foods, especially in *nham* collected in Utaradit province, the spore-forming bacteria, *B. methylotropicus* and *P. pasadenensis* were isolated; in *pla-ra* collected in Buriram province, *B. pumilus* and *B. flexus* strains were isolated; in *kung-chom* collected in Buriram province, *B. cereus* strain was isolated; in *sai-krog-prieo,* collected in Mahasarakram province, *B. cereus*, *B. subtilis*, and *B. anthracis* strains were found (Table 1). All of them exhibited lipase activity compared to the strains of *B. amylolicefacieus*, *B. cereus*, *B. megaterium, B. mesenterichs*, *B. mycoides*, *B. popilliae*, *B. stearothermophilus*, *B. subtilis* as previously reported (Achamma *et al*., 2003; Gupta *et al.*, 2004; Gayathri *et al*., 2013; Miettinen *et al*., 2013). These isolates may involve in the changes in lipid composition during fermentation that provide the flavor of the products. This study was the first report on the lipolytic spore forming Gram-positive bacteria in genera *Bacillus* and *Paenibacillus* isolated from Thai fermented foods including *nham*, *sai-krog-prieo, pla-ra* and *kung-chom*.

CONCLUSION

Eight lipolytic bacteria were isolated from Thai fermented foods. Seven isolates which belong to the genus *Bacillus* are closely related to *B. methylotropicus*, B*. pumilus*, B*. flexus*, B*. cereus*, *B. subtilis* and B*. anthracis* based on16S rRNA gene sequence analyses (99.90-100% similarity). An isolate which belongs to the genus *Paenibacillus* is closely related to *P. pasadenensis*. A potential isolate SS48-5 (Group 5) from *sai-krog-prieo* collected in Mahasarakram province, was identified as *B. subtilis* based on the phenotypic characteristics and 16S rRNA gene sequence analysis. The strain showed the highest lipase production when cultivated in NB with Tween 80, at pH 7.5 and incubated at 40 °C for 30 h.

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