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CHARACTERIZATION OF XYLANOLYTIC *PAENIBACILLUS* STRAINS ISOLATED IN THAILAND

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Abstract: Seven xylanolytic bacteria were isolated from soils and muddy shore sediments collected in Thailand. All isolates were Gram-positive, spore-forming, rod-shaped bacteria. They were belonged to genus *Paenibacillus* and were divided into 5 groups based on their phenotypic and chemotaxonomic characteristics including 16S rRNA gene sequence analyses. They contained *meso*-diaminopimelic in cell wall peptidoglycan and menaquinone with seven isoprene units. An isolate (Group 1) was identified as *P. macerans*, 3 isolates (Group 2) were *P. barengoltzii*, each isolate in Group 3, 4 and 5, was *P. timonensis*, *P. montaniterrae* and *P. dendritiformis*, respectively. The xylanase producing isolate in Group 1 showed clear zone with 6.5 mm in diameter and produced xylanase activity with 3.20±0.7 unit/ml while Group 2 to Group 5 isolates showed clear zone with 1.5-5.0 mm in diameter and produced xylanase activity ranged from 1.07±0.15 to 1.86±0.20 unit/ml.

Keywords: *Paenibacillus*, *P. macerans*, *P. barengoltzii*, *P. timonensis*, *P. montaniterrae*, *P. dendritiformis*, Xylanolytic bacteria

INTRODUCTION

Xylan, the most abundant of the hemicelluloses containing hetero-polysaccharides, consists of a backbone of β-1, 4-linked xylopyranose residues with substitutions of *O*-acetyl, arabinosyl and methylglucuronosyl substitutents[1-3]. The combined action of various enzymes such as endoxylanase, β-xylosidase and several accessory enzymes are required to hydrolyse substituted xylan. Xylanolytic enzymes occur widely in bacteria, yeasts and fungi. Many microorganisms are known to produce different type of xylanases and the nature of the enzymes varies between different organisms. The xylan degrading bacteria such as the strains of *Aeromonas*, *Bacillus*, *Bacteroides*, *Cohnella*, *Cellulomonas*, *Microbacterium*, *Paenibacillus*, *Ruminococcus*, *Streptomyces*, *Xylani bacterium* and *Xylanibacter* have been reported[4-11]. Recently, xylanase is an enzyme in high demand for various industrial applications such as those in the saccharification of hemicellulosic wastes[12], clarification of juices and wines, extraction of plant oils and coffee[13-14], in pulp and paper fields[15-16] and in flour improvement for bakery products[17]. This work deals with the screening, identification and characterization of the xylanase-producing Gram-positive, spore-forming, rod-shaped bacteria isolated from soils and muddy shore sediments in Thailand based on their phenotypic and chemotaxonomic characteristics including 16S rRNA gene sequence similarity.

MATERIALS AND METHODS

Isolation and screening of xylanase activity: Seven xylanolytic bacteria were isolated from soils and muddy shore sediments collected in Thailand (Table.1), by the spread plate method on XC agar medium [containing (l^{-1}): 10g oat spelt xylan, 5g peptone, 1g yeast extract, 4g K_2HPO_4, 1g $MgSO_4.7H_2O$, 0.2g KCl, 0.02g $FeSO_4.7H_2O$, 15 g agar; at pH 7.0]. In this screening step for the spore-forming strains, the agar plates were incubated at 40 °C for 2 days. Xylanase-producing capacity of the cultures was detected by using a Congo red overlay method, as reported previously[18-19]. Isolates showing xylanase-producing capacity were transferred to C agar medium. This medium had the same composition of XC medium apart from the omission of the oat spelt xylan. They were assayed for xylanase activity by DNS method using 1% Oat spelt xylan as substrate[20]. The reaction mixtures were incubated at 37 °C for 10 min.

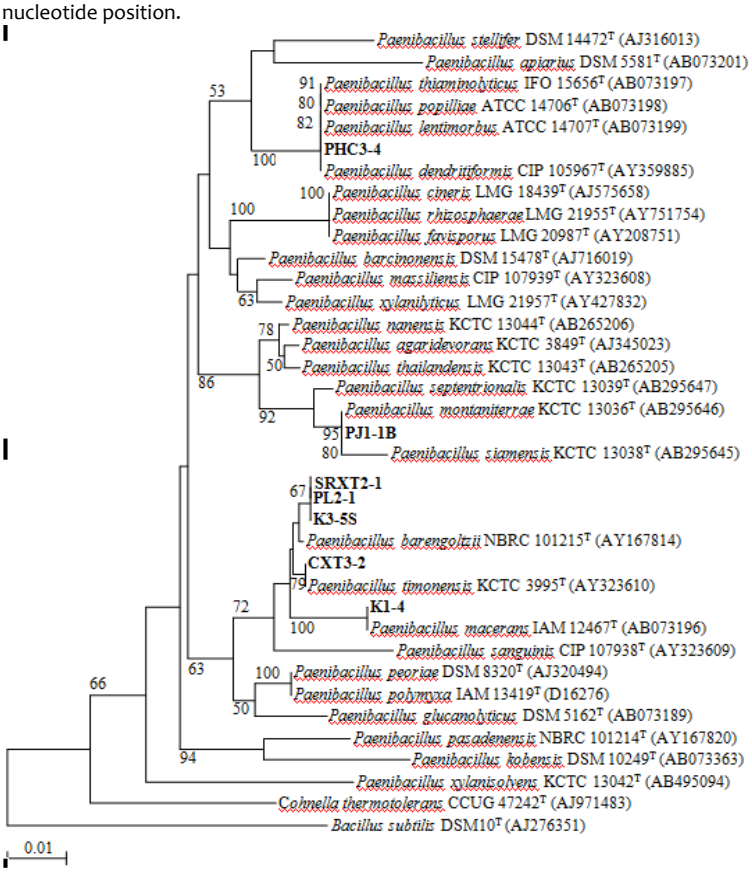
Identification methods: Cells grown on C agar medium were examined for their morphological and cultural characteristics, including cell shape, colony appearance, endospore formation and pigmentation, after incubation at 37 °C for 2 days. Physiological and biochemical characterization was performed using the API 20NE and API 50CH (combined with API 50CHB/E medium) strips (bioMérieux), in accordance with the manufacturer's directions. Catalase and oxidase, hydrolysis of casein, DNA, starch, Tween 80 and L-tyrosine, urea, the methyl red/Voges-Proskauer reactions, indole production, citrate utilization and hydrogen sulfide (H_2S) production were determined [21]. Growth at different pH (5, 6, 7, 8 and 9), in 3 and 5% (w/v) NaCl and at different temperatures (10, 15, 20, 25, 30, 40, 45, 50, 55 and 60 °C) was tested by using C agar medium. All tests were carried out by incubating the cultures at 37 °C, except for investigations into the effect of temperature on growth. Diaminopimelic acid in the cell wall and quinone system were determined[22]. DNA was prepared as previous reported[23]. DNA base composition was determined by reversed-phase HPLC[24]. The 16S rRNA genes of the strains were amplifed and PCR products were purified and sequenced as described previously[25]. The sequences of strains were aligned with selected sequences obtained from GenBank by using CLUSTAL_X version 1.83[26]. The alignment was edited manually to remove gaps and ambiguous nucleotides prior to the construction of phylogenetic trees. The phylogenetic trees were constructed by using the neighbour-joining[27] method in MEGA4 software[28]. The confidence values of branches of the phylogenetic trees were determined using bootstrap analysis[29] based on 1000 resample datasets.

RESULTS AND DISCUSSION

Isolation and screening of xylanase activity: Seven isolates showed xylanase clear zone 1.5-6.5 mm in diameter, surrounded their colonies. The xylanase producing bacteria of Group 1 isolate showed clear zone with 6.5 mm in diameter and produced xylanase activity with 3.20±0.7 unit/ml while Group 2 to Group 5 isolates showed clear zone with 1.5-5.0 mm in diameter and produced xylanase activity ranged from 1.07±0.15 to 1.86±0.20 unit/ml as shown in Table.1. In this study, the isolates showed a wide ranges of xylanolytic activity and were better than the non spore-forming, Gram-positive irregular rods, *Microbacterium barkeri* (0–0.13 units/ml) and the isolates of Gram-positive spore-forming rods, *Bacillus niabensis*, *B. funiculus*, *B. megaterium*; the isolates of Gram-negative rods, *Pseudoxanthomonas suwonensis*, *Cupriavidus gilardii*; and an isolate of Gram-positive rods/cocci, *Rhodococcus rhodochrous* (0–0.17 units/ml) as previous reported[30]. In addition, the xylanolytic strains were reported in the novel species *Paenibacillus septentrionalis*, *Paenibacillus montaniterrae*, *Paenibacillus thailandensis*, *Paenibacillus nanensis* and *Paenibacillus xylanisolvens* isolated from Thai soils [8,9,11].

Identification and characterization of isolates: Seven isolates were Gram-positive, spore-forming, rod-shaped bacteria. Six isolates were facultatively anaerobic and one isolate was aerobic. They contained *meso*-diaminopimelic in cell wall peptidoglycan and menaquinone with seven isoprene units. They were divided into 5 groups and were clustered within a clade of the genus *Paenibacillus* based on their phenotypic properties and 16S rRNA gene sequence and phylogenetic analyses (Fig.1 and Table.1).

Figure.1: Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between the isolates in group 1 to 5 and *Paenibacillus* species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position.



Group 1 contained isolate K1-4. Colonies were 1.5-4 mm in diameter, round, curled, concentric, flat and white after 2 days of incubation at 37 °C on C medium. The isolate grew in 3-5% NaCl, at pH 7-9 and at 25-50 °C. The isolate showed positive reaction for catalase, oxidase and hydrolysis of aesculin, L-arginine, casein, gelatin and starch, but negative for hydrolysis of DNA, Tween 80 and urea, methyl red, Voges-Proskauer, citrate utilization, H_2S production, indole production and nitrate reduction. It produced acid from various carbohydrates (Table.2), but did not produce acid from inositol, sorbitol and sorbose.

On the basis of 16S rRNA gene sequence and phylogenetic analyses, isolate K1-4 (1,432 nt) was closely related to *P. macerans* IAM 12467^T (Fig.1) with 99.6% 16S rRNA gene sequence similarity. Therefore, the isolate K1-4 was identified as *P. macerans* [31].

Table.1: Location, sample, isolate number, group, xylanase activity and identification of the isolates

Location (Province)	Sample	Isolate no.	Group	Clear zone (mm)	Xylanase activity (unit/ml)*	Identification
Kanchanaburi	Soil	K1-4	1	6.5	3.20±0.07	*P. macerans*
	Soil	K3-5S	2	2.0	1.86±0.20	*P. barengoltzii*
Phetchaburi	Muddy shore sediment	PHC3-4	5	1.5	1.32±0.17	*P. dendritiformis*
	Muddy shore sediment	PL2-1	2	1.95	1.64±0.10	*P. barengoltzii*
Prachuapkhirikhan	Muddy shore sediment	PJ1-1B	4	1.5	1.31±0.13	*P. montaniterrae*
Suratthani	Soil	SRXT2-1	2	5.0	1.16±0.12	*P. barengoltzii*
Nakhonsithammarat	Soil	CXT3-2	3	1.9	1.07±0.15	*P. timonensis*

*Note: one unit of xylanase activity was defined as 1 µmol of xylose released per min under the condition assayed

Group 2 contained 3 isolates, SRXT2-1, K3-5S and PL2-1. Colonies were 2-20 mm in diameter, circular, entire, smooth, raise or flat and yellow after 2 days of incubation at 37 °C on C medium. All isolates grew in 3% NaCl, at pH 7-9 and at 25-55 °C but did not grow in 5% NaCl and at 10-15 °C. They showed positive for catalase,oxidase and hydrolysis of aesculin, but negative for Voges-Proskauer, H_2S production, indole production, citrate utilization and hydrolysis of L-tyrosine and urea. They did not produce acid from D-amygdalin, D-galactose, D-glucose, glycerol, inositol, inulin, D-maltose, D-melezitose, α-glucopyranoside, L-rhamnose, sorbitol, sucrose and sorbose. Variable characteristics were found in nitrate reduction and other reactions as shown in Table.2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, the isolates SRXT2-1 (1,026 nt), K3-5S (1,016 nt), PL2-1 (981 nt) were closely related to *P. barengoltzii* NBRC 101215^T (Fig.1) with 99.8%, 99.8%, and 99.7% 16S rRNA gene sequence similarity, respectively. Therefore, the isolates SRXT2-1, K3-5S and PL2-1 were identified as *P. barengoltzii* [32].

Group 3 contained isolate CXT3-2. Colonies were 2-20 mm in diameter, circular, entire, smooth, raise or flat and yellow after 2 days of incubation at 37 °C on C medium. This isolate grew in 3% NaCl, at pH 6-9 and 25-50 °C, but did not grow in 5% NaCl, pH 5 and at 10-15 and 55-60 °C. The isolate showed positive for catalase, oxidase, methyl red and hydrolysis of aesculin and gelatin, but did not hydrolyse L-arginine, casein, DNA, starch, L-tyrosine, Tween 80 and urea, and showed negative for Voges-Proskauer, citrate utilization, H_2S production, indole production and nitrate reduction. Acid was produced from D-galactose, D-glucose, gluconate, D-maltose, D-melibiose, D-ribose (weak) and D-xylose but was not from D-amygdalin, L-arabinose, D-cellobiose, D-fructose, glycerol, inulin, lactose, inositol, D-mannitol, D-mannose, D-melezitose, α-glucopyranoside, raffinose, L-rhamnose, salicin, sorbitol, sorbose, sucrose and D-trehalose as shown in Table.2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, the isolate CXT3-2 (979 nt), showed 100% similarity of 16S rRNA gene sequence when compared with *P. timonensis* KCTC 3995^T. Therefore, the isolate CXT3-2 was identified as *P. timonensis*[33].

Group 4 contained isolate PJ1-1B. Colonies were 1-5 mm in diameter, circular, entire or curled, smooth or concentric, flat, opaque and yellow after 2 days of incubation at 37 °C on C medium. This isolate grew in 3% NaCl (weak), at pH 7-9 and 25-45 °C, but did not grow in 5% NaCl, pH 5-6 and at 10-15 and 50-60 °C. It showed positive for catalase, oxidase and hydrolysis of aesculin, starch, DNA and urea, but showed negative for methyl red, Voges-proskauer, citrate utilization, H_2S production, indole production, nitrate reduction and hydrolysis of L-arginine, casein, gelatin, L-tyrosine and Tween 80. Acid was produced from L-arabinose, D-cellobiose, D-galactose, D-glucose (weak), gluconate, L-rhamnose (weak), D-trehalose and D-xylose (weak) but was not produce acid from D-amygdalin, D-fructose, glycerol, inositol, inulin, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-glucopyranoside, raffinose, D-ribose, salicin, sorbitol, sorbose and sucrose as shown in Table 2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, the isolate PJ1-1B (919 nt) was closely related to *P. montaniterrae* KCTC 13036^T (Fig. 1) with 99.7% 16S rRNA gene sequence similarity. Therefore, the isolate PJ1-1B was identified as *P. montaniterrae* [8].

Table.2: Differential characteristics of *Paenibacillus* isolates in Group 1 to 5

Characteristics	1	2	3	4	5
Growth in: 3% NaCl	+	+	+	w	-
Growth at pH 6	-	+(-1)	+	-	+
Growth at pH 9	+	+	+	+	+
Growth at 50 °C	w	+	+	-	+
Growth at 55 °C	-	+	-	-	-
Facultative anaerobic	+	+	+	-	+
Nitrate reduction	-	-(+1)	-	-	-
Hydrolysis:					
L-Arginine	+	-(+1)	-	-	+
Casein	+	-(+1)	-	-	+
DNA	-	+(-1)	-	+	+
Gelatin	+	-(+1)	+	-	-
Starch	+	+(-1)	-	+	-
Tween 80	-	-(+1)	-	-	-
Urea	-	-	-	+	+
Acid production:					
D-Amygdalin	+	-	-	-	+
L-Arabinose	+	-(+1)	-	+	-
D-Cellobiose	+	-(+1)	-	+	-
D-Fructose	+	+(-1)	-	-	-
D-Galactose	+	-	+	+	-
D-Glucose	+	-	+	w	-
Gluconate	+	-(+1)	+	+	-
Glycerol	+	-	-	-	-
Inulin	+	-	-	-	-
Lactose	+	-(+1)	-	-	-
D-Maltose	+	-	+	-	-
D-Mannitol	+	-(+1)	-	-	-
D-Mannose	+	-(+1)	-	-	-
D-Melibiose	+	-(+1)	+	-	-
D-Melezitose	+	-	-	-	-
α-Glucopyranoside	+	-	-	-	-
Raffinose	+	-(+1)	-	-	-
L-Rhamnose	+	-	-	w	-
D-Ribose	+	-(+1)	w	-	-
Sucrose	+	-	-	-	+
D-Trehalose	+	-(+1)	-	+	-
D-Xylose	+	-(+1)	+	w	-

Note: Group 1, K1-4; Group 2, K3-5S, PL2-1, SRXT2-1; Group 3, CXT3-2; Group 4, PJ1-1B; Group 5, PHC3-4. +, positive; –, negative ; w, weakly positive. Number in parentheses indicates the number of isolate shows positive or negative reaction.

Group 5 contained isolate PHC3-4. Colonies were 1-5 mm in diameter, round, entire, smooth, flat, opaque and creamy-white after 2 days of incubation at 37 °C on C medium. The isolate grew at pH 5-9 and at 25-50 °C but did not grow in 3% and 5% NaCl and at 10-15, 55-60 °C. It showed positive for oxidase, indole production and hydrolysis of aesculin, L-arginine, casein, DNA and urea but showed negative for catalase methyl red, Voges-Proskauer, citrate utilization, H_2S production, nitrate reduction and hydrolysis of gelatin, starch, L-tyrosine and Tween 80. The isolate produced acid from D-amygdalin, salicin and sucrose, and showed negative reactions as shown in Table 2. On the basis of 16S rRNA gene sequence and phylogenetic analyses, isolate PHC3-4 (904 nt) was closely related to *P. dendritiformis* CIP 105967^T (Fig.1) with 99.7% 16S rRNA gene sequence similarity. Therefore, the isolate PHC3-4 was identified as *P. dendritiformis*[34].

CONCLUSION

The phenotypic and chemotaxonomic characteristics including the 16S rRNA gene sequencing results were useful to indicate the species level of the xylanolytic *Paenibacillus* isolated in Thailand. An isolate (Group 1), *P. macerans* from soil, 3 isolates (Group 2), *P. barengoltzii* from soil and muddy shore sediment, each isolate in Group 3, 4 and 5, *P. timonensis*, *P. montaniterrae* and *P. dendritiformis* from soil and muddy shore sediment, were isolated, respectively. The xylanase producing isolate in Group 1 showed the best clear zone and produced xylanase activity with 3.20±0.7 unit/ml. In this study, the isolates are the most likely source of enzymes and constitute a heterogeneous group of xylanase producing bacteria belonging to different *Paenibacillus* species. The isolated bacteria that are able to produce extracellular enzymes will provide the possibility to have optimal activities at different temperature and pH. Thus, the applications of the isolates are required for further study.

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