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Screening and identification of cellulase producing bacteria isolated from oil palm meal

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ABSTRACT

Oil palm (*Elaeis guineensis*) meal, a by-product of palm oil, is rich in fiber and contains lignocelluloses, which inhibits the absorption of the nutrients has been widely used for animal feed. The improvement of the nutrient absorption is required treating with cellulase enzyme. This study was aimed to isolate, screen and characterize the cellulase producing bacteria. Ten strains of cellulolytic bacteria were isolated from 7 oil palm meal samples collected in Phetchaburi, Prachuap Khiri Khan and Pattani provinces, Thailand. They exhibited the ability to degrade carboxymethyl-cellulose (CMC) based on the decolorization of CMC-basal agar medium using Congo red as a color indicator. They showed the cellulase hydrolysis capacity ranged from 1.56 to 4.14. All isolates were Gram positive rod-shaped bacteria and belonged to *Bacillus* (8 isolates), *Paenibacillus* (1 isolate) and *Lysinibacillus* (1 isolate) based on the phenotypic characteristics and 16S rRNA gene sequence analysis. Their cellulase activity ranged from 0.039±0.002 to 0.233±0.005 IU/ml when they were cultivated in broth.

INTRODUCTION

Cellulose is the most abundant biological compound on terrestrial and aquatic ecosystem and is the main component of plant biomass (Shankar *et al*., 2011). It is the dominant waste material from agricultural industry in the form of stalks, stems and husk, there has been great interest in utilizing cellulose as an energy resource and feed (Balachandrababu *et al*., 2012). The cellulose is composed of D-glucose units linked together to form linear chain via ß-1,4-glycosidic linkages (Salmon and Hudson, 1997).

Cellulose is commonly degraded by cellulase. Cellulolytic enzyme system consists of three major components such as endoglucanases, exoglucanases and ß-glucosidases. Cellulases have a potential to use in biotechnology and industry such as, starch processing, alcoholic beverage, malting and brewing, clarify of juice, pulp bleaching, textile industry and animal feed (Sreeja *et al*., 2013).

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Oil palm (*Elaeis guineensis*) meal is an oil palm by-product, which is obtained from palm after the oil has been extracted. The predominant constituents of oil palm meal are cellulose, hemicellulose and lignin. Oil palm meal consists roughly 30% of cellulose (Yan *et al*., 2009; Rupani *et al*., 2010; Shahriarinour *et al*., 2011). These products are significant enough to consider as the bioresources of raw materials for agriculture, such as animal feed and compost. In our investigation of cellulolytic bacteria, the isolates from oil palm meal were screened and identified based on the phenotypic characteristics and 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Sources and isolation methods

Oil palm meal samples were collected from Phetchaburi, Prachuap Khiri Khan and Pattani provinces, the southern part of Thailand (Table 1). One gram of the sample was enriched in 100 ml of Berg's medium [containing (l^{-1}): 2 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$, 0.05 g K_2HPO_4, 0.01 g $FeSO_4.7H_2O$, 0.02 g $CaCl_2$ and 0.02 g $MnSO_4$, pH 7.0] (Immanuel *et al.*, 2006) and incubated at 37 °C on a rotator shaker (200 rpm) for 2 days.

The 0.1 ml suspension of 10^{-6} dilution in sterile 0.85% NaCl solution was transferred to Carboxymethyl Cellulose (CMC) screening medium [contain (l^{-1}): 3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7H_2O$, 1.0 g CMC and 15 g agar, pH 7.0] (Shankar *et al*., 2011) and incubated at 37 °C for 2 days. The colonies were isolated and purified by streaking on CMC agar plate. The pure cultures were maintained on LB slant at 4 °C for further analysis.

Screening of cellulase activity

Bacterial isolates were individually inoculated on CMC agar plates and incubated for 2 days. The plates were flooded with 0.1% Congo red for 20 min and washed with 1 M NaCl for 15 min. The clear zone formed by the isolates was indicated their cellulase activity (Lisdiyanti *et al*., 2012). Hydrolysis capacity (HC) value was calculated as described by Sreeja *et al.* (2013).

Identification methods

Phenotypic characterization

Cells grown on Luria-Bertani (LB) agar medium were examined for their morphological and cultural characteristics, including cell shape, colonial appearance, endospore formation and pigmentation, after incubated at 37 °C for 2 days. Catalase and oxidase, the methyl red (MR) and Voges-Proskauer (VP) reactions, indole production, nitrate reduction, hydrolysis of aesculin, L-arginine, casein, starch, and Tween 80, citrate utilization, H_2S production and acid formation from carbohydrates were determined as described by Barrow and Feltham (1993). Growth under anaerobic condition on agar plates was investigated using a Gaspak (BBL) anaerobic jar. Growth at different pH (5, 6, 7, 8 and 9), in 3 and 5 % (w/v) NaCl and at different temperatures (37, 45, 50, 55 and 60 °C) were tested by using LB agar medium. All tests were carried out by incubating the cultures at 37 °C, except for investigations into the effect of temperature on growth.

Genotypic characterization

The 16S rRNA gene sequencing was carried out by using the primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR product was purified and sequenced as described previously (Tanasupawat *et al*., 2004). The nucleotide sequence of isolates were aligned with selected sequences obtained from GenBank by using CLUSTAL_X version 1.83 (Thompson *et al*., 1997). The alignment was edited manually to remove gaps and ambiguous nucleotides prior to the construction of phylogenetic trees. The phylogenetic tree was constructed by using the neighbour-joining (Saitou and Nei, 1987). The confidence values of branches of the phylogenetic trees were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Measurement of crude cellulase

The 2% inoculum of bacterial isolates were inoculated into production medium containing (g/L), 10g CMC, 0.2g $MgSO_4.7H_2O$, 0.75g KNO_3, 0.5g K_2HPO_4, 0.02g $FeSO_4.7H_2O$, 0.04 $CaCl_2$, 2 g yeast extract and 1g D-glucose, at pH 7.0 (Lisdiyanti *et al*., 2012) and incubated at 37 °C on rotator shaker (200 rpm) for 2 days. After incubation, the fermentation broth was harvested by centrifugation at 5,000 g for 20 min at 4 °C. The cell free extract was used as crude enzyme to measure the cellulase activity.

Cellulase activity was determined by estimating the reducing sugar produced during enzymatic reaction by dinitrosalicylic acid (DNS) (Miller, 1959). A reaction mixture composed of 0.5 ml of crude enzyme, 0.5 ml of 0.05 M citrate-phosphate buffer (pH 7.0) and 1.0 ml of 1% (w/v) CMC (Sigma-Aldrich) in 0.05 M citrate-phosphate buffer (pH 7.0) was incubated at 37 °C for 15 min. The reaction was terminated by adding 1.5 ml of DNS reagent. After boiling the mixture for 5 min, the color was developed and the samples were measured using a colorimeter as the absorbance at 540 nm against a blank containing all the reagents minus the crude enzyme. One unit (IU) of cellulase activity was defined as the amount of enzyme required to releasing 1 µmole of glucose produce per min per ml of crude supernatant under assay condition. All the experiments were carried out in triplicates. The mean values are shown in Figure 2.

RESULTS AND DISCUSSION

Isolation and Screening of isolates

Ten bacterial isolates, PH3-48, PH4-34, PH4-36, PH5-42, PH21, PH27 and PH32 were isolated from oil palm meal samples collected in Phetchaburi province, PJ1-24B and PJ1-24S were from samples in Prachuap Khiri Khan province and the isolate PT31 was selected from sample in Pattani province, Thailand (Table 1). All the isolates had capability to degrade CMC. They showed the cellulase hydrolysis capacity ranged from 1.56 to 4.14 when screened by Congo red method (Table 1).

Table. 1: Isolate number, location, Group, hydrolysis capacity (HC), sequence similarity (%) and closest species.

Isolate no.	Province	Group	HC*	Similarity (%)	Closest species
PH3-48	Phetchaburi	1	1.95	99.1	*B. tequilensis* ATCC BAA-819^T
PH4-34	Phetchaburi	2	1.92	98.8	*B. cereus* IAM 12605^T
PH4-36	Phetchaburi	2	2.12	98.8	*B. cereus* IAM 12605^T
PJ1-24B	Prachuap Khiri Khan	2	1.88	99.2	*B. cereus* IAM 12605^T
PH5-42	Phetchaburi	3	2.44	98.5	*B. subtilis* DSM 10^T
PH21	Phetchaburi	3	1.56	98.8	*B. subtilis* DSM 10^T
PH27	Phetchaburi	4	2.80	99.7	*B. altitudinis* DSM 21631^T
PJ1-24S	Prachuap Khiri Khan	5	4.14	99.9	*B. safensis* NBRC 100820^T
PH32	Phetchaburi	6	2.12	96.2	*P. panacisoli* KCTC 13020^T
PT31	Pattani	7	3.00	99.1	*L. fusiformis* NBRC 15717^T

*Hydrolysis capacity (HC), the ratio of the clear zone diameter to the colony diameter.

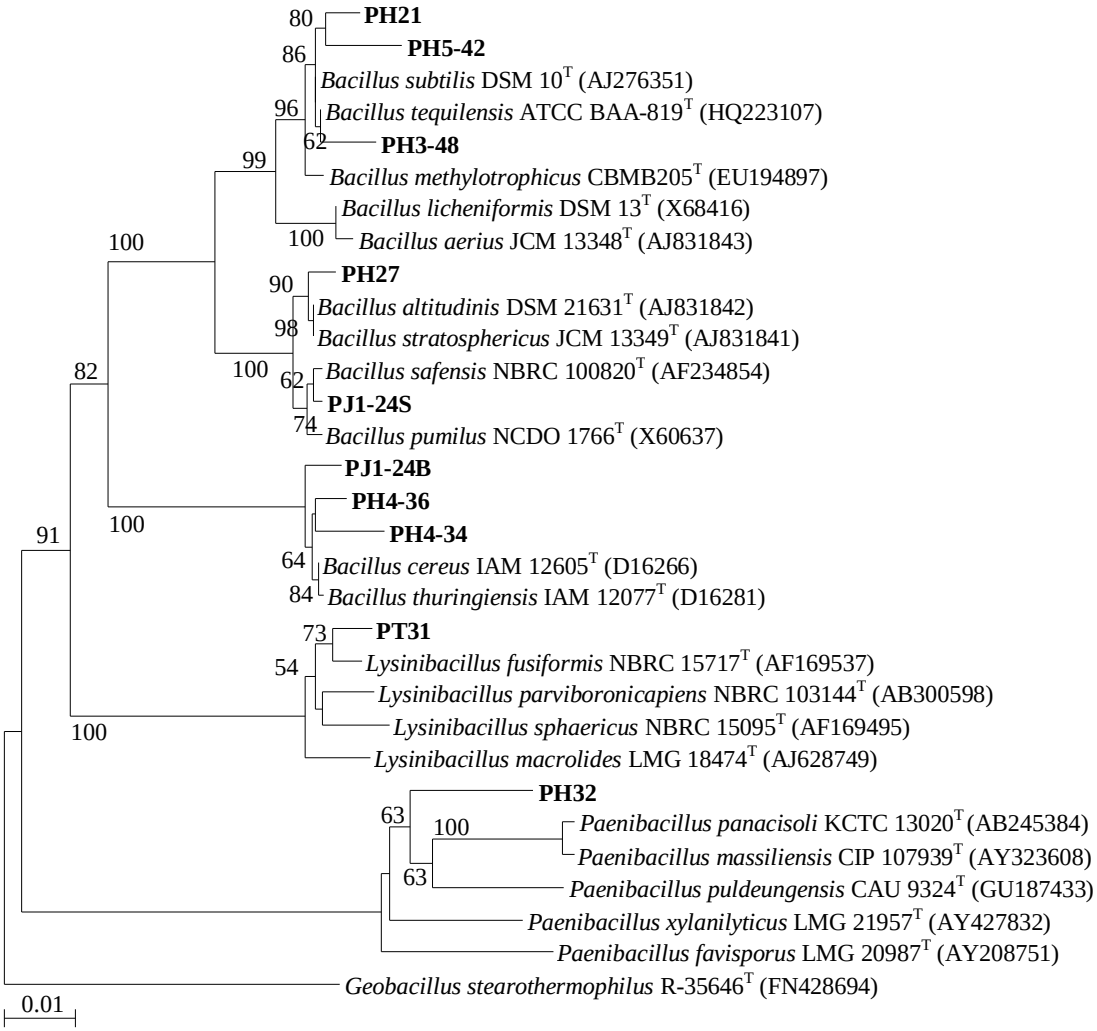

Fig.1: Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of isolates and related species. Based on 1000 replications, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position.

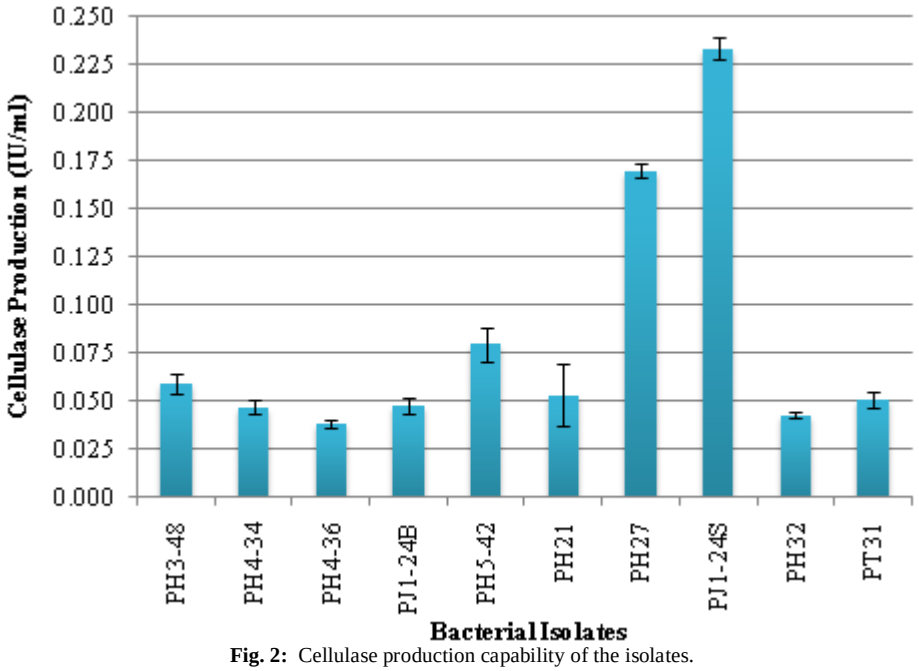

Fig. 2: Cellulase production capability of the isolates.

Table. 2: Differential characteristics of isolates in Group 1 to 7.

Characteristics	1	2	3	4	5	6	7
Cell form	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Oxidase	+	+	+	+	+	-	+
Growth at pH:							
5	+	-	+	+	-	+	-
9	+	+	+	+	+	+	-
Growth at :							
45 ℃	+	+	+	+	+	+	-
50 ℃	+	-	+	+	+	+	-
55 ℃	+	-	+	-	-	-	-
Growth in:							
3% NaCl	+	+	+	+	+	+	-
5% NaCl	+	+	+	+	+	-	-
Methyl red	-	+(-1)	-	-	-	-	-
Nitrate reduction	-	+(-1)	+	-	-	+	-
Hydrolysis of:							
L-Arginine	+	+	+	w	-	-	-
Casein	+	w	+	w	+	-	-
Starch	+	+(-1)	+	-	-	+	+
Citrate utilization	+	-	w	+	-	-	-
Acid from:							
L-Arabinose	W	-	-	-	-	+	-
D-Cellobiose	-	-	+	w	+	+	-
D-Fructose	+	+	+	+	+	+	-
D-Galactose	+	-	-	+	+	+	-
D-Glucose	+	+	+	+	+	+	-
Inositol	-	-	-	-	-	w	-
Inulin	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	+	-
D-Maltose	-	+	-	-	-	+	-
D-Mannitol	-	-	-	-	-	+	-
D-Mannose	+	+	+	+	+	+	-
D-Melibiose	+	-	-	-	-	+	-
D-Melezitose	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	+	-
L-Rhamnose	-	-	-	-	-	+	-
D-Ribose	w	+	-	+	w	+	-
Salicin	-	+(-1)	+	+	+	+	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	+	+(-1)	+	+	+	w	-
D-Trehalose	-	+	+	-	-	+	-
D-Xylose	+	-	-	-	-	+	-

+, positive; -, negative; w, weakly positive. Number in parentheses indicates the number of isolate shows positive or negative reaction.

Identification of isolates

Ten cellulolytic bacteria were Gram-positive, facultative anaerobic, spore forming rod-shaped bacteria. They were divided into seven groups based on their phenotypic characteristics and the 16S rRNA gene sequence analysis. All isolates showed positive reaction for catalase, hydrolysis of aesculin and Tween 80 but did negative for Voges-Proskauer (VP) reaction, indole, and hydrogen sulfide formation. They grew in anaerobic condition, grew at pH 6-8, but they did not grow at 60 °C. Their differential phenotypic characteristics are described below and in Table 2.

Group 1 contained isolate PH 3-48. Colonies of this strain were 1-6 mm in diameter, round, smooth and creamy-white after incubation at 37 °C on LB agar plate for 2 days. It grew in 3% and 5% NaCl, at pH 5-9 (optimally at 7) and 37-55 °C (optimally at 37 °C). The strain showed positive for oxidase, hydrolysis of L-arginine, casein, starch and citrate utilization, but negative for Methyl-Red and nitrate reduction. The strain produced acid from L-arabinose (weakly), D-fructose, D-galactose, D-glucose, D-mannose, D-melibiose, D-ribose (weakly), sucrose and D-xylose. On the basis of 16S rRNA gene sequence, isolate PH3-48 (1,398 nt) was closely related to *Bacillus tequilensis* ATCC BAA-819^T (Figure 1) with 99.1% sequence similarity. This isolate was different from *B. tequilensis* ATCC BAA-819^T in colony pigment, indole formation, nitrate reduction, acid production from D-cellobiose, inositol, lactose, D-mannitol, D-melezitose, D-sorbitol, and D-trehalose (Gatson *et al.*, 2006). The DNA-DNA hybridization with *B. tequilensis* ATCC BAA-819^T is required to clarify its taxonomic position at a species level.

Group 2 contained isolates PH4-34, PH4-36 and PJ1-24B. Their colonies were 1-5 mm in diameter, irregular, lobate and creamy-white. They grew in 3% and 5% NaCl, at pH 6-9 (optimally at 7) and 37-45 °C (optimally at 37 °C) but did not grow at pH 5, at 50-60 °C. They showed positive reaction for oxidase, hydrolysis of L-arginine, and casein (weakly), but negative for citrate utilization. They produced acid from D-fructose, D-glucose, D-maltose, D-mannose, D-ribose and D-trehalose. On the basis of 16S rRNA gene sequence, isolates PH4-34 (1,429 nt), PH4-36 (1,426 nt) and PJ1-24B (1,395 nt) were

closely related to *Bacillus cereus* IAM 12605^T (Figure 1) with 98.8, 98.8 and 99.2% sequence similarity, respectively. The isolates showed almost the same characteristics as *B. cereus* strains, except the difference in acid production from D-mannose (Logan *et al*., 2002; Yoon *et al.*, 2005). They are required for DNA-DNA hybridization experiment to clarify its taxonomic position at a species level.

Group 3 contained isolates PH5-42 and PH21. Their colonies were 1-3 mm in diameter, irregular, lobate and creamy-white. They grew in 3% and 5% NaCl, at pH 5-9 (optimally at 7) and 37-55 °C (optimally at 37 °C). They showed positive reaction for oxidase, nitrate reduction, hydrolysis of L-arginine, casein, and starch; and citrate utilization (weakly), but negative for methyl-red. They produced acid from D-cellobiose, D-fructose, D-glucose, D-mannose, salicin, sucrose and D-xylose. On the basis of 16S rRNA gene sequence, isolates PH5-42 (1,402 nt) and PH21 (1,437 nt) were closely related to *Bacillus subtilis* DSM 10^T (Figure 1) with 98.5 and 98.8% sequence similarity, respectively. They were different from *B. subtilis* strains (Gatson *et al.*, 2006; Lim *et al.*, 2006) in growth at 55 °C, acid production from L-arabinose, D-maltose, D-mannitol, D-melibiose, raffinose, D-sorbitol and D-xylose. They are required for DNA-DNA hybridization experiment to clarify its taxonomic position at a species level.

Group 4 contained isolate PH27. The colonies of this isolate were 2-5 mm in diameter, irregular, lobate and creamy-white. It grew in 3% and 5% NaCl, at pH 5-9 (optimally at 7) and 37-50 °C (optimally at 37 °C) but did not grow at 55 and 60 °C. They showed positive reaction for oxidase, hydrolysis of L-arginine (weakly), casein (weakly) and citrate utilization, but negative for methyl-red and nitrate reduction. The strain produced acid from D-cellobiose (weakly), D-fructose, D-galactose, D-glucose, D-mannose, D-ribose, salicin and sucrose. On the basis of 16S rRNA gene sequence, isolate PH27 (1,437 nt) was closely related to *Bacillus altitudinis* DSM 21631^T (Figure 1) with 99.7% sequence similarity. This isolate showed positive on acid production from D-fructose, D-galactose, D-glucose, D-mannose and sucrose the same as *B. altitudinis* DSM 21631^T but it was different on growth in 3% NaCl, nitrate reduction, Voges-Proskauer test, hydrolysis of casein and starch, and citrate utilization (Shivaji *et al.*, 2006). However, it exhibited high similarity to *B. altitudinis* DSM 21631^T, therefore, it was identified as *B. altitudinis*.

Group 5 contained isolate PJ1-24S. The colonies of isolate were 0.5-6 mm in diameter, irregular or smooth, lobate and creamy-white. The strain grew in 3% and 5% NaCl, at pH 6-9 (optimally at 7) and 37-50 °C (optimally at 37 °C). They showed positive reaction for oxidase, hydrolysis of casein, but negative for hydrolysis of L-arginine and starch, citrate utilization, methyl-red and nitrate reduction. The strain produced acid from D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, D-ribose (weakly), salicin and sucrose. On the basis of 16S rRNA gene sequence, isolate PJ1-24S (1,399 nt) were closely related to *Bacillus safensis* NBRC 100820^T (Figure 1) with 99.9% sequence similarity. This isolate showed almost the same characteristics as *B. safensis* NBRC 100820^T but it was different in Voges-Proskauer test, acid production from L-arabinose, D-mannitol and D-trehalose (Satomi *et al*., 2006). This isolate exhibited high similarity to *B. safensis* NBRC 100820^T, therefore, it was identified as *B. safensis*.

Group 6 contained isolate PH32. The colonies of isolate were 1-3 mm in diameter, irregular or smooth, lobate and creamy-white. The strain grew in 3% NaCl, at pH 5-9 (optimally at 7) and 37-50 °C (optimally at 37 °C) but did not grow in 5% NaCl, at 55 and 60 °C. They showed positive reaction for hydrolysis of starch but negative for oxidase, hydrolysis of L-arginine and casein and citrate utilization, methyl-red, and nitrate reduction. The strain produced acid from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, inositol (weakly), lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, raffinose, L-rhamnose, D-ribose, salicin, sucrose (weakly), D-trehalose, and D-xylose. On the basis of 16S rRNA gene sequence, isolate PH32 (1,454 nt) was closely related to *Paenibacillus panacisoli* KCTC 13020^T reported by Ten *et al.* (2006) with 96.2% sequence similarity (Figure 1). The isolate 16S rRNA gene sequence similarity showed less than 97% as an accepted criterion for differentiation of bacterial species (Stackebrandt and Goebel, 1994). In addition, this isolate was different from *P. panacisoli* KCTC 13020^T in growth at 50 °C and in 5% NaCl, hydrolysis of casein and starch, acid production from D-glucose, inositol, L-rhamnose and sucrose (Ten *et al.*, 2006). Therefore, isolate PH32 should represent the novel species of the genus *Paenibacillus*.

Group 7 contained isolate PT31. The colonies of isolate were 3-6 mm in diameter, round, smooth and yellow. The strain grew at pH 6-8 (optimally at 7) and 37 °C but did not grow in 3% and 5% NaCl, at pH 5 and 9, at 45-60 °C. They showed positive reaction for oxidase, hydrolysis of starch but negative for hydrolysis of L-arginine and casein, citrate utilization, methyl-red and nitrate reduction but it produced no acid from all carbohydrates. On the basis of 16S rRNA gene sequence, isolate PT31 (1,400 nt) was closely related to *Lysinibacillus fusiformis* NBRC 15717^T (Figure 1) with 99.1% sequence similarity. However, the phenotypic characteristics of this isolate were different from *L. fusiformis* (Ahmed *et al*., 2007). Therefore, it is required for DNA-DNA hybridization experiment to clarify its taxonomic position at a species level.

As mentioned above, our isolates very closely related to *B. tequilensis*, *B. cereus*, *B. subtilis*, *B. altitudinis* and *Paenibacilus* strains were isolated from the samples collected in Phetchaburi province, while *B. cereus*, *B. safensis* strains were isolated from the samples collected in Prachuap Khiri Khan province, and *L. fusiformis* was from Pattani province.

Quantification of cellulase activity

The isolate in Group 5, PJ1-24S was exhibited the highest cellulase activity with 0.233±0.005 IU/ml, which are

significantly different from the other isolates. The isolate PH27 in Group 4 exhibited 0.170±0.004 IU/ml of cellulase activity. The minimum cellulase activity were evaluated by the isolates in Group 1, 2, 3, 6 and 7 (0.039±0.002 to 0.079±0.009 IU/ml) as shown in Figure 2. Cellulolytic enzymes produced by several microorganisms, commonly in bacteria in the genera, *Acetivibrio*, *Bacillus*, *Bacteroides*, *Cellulomonas*, *Cellvibrio*, *Clostridium*, *Micrococcus*, *Paenibacillus*, *Pseudomonas*, *Ruminococcus*, especially in *B. subtilis*, *B. cereus*, *B. altitudinis* and *Paenibacillus* sp. and in fungi, *Aspergillus*, *Fusarium*, *Penicillium*, *Rhizopus* and *Trichoderma* which they were isolated from soil, sediment, intestine of the fish, cow dung, paper sludge and poultry manure compost have been reported (Wang *et al*., 2008; Shahriarinour *et al*., 2011; Balachandrababu *et al*., 2012; Kumar *et al.*, 2012; Shanmugapriya *et al*., 2012; Sethi *et al*., 2013). This study, we found the isolates closely related to *B. tequilensis*, *B. cereus*, *B. subtilis*, *B. altitudinis*, *B. safensis*, *L. fusiform*, and *Paenibacillus* have showed the same cellulase activity.

CONCLUSION

In conclusion, 10 cellulase producing bacteria were isolated from oil palm meal samples from Phetchaburi, Prachuap Khiri Khan and Pattani, Thailand. They were screened for their cellulase activity by Congo red test and they showed hydrolysis capacity value ranged from 1.56-4.14. When the isolates cultivated in the CMC broth, the isolate, PJ1-24S in Group 5, identified as *B. safensis*, showed the best cellulase activity (0.233±0.005 IU/ml). The isolates in Group 1, 2, 3, 4, 6 and 7 exhibited lower cellulase activity ranged from 0.039±0.002 to 0.170±0.004 IU/ml. On the basis of their phenotypic characteristics and phylogenetic analyses, 9 isolates were closely related to *B. tequilensis*, *B. cereus*, *B. subtilis*, *B. altitudinis*, *B. safensis*, and *L. fusiformis*. Only the isolate PH32 showed low 16S rRNA gene sequence similarity (96.2%) with the closest type strain. Thus, this isolate should represent a novel species of the genus *Paenibacillus*. In this study, the isolate PJ1-24S that produced high cellulase will be useful to improve the absorption of nutrients of the oil palm meal as the animal feed, however the further study for this isolate is required.

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