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Screening and characterization of protease-producing *Virgibacillus*, *Halobacillus* and *Oceanobacillus* strains from Thai fermented fish

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ABSTRACT

Halophilic bacteria from fermented fish (*pla-ra* and *pla-chom*), shrimp paste (*ka-pi*) and fermented crab] were isolated and screened for protease activities. Twenty-seven isolates exhibited protease specific activities ranged from 0.12 to 3.62 unit/mg protein. Isolate TPPN2-1 from *pla-ra* produced maximal protease specific activity (3.62 units/mg protein). They were Gram-positive, moderately halophilic rod-shaped bacteria and were divided into 5 groups on the basis of their phenotypic and chemotaxonomic characteristics, DNA-DNA relatedness including the 16S rRNA gene sequences analyses. Three isolates (Group I) were identified as *Virgibacillus halodenitrificans*, 3 isolates (Group II) as *V. marismortui*, 7 isolates (Group III) as *V. dokdonensis*, 10 isolates (Group IVA & IVB) as *Halobacillus* species and 4 isolates (Group V) as *Oceanobacillus iheyensis*.

INTRODUCTION

Moderately halophilic microorganisms are phylogenetically very diverse, including a great variety of microorganisms.They grow either in an absence or up to 20% (w/v) NaCl (Ventosa *et al.*, 1998) because they have an excellent adaptation capability to frequent change of extracellular osmolarity. Moderately halophilic bacteria show important advantages for being used as a source of halophilic enzymes, such as proteases, amylases, DNases, pullulanases and lipases (Mellado *et al*., 2004 & 2005). These characteristics exhibited by moderately halophilic bacteria make them a group with great potential bio-technological applications (Ventosa *et al.*, 1998). Halophilic bacteria are a good source of such protease, because their enzymes not only salt-tolerant but many are also themotolerant (Sanchez-Perro, 2003). In Thailand, the protease-producing halophilic bacteria were fund in *Filobacillus* sp. RF2-1 (Hiraga *et al.*, 2005), *Halobacillus* sp. SR5-3 (Namwong *et al.*, 2006), *Virgibacillus* sp. SK37 (Sinsuwan *et al*., 2007; Phrommao *et al.*, 2010) and *Virgibacillus* sp. SK33 (Sinsuwan *et al*., 2010) from fish sauce and *V. marismortui* NB2-1 from *pla-ra* (Chamroensaksri *et al.*, 2008), however the distribution of halophiles are still interesting in Thai salted fermented foods. This present study deals with the isolation, screening and identification of protease-producing moderately halophilic bacteria from fermented fish products based on their phenotypic and chemotaxonomic characteristics including the phylogenetic analysis using16S rRNA gene sequences.

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MATERIALS AND METHODS

Screening of protease-producing halophilic bacteria

Salt fermented foods collected from markets in Thailand were suspensed in 10% (w/v) NaCl water and were used for screening of protease-producing bacteria by spreading on JCM No. 377 agar medium and 1% (w/v) skim milk (Namwong et al., 2006), and incubated at 37 ºC for 3-5 days. Colonies surrounded by clear zone were selected and further purified by streak plate on JCM No. 377 agar plates. A loopful of the purified isolates was inoculated

in 125-ml Erlenmeyer flask containing 20 ml JCM No. 377 broth, incubated at 37 ^{o}C (200 rpm) for 3 days. Cell-free supernatant recovered by centrifugation at 4 ^{o}C, 10,000 rpm (13,300 g) for 20 min was used as crude enzyme for protease activity (caseinolytic activity) assay by method described by Hiraga *et al*. (2000).

Identification methods

Cell morphology, colonial appearance, spore formation, and pigmentation of bacteria isolated grown on JCM No.377 agar plates at 37 ^{o}C for 1-2 days were examined. Catalase, oxidase, hydrolysis of L-arginine, casein, gelatin, starch and L-tyrosine; MR-VP, nitrate reduction, Simmon citrate test and acid from carbohydrates were determined as described by Barrow and Feltham (1993). Growth at different pH (5, 6, 8 and 9), in 0, 1, 2, 10, 15 and 20% (w/v) NaCl and at different temperatures (37 ^{o}C and 50 ^{o}C) were investigated. All tests were carried out by using JCM No. 377 containing 10% (w/v) NaCl as a basal medium and incubated at 37 ^{o}C for 3 days, except for the investigation of the effect of temperatures. Cell wall composition was determined as described by Komagata and Suzuki (1987). Bacterial DNA isolated by method recommended by Saito and Miura (1963). The 16S rRNA gene was PCR amplified using 9F (5'GAGTTTGATCCTGGCTCAG '3), *Escherichia coli* numbering) and 1541R (5'AAGGAGGTGATCCAGCC'3) as forward and reverse primers, respectively. The amplified 16S rRNA gene sequence was analyzed by automated DNA sequencer (Applied Biosystems, USA) using the following primers: 339F (5'CTCCTACGGGAGGCAGCAG'3), 785F (5'GGATTAGATACCC TGGTAGTC'3), 1099F (5'GCAACGAGCGCAACCC'3), 357R (5'CTGCTGCCTCCCGTAG'3) and 802R (5'TACCAGGGTATCTAATCC'3). The sequence was multiply aligned with the CLUSTAL_X program (version 1.83; Thompson *et al*., 1997), then the alignment was manually verified and edited prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by the neighbour-joining method (Saito and Nei, 1987) in MEGA version 5.05 (Tamura *et al.,* 2011). The confidence value of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The sequence similarity value among the closest strains were calculated manually after pairwise alignment obtained. Gap and ambiguous nucleotides were eliminated from the calculations. DNA-DNA hybridization was performed by using photobiotin-labelled DNA probes and micro-dilution wells (Ezaki *et al*., 1989).

RESULTS AND DISCUSSION

Screening of protease-producing halophilic isolates

From 17 salt fermented food samples, 27 halophilic bacteria which exhibited protease activity on skim milk agar plate containing 10% (w/v) NaCl were isolated. The halophilic bacterial isolates were obtained from *pla-ra* (13 isolates), *ka-pi* (shrimp paste) (5 isolates), *pla-chom* (4 isolates) and fermented crab (5 isolates) (Table 1). The isolates exhibited caseinolytic activity on JCM No. 377 agar with 10% NaCl containing 1% skim milk. Protease activity in the cell-free supernatant of isolates ranged from 0.12 - 3.62 units/mg protein (Figure 1). The isolates TCR11-1, TCR11-2 and TP4-4 produced high amount of protease activity (3.51-3.60 units/mg protein), however isolate TPPN2-1 from *pla-ra* produced maximal protease specific activity (3.62 units/mg protein).

Identification of isolates

Twenty-seven halophilic bacteria were Gram-positive, spore forming rod-shaped bacteria. They contained *meso*-diaminopimelic acid (*meso*-DAP) in the cell-wall peptidoglycan. They were divided into five groups based on the phenotypic and chemotaxonomic characteristics, DNA-DNA relatedness including the 16S rRNA gene sequence analyses of the representative strain in each of groups (Figure 2 and Table 1). All required NaCl for growth. Catalase and oxidase activity were positive but negative for Voges-Proskauer reaction and citrate utilization. Their phenotypic characterizations and group differentiation are described here and listed in Table 2. Group I contained 3 isolates, TCR11-1, TCR11-2 and TPSK2-3. Colonies were circular, slight irregular, raised, translucent and cream in colour. They grew in anaerobic condition but not at 50^{o} C. Positive for nitrate reduction. Their phenotypic characteristics were shown in Table 2. The isolate TCR11-1 showed 99.4% (1,456 bp) sequence similarity to *Virgibacillus halodenitrificans* DSM 10037^{T} based on 16S rRNA gene sequence (Figure 2). In addition, isolates TCR11-1, TCR11-2 and TPSK2-3 showed high DNA-DNA relatedness (70.2-79.0%) to *V. halodenitrificans* DSM 10037^{T}. Therefore, they were identified as *V. halodenitrificans* (Yoon *et al*., 2004; Wyne *et al*., 1987). These isolates were isolated from fermented crab and *pla-ra*. Group II contained 5 isolates CC7-1, J4 and TPPN1-1. Colonies were circular, smooth, slightly irregular, slightly raised and cream in colour. They showed positive for nitrate reduction. Their phenotypic characteristics were shown in Table 2. TPPN1-1 (1,540 bp) showed 99.9 % sequence similarity to *V. marismortui* DSM 12325^{T} based on 16S rRNA gene sequence (Figure 2). In addition, isolates CC7-1, J4 and TPPN1-1 showed high DNA-DNA relatedness (70.3-105%) to *V . marismortui* DSM 12325^{T}. Therefore, they were identified as *V. marismortui* (Arahal *et al*., 1999; Wyne *et al*., 1987). These isolates were found in *pla-ra*. Group III contained 7 isolates, TCN24, TCN24-1, TCN4, J1, TPC5-1, TPC5-2 and TKNR12-8. Colonies were irregular, flat, translucent, raised and milky white in colour. They showed positive for hydrolysis of tween 80 but negative for nitrate reduction. Isolates TCN24-1, TCN4, J1, TPC5-1, TPC5-2 and TKNR12-8 showed almost the same phenotypic characteristics as isolate TCN24 as shown in Table 2. TCN24 (1,525 bp) showed sequence similarity 99.3% to *V. dokdonensis* DSW-10^{T} based on 16S rRNA gene sequence (Figure 2). Therefore, isolate TCN24 was identified as *V. dokdonensis* and isolates TCN24-1, TCN4, J1, TPC5-1, TPC5-2 and TKNR12-8 were belonged to the same species based on their phenotypic and chemotaxonomic

characteristics (Yoon *et al.*, 2005). These isolates were found in fermented crab, *pla-ra* and shrimp paste. Group IVA Group IVA contained 6 isolates TP4-4, TP4-5, TP4-6, TP4-7, TPP1-1 and TPSK2-2. They were spore forming, Gram-positive rods. Colonies were circular, smooth, slightly irregular, raised and yellow in colour. They were positive for hydrolysis of casein, gelatin and starch but negative for MR reaction and nitrate reduction. They could grow at pH 6.0-9.0, at 15-50^{o}C, and in 0-20% NaCl. Their phenotypic characteristics were shown in Table 2. Isolates TP4-5, TP4-6, TP4-7, TPP1-1 and TPSK2-2 showed high DNA-DNA relatedness (70-107%) to isolate TP4-4. Therefore, these isolates are belonged to the same species (Wyne et al., 1987). However, the isolate TP4-4 (1,529 bp) showed 98.4% sequence similarity to *Halobacillus trueperi* DSM 10404^{T} based on 16S rRNA gene sequence (Figure 2). Therefore, there were identified as *Halobacillus* sp. but were differentiated from *Halobacillus trueperi* DSM 10404^{T} (Spring *et al.*, 1996). The further study is required to propose them as a novel *Halobacillus* species. These isolates were found in *pla-chom* and *pla-ra*. Group IVB contained 4 isolates TSN17, TSN17-2, TSN17-4 and TSN2. They were spore forming, Gram-positive rods. Colonies were circular, smooth, slightly irregular, raised and pale yellow or yellow colonies. They were positive for hydrolysis of casein, gelatin and starch but negative for MR reaction and nitrate reduction. They could grow at pH 6.0-9.0, at 15-40^{o}C and in 2-20% NaCl. Their phenotypic characteristics were shown in Table 2. Isolates TSN17-2, TSN17-4 and TSN2 showed low DNA-DNA relatedness (15.6-45%) to isolate TSN17. The representative strain TSN17 (1,529 bp) in this Group showed 97.5% sequence similarity to *Halobacillus dabanensis* D-8^{T} (Liu *et al.*, 2005) based on 16S rRNA gene sequence (Figure 2). The isolates in this Group showed different characteristics from *Halobacillus salinus* JCM 11546^{T}.

However, they showed the characteristics of the genus *Halobacillus* and they were identified as *Halobacillus*. The further study is required to propose them as a novel *Halobacillus* species. These isolates were isolated from shrimp paste. Group V contained 4 isolates, TPS12, TPS12-1, TPS12-2 and TPPN2-1. Colonies were circular, raised and light cream-beige in colour. They grew at 50^{o}C but not in anaerobic condition. They showed negative for starch and tyrosine hydrolysis and nitrate reduction. Isolates TPS12, TPS12-1 and TPS12-2 showed almost the same phenotypic characteristics as isolate TPPN2-1 as shown in Table 2. TPPN2-1 (1,454 bp) showed 99.5 % sequence similarity to *Oceanobacillus iheyensis* HTE831^{T} based on 16S rRNA gene sequence (Figure 2). Therefore, the isolate TPPN2-1 was identified as *O. iheyensis* HTE831^{T} and isolates TPS12, TPS12-1 and TPS12-2 were belonged to the same species based on their phenotypic and chemotaxonomic characteristics (Lu *et al*., 2001). These isolates were found in *pla-ra*. Our work revealed that the halophilic Gram-positive, spore forming rod-shaped bacterial isolates in genera could produced protease activity as previous reported (Hiraga *et al.*, 2005; Namwong *et al.*, 2006; Sinsuwan *et al*., 2007, 2010; Chamroensaksri *et al.*, 2008; Phrommao *et al.*, 2010). They were distributed in many kinds of salt fermented foods (*pla-ra, pla-chom*, shrimp paste and fermented crab) in Thailand. In contrast, the halophilic Gram-negative rod-shaped bacteria such as C*hromohalobacter* sp. TVSP101 from solar saltern samples (Vidyasagar *et al*., 2007; 2009) *Pseudoalteromonas* sp. CP76 (Sanchez-Porro *et al*., 2003) and *Salinivibrio costicola* (Lama *et al*., 2005) are reported to produced proteases. However, the isolates found in fermented products may play the important role in the fermentation. Some of the isolates in Group IVA and IVB are the novel species; therefore, DNA-DNA hybridization experiments should be done for further study.

Table. 1: Sample, sample location, isolate number and identification.

Sample	Province	Isolate no.	Group	Identification
Fermented crab	Ratchaburi	TCR11-1	I	*V. halodenitrficans*
Fermented crab	Ratchaburi	TCR11-2	I	*V. halodenitrficans*
Pla-ra	Sukothai	TPSK2-3	I	*V. halodenitrficans*
Pla-ra	Pisanulok	TPPN1-1	II	*V. marismortui*
Pla-ra	Samutsakhon	J4	II	*V. marismortui*
Pla-ra	Chachoengsao	CC7-1	II	*V. marismortui*
Fermented crab	Nan	TCN4	III	*V. dokdonensis*
Fermented crab	Nan	TCN24,	III	*V. dokdonensis*
Fermented crab	Nan	TCN24-1	III	*V. dokdonensis*
Pla-ra	Chonburi	TPC5-1	III	*V. dokdonensis*
Pla-ra	Chonburi	TPC5-2	III	*V. dokdonensis*
Pla-ra	Samut Sakhon	J1	III	*V. dokdonensis*
Shrimp paste	Nakhonratchasima	TKNR12-8	III	*V. dokdonensis*
Pla-chom	Nakhonayok	TP4-4	IVA	*Halobacillus* sp.
Pla-chom	Nakhonayok	TP4-5	IVA	*Halobacillus* sp.
Pla-chom	Nakhonayok	TP4-6	IVA	*Halobacillus* sp.
Pla-chom	Nakhonayok	TP4-7	IVA	*Halobacillus* sp.
Pla-ra	Petchaburi	TPP1-1	IVA	*Halobacillus* sp.
Pla-ra	Sukothai	TPSK2-2	IVA	*Halobacillus* sp.
Shrimp paste	Nan	TSN17	IVB	*Halobacillus* sp.
Shrimp paste	Nan	TSN172	IVB	*Halobacillus* sp.
Shrimp paste	Nan	TSN17-4	IVB	*Halobacillus* sp.
Shrimp paste	Nan	TSN2	IVB	*Halobacillus* sp.
Pla-ra	Suphanburi	TPS12	V	*O. iheyensis*
Pla-ra	Suphanburi	TPS12-1	V	*O. iheyensis*
Pla-ra	Suphanburi	TPS12-2	V	*O. iheyensis*
Pla-ra	Pisanulok	TPPN2-1	V	*O. iheyensis*

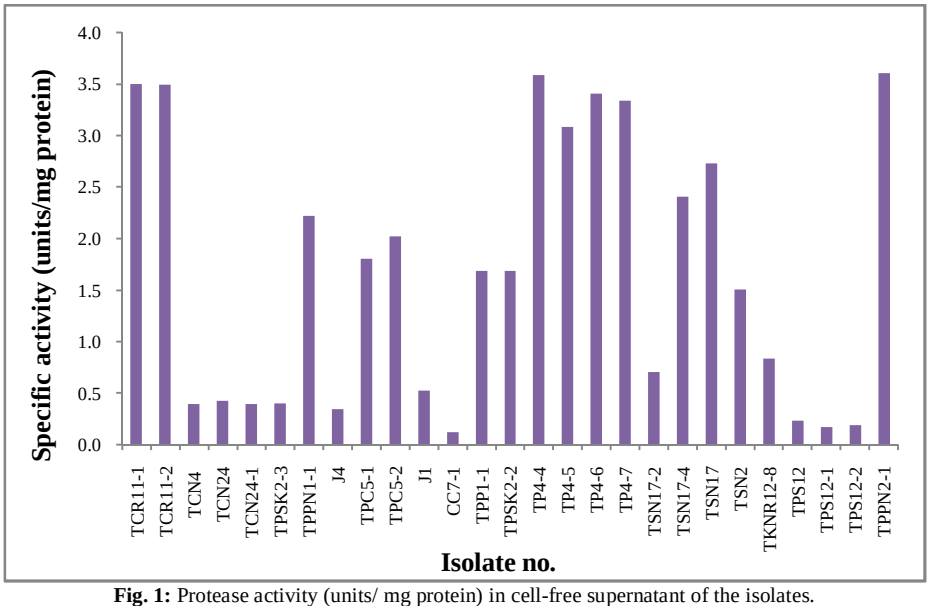

Fig. 1: Protease activity (units/ mg protein) in cell-free supernatant of the isolates.

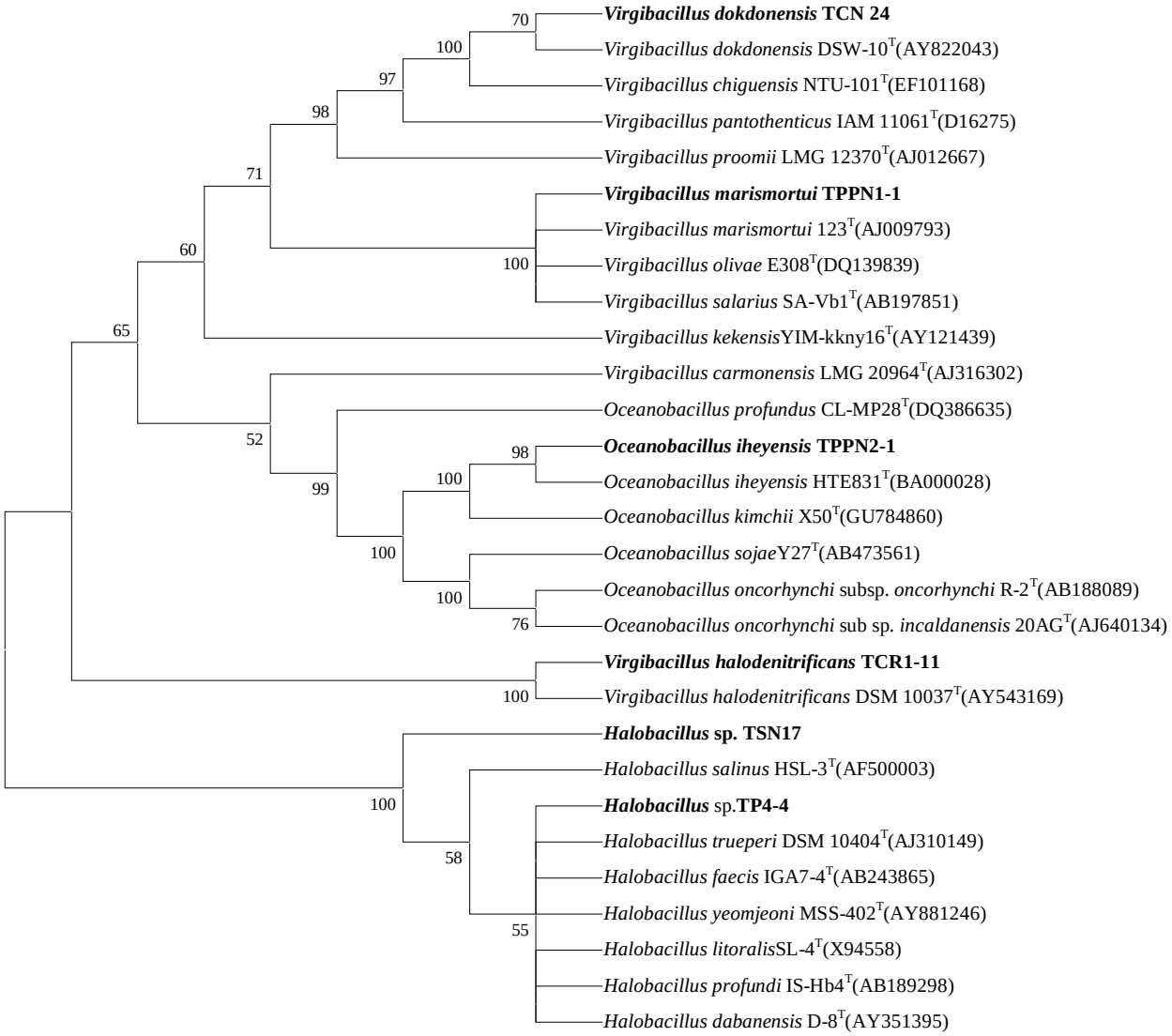

Fig.2: Neighbour-joining-tree showing the phylogenetic position of isolates and related taxa based on 16S rRNA gene sequences.

Table. 2: Differential characteristics of the isolates in Group I to Group V .

Characteristics	I	II	III	IVA	IVB	V
Growth at pH 5	+	+	-	-	-	-
Growth at pH 6	+	+	+(3w)	+	+	+(-1)
Growth at pH 8	+	+	+	+	+	+
Growth at pH 9	+	+	+	+	+	+
Growth at 15 ºC	-	-	-	+	+	+
Growth at 50 ºC	-	+(-1)	+	+	-	+
Growth in 0% NaCl	+	+	+(-2)	+	-	+
Growth in 1% NaCl	+	+	+	+	+	+
Growth in 15% NaCl	+	+	+	+	+	+
Growth in 20% NaCl	+	-	+(-1)	+	+	+
Nitrate reduction	+	+(-1)	-	-	-	-
Citrate utilization	-	-	-	-	-	-
L-Arginine hydrolysis	-	-	+(-1)		-(+1)	-
Casein hydrolysis	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+
Starch hydrolysis	-	-	-(+1)	+	+	-
L-Tyrosine hydrolysis	-	-	-	-	-	-(+1)
Tween 80 hydrolysis	-	-	+(-2)	-	-	-
Acid from						
L-Arabinose	-	-	-(+2)	-	-	-
D-Cellobiose	-	+(-1)	+	-	-	-
D-Fructose	+	+	+	+	+	+
D-Galactose	+	-	+(-1)	+	+	+
D-Glucose	+	+	+	+	+	+
Glycerol	+	+(-1)	+	+(-2)	+(-1)	-(+1)
Inositol	-	+(-1)	+	-	-	-
Inulin	-	-	+(-1)	-	-(+1)	-
Lactose	+	-	+(-1)	-	-	-
D-Maltose	+	+(-1)	+	+	-	+
D-Mannitol	+(-1)	-	+(-1)	+(-1)	+(-1)	+
D-Mannose	+	+(-1)	+(-3)	+(-1)	+(-1)	+
D-Melibiose	-	-	-(+2)	-	-	+
D-Melezitose	-	-	-(+2)	-	-	-
Raffinose	-	-	-(+2)	-	-(+1)	-
L-Rhamnose	-	-	+(-3)	-	+(-2)	-
D-Ribose	+	-	+	+	-(+1)	+
Salicin	-	+(-1)	+	+(-1)	+(-1)	+
Sorbitol	-	-	+(-1)	-	-	-
Sucrose	+	-	+	+	+	-
D-Trehalose	+(-1)	-	-(+2)	+	+(-1)	+
D-Xylose	-	-	-	-	-(+1)	+(-1)

I, TCR11-1, TCR11-2 and TPSK2-3; II, TPPN1-1, J4 and CC7-1; III, TCN4, TCN24-1, TPC5-1, TPC5-2, J1 and TKNR12-8; IVA, TP4-4, TP4-5, TP4-6, TP4-7, TPP1-1 and TPSK2-2; IVB, TSN17, TSN17-2, TSN17-4 and TSN2; V, TPS12, TPS12-1, TPS12-2 and TPPN2-1. +, positive; w, weak positive; –, negative. Number in parentheses indicates the number of isolate shows positive, weak or negative reaction.

CONCLUSION

In this study, the isolates TCR11-1 and TCR11-2 identified as *V. halodenitrificans* and isolate TP4-4 identified as *Halobacillus* sp. could produced high amount of protease activity (3.51-3.60 units/mg protein) while isolate TPPN2-1 identified as *O. iheyensis* and isolated from *pla-ra* could produce a maximal protease specific activity (3.62 units/mg protein). They were divided in to 5 groups including *V. halodenitrificans* (Group I), *V. marismortui* (Group II), *V. dokdonensis* (Group III), *Halobacillus* sp. (Group IVA and IVB) and *O. iheyensis* (Group V) based on the phenotypic and chemotaxonomic characteristics including the phylogenetic analysis using 16S rDNA sequences.

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