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Full Length Research Paper

Characterization of *Virgibacillus* strain TKNR13-3 from fermented shrimp paste (*ka-pi*) and its protease production

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A moderately halophilic bacterium, TKNR13-3, isolated from fermented shrimp paste (*ka-pi*) in Thailand was identified as *Virgibacillus halodenitrificans* based on its phenotypic and chemotaxonomic characteristics, 16S ribosomal ribonucleic acid (Rrna) gene sequence and deoxyribonucleic-deoxyribonucleic (DNA-DNA) relatedness. The strain TKNR13-3 produced substantial amount of extracellular protease. Its protease production was maximum when grown in JCM No.377 medium without casamino acids but containing 2% (w/v) yeast extract and 5% (w/v) NaCl at 37°C, pH 6.5 for 3 days. The optimum pH, salt concentration and temperature for the protease activity were pH 8, 15% (w/v) NaCl and 60°C, respectively. Thus, the enzyme was salt-tolerant and slightly thermoalkaliphilic. The protease activity was strongly inhibited (77.62%) by chymostatin. Therefore, the protease of strain TKNR13-3 was serine protease type chymotrypsin. Activity stained-substrate gel of the crude protease indicated the presence of several proteases with the estimated molecular mass of 12, 21, 29, 39 and 49 kDa.

Key words: Halophilic bacteria, *Virgibacillus halodenitrificans*, protease, fermented shrimp paste.

INTRODUCTION

Halophilic bacteria are salt-loving bacteria that distribute in hypersaline environment. They grow in a wide range of salinities, 2-5, 5-20 and 20-30% (w/v) NaCl and therefore, their salt concentration requirement is used as a criteria for their classification, slight, moderate and extreme halophiles, respectively (Kushner, 1985). Moderately halophilic bacteria grow either in an absence of or in up to 20% (w/v) NaCl, it is an interesting group from a biotechnological point of view (Ventosa, 1998) because they have an excellent adaptation capability to frequent change of extracellular osmolarity. Protease is a group of hydrolases that catalyze total hydrolysis of protein. Besides their physiological importance, they have great industrial applications (Rao et al., 1998). Although, a variety of bacteria produce protease, only a few of them are recognized as commercial protease producer, for this reason, it is important to search for protease with an industrial-desired characteristics, such as having optimal activity in high salt concentration and/or at high temperature. For example, industrial processes of laundry and dishwashing detergents manufacturing, seafood industrial waste treatment, and deproteinization of crustacean waste for chitin preparation (Yang et al., 2000) are operated at high salt concentration. Halophilic bacteria are a good source of such protease, because

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Abbreviations: rRNA, ribosomal ribonucleic acid; **DNA,** deoxyribonucleic; **TCA,** trichloroacetic acid; **DNAse,** deoxyribonuclease.

their enzymes are not only salt-tolerant but many are also themotolerant (Sánchez-Porro, 2003). There are several previous reports on protease-producing halophilic bateria, *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, *Virgibacillus marismortui* NB2-1 from *pla-ra* (Chamroensaksri et al., 2008), C*hromohalobacter* sp. TVSP101 from solar saltern samples (Vidyasagar et al., 2007; 2009) but still only few from salt fermented foods in Thailand. In this present study, we isolated and identified protease-producing moderately halophilic bacterium from fermented shrimp paste (*ka-pi*), optimized its protease production and characterized some properties of the crude protease.

MATERIALS AND METHODS

Screening of protease-producing halophilic bacteria

Salt fermented foods collected from markets in Thailand were suspended in 10%(w/v) NaCl water and screened for protease-producing bacteria by spreading on JCM No. 377 agar medium; [0.5% (w/v) yeast extract, 0.1% (w/v) casamino acids, 0.1% (w/v) sodium glutamate, 0.3% (w/v) tri-sodium citrate, 2.0% (w/v) $MgSO_4.7H_2O$, 0.2% (w/v) KCl, 0.0362% (w/v) $FeCl_2.4H_2O$, 0.00362% (w/v) $MnCl_2.4H_2O$, 2% (w/v) agar, pH 7.2] containing 10% (w/v) NaCl and 1%(w/v) skim milk, and incubated at 37°C for 7-10 days. Colonies surrounded by clear zone were selected and further purified by streaking on JCM No. 377 agar plates. A loopful of the purified isolates were inoculated in 125 ml Erlenmeyer flask containing 20 ml broth of the same medium, incubated at 37°C (200 rpm) for 3 days. Cell-free supernatant recovered by centrifugation at 12,000 × g, 4°C for 20 min was used as crude enzyme for protease activity (caseinolytic activity) assay by method described by Hiraga et al. (2005).

Protease activity assay

Sample (0.5 ml) was incubated with 1.5 ml of 1.33% (w/v) Hammarsten casein (Research Organics, Inc., OH, USA) in 50 mM Tris-HCl buffer pH 7.5 containing 10% (w/v) NaCl at 37°C for 60 min. The enzymatic reaction was stopped by adding 2 ml of 0.44 M trichloroacetic acid (TCA), and then centrifuged at 2,000 × g, 4°C for 10 min to remove precipitate. The supernatant obtained (0.5 ml) was mixed with 2.5 ml of 0.44 M sodium carbonate and 0.5 ml of Folin-Ciocalteu's phenol reagent, and then incubated at 37°C for 20 min. The color of the above mixture developed was measured at 660 nm. A correlation between an absorbance at 660 nm and tyrosine concentration was constructed for an estimation of tyrosine liberated. One unit of protease was defined as an amount of the enzyme yielding an equivalent of 1 µg of tyrosine per minute under the defined assay conditions. Blank control was performed in the same manner except the enzyme was added after the addition of TCA solution. Total protein in the supernatant was measured by the method described by Lowry et al. (1951) using serum albumin as standard protein.

Bacterial identification methods

Cells morphology, colonial appearance, spore formation, and pigmentation of bacterial isolate grown on JCM No.377 medium containing 10% (w/v) NaCl at 37°C for 1 day were examined. Catalase, oxidase, hydrolysis of L-arginine, aesculin, casein, gelatin, starch, Tween 80, tyrosine and deoxyribonuclease (DNAse) activity; urease activity; MR-VP, indole test, nitrate reduction, Simmon citrate test, and acid from carbohydrates were determined as described by Barrow and Feltham (1993). All the tested media were supplemented with 10% (w/v) NaCl. 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 and 50°C) were investigated by using JCM No.377 medium and incubated at 37°C, except for the investigation of the effect of temperatures.

Cell wall composition and quinone system were determined using dried cells as described by Komagata and Suzuki (1987). Bacterial deoxyribonucleic (DNA) was isolated by the method recommended by Saito and Miura (1963). The 16S ribosomal ribonucleic acid (rRNA) gene was PCR amplified using 9F (5'GAGTTTGATCCTGGCTCA G'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'GGATTAGATACCCTGGTAGTC'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), and then the alignment was manually verified and edited prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) in MEGA 4 software version 4 (Tamura et al., 2007). The confidence value of branches of the phylogenetic tree was determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The sequence similarity value among the closest strains was calculated manually after pairwise alignment obtained. Gap and ambiguous nucleotides were eliminated from the calculations. DNA-DNA hybridization was performed as described by Ezaki et al. (1989).

Optimization of protease production

One loopful of bacterial culture grown in 50 ml of JCM No.377 medium containing 10% (w/v) NaCl in 250 ml Erlenmeyer flask and incubated with shaking (200 rpm) at 37°C for 2 days was used as inoculum. A 2.5 ml of the inoculum was inoculated into 50 ml of the same medium in 250 ml Erlenmeyer flask and incubated at the same above condition for 7 days. Protease production was quantified daily by the method described previously. The influences of incubation time, medium composition, NaCl concentration, initial pH, and incubation temperature on protease production were determined by varying each parameter. An optimal condition of prior experiment was used as the basis in the latter experiment to optimize the conditions.

Characterization of protease activity

Protease produced by selected strain at the optimal conditions was used in this study. The protease activity was assayed by the method described previously at various pH (4-9) using 50 mM of acetate buffer (pH 4-5), phosphate buffer (6-7) and Tris-HCl buffer (pH 7-9), at various temperature (30-90°C) and various NaCl concentration (0-25% (w/v) NaCl) to determine effect of pH, temperature and salt concentration on activity. The effect of various kind of protease inhibitors including 10 µM E-64, 1 mM EDTA, 10 µM leupeptin, 1 mM PMSF, 1 µM pepstatin A, 10 µM chymostatin, 10 µM TLCK, 10 µM TPCK, 10 µM phosphoramidon and 0.1 mg/ml trypsin from soybean on protease activity were analyzed after

incubation of crude protease and the above protease inhibitors at 30°C for 30 min. Culture broth of the selected strain grown at optimal condition for protease production was centrifuged at 12,000 × g, 4°C for 10 min. Protein in the obtained supernatant was precipitated by addition of ammonium sulfate to 80% saturation and allowed to stand at 4°C overnight. The resulting precipitate was collected by centrifugation at 10,000 × g, 4°C for 20 min. The precipitate was dissolved in 50 mM Tris-HCl buffer pH 7.5 and dialyzed against the same buffer. Sample was subjected to electrophoresis (Laemmli, 1970) and stained for protease activity by the modified of Garciacarreno et al. (1993). Protein (100 µg) was loaded into the gel made of 4% stacking gel and 12% separating gel and then subjected to electrophoresis at a constant voltage of 75 V using a mimi vertical Hoefer apparatus. (Hoefer Pharmacia Biotech Inc., Sweden). After electrophoresis, the gel was immersed in 100 ml of 1.675% (w/v) sodium caseinate in 50 mM Tris-HCl buffer pH 7.5 for 1 h at 0°C to allow the substrate to penetrate into the gel. The gel was then immersed in 1.675% sodium caseinate in 50 mM Tris-HCl buffer pH 7.5 containing 10% (w/v) NaCl at 0°C for 1 h. The gel was fixed and stained with 0.125% Coomassie blue R-250 in 50% ethanol and 10% acetic acid and destained in 25% ethanol. Development of clear zone on blue background indicated proteolytic activity.

RESULTS AND DISCUSSION

Screening of protease-producing halophilic bacteria

From 30 salt fermented food samples, 54 halophilic bacteria which exhibited protease activity on skim milk agar plate containing 10% (w/v) NaCl were isolated. It was found that a number of the halophilic bacteria was obtained from *pla-ra* (25 isolates), *ka-pi* (shrimp paste) (15 isolates), *pla-chom* (6 isolates), fermented crab (5 isolates), *mang-da dong* (2 isolates) and *nam-pla* (fish sauce) (1 isolate). Protease production of the bacteria isolated was quantitatively analyzed in cell-free supernatant of each isolate grown in halobacterium medium JCM No. 377 containing 10%(w/v) NaCl at 37°C (200 rpm) for 3 days. Range of protease produced by the halophilic isolated bacteria was 0.01-4.23 units/mg protein. Strain TKNR13-3 isolated from shrimp paste produced both maximal protease activity (0.55 units/ml), and maximal protease specific activity (4.23 units/mg protein). This strain TKNR13-3 was selected for further studies.

Identification of strain

Strain TKNR13-3 was a rod-shaped, spore-forming, aerobic, Gram-positive bacteria (Figure 1). Colonies were circular or slight irregular, raised, translucent, and cream color. It grew in the medium containing 10-20% (w/v) NaCl and optimally at 15% (w/v) NaCl. Growth at pH 5 to pH 9, in anaerobic condition but not at 50°C. Positive for catalase, oxidase, nitrate reduction, and hydrolysis of DNA, casein and gelatin. Hydrolysis of L-arginine, starch, Tween 80, and L-tyrosine; urease, MR-VP, indole test and citrate utilization were negative. Acids were produced

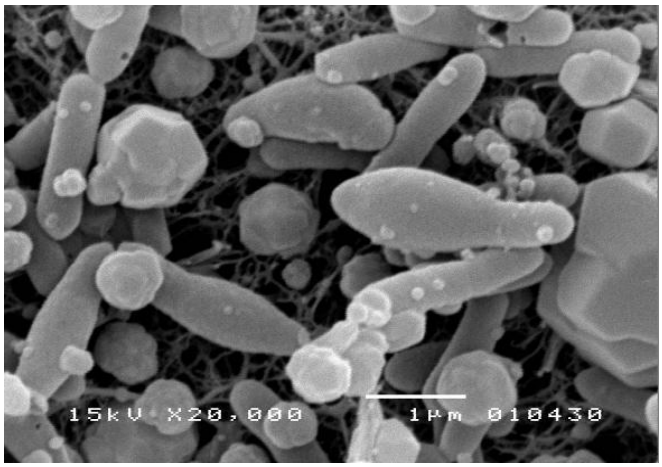

Figure 1. Scanning electron micrograph of strain TKNR13-3 grew on JCM No. 377 agar medium at 37°C for 5 days.

from D-fructose, D-galactose, D-glucose, glycerol, lactose, maltose, D-mannitol, mannose, D-ribose, sucrose, D-trehalose but not L-arabinose, D-cellobiose, inulin, *myo*-inositol, melibiose, melizitose, raffinose, rhamnose, salicin, D-sorbitol and D-xylose. Strain TKNR13-3 was moderately halophilic bacteria based on their optimal growth in 15% (w/v) NaCl. It contained *meso*-diaminopimelic acid as the diagnostic diamino in the cell wall peptidoglycan. The predominant menaquinone was menaquinone with seven isoprene units (MK-7). The 16S rRNA gene sequence (1550 bp) of strain TKNR13-3 showed 99.4% similarity to *V. halodenitrificans* JCM 12304^T (Yoon et al., 2004) (=DSM 1037^T). Phylogenetic tree indicated taxonomic position of strain TKNR13-3 was shown in Figure 2. DNA-DNA relatedness of this strain to *V. halodeniitrificans* JCM 12304^T was 93.4%. Therefore, it was identified as *V. halodeniitrificans* (Yoon et al., 2004; Wayne et al., 1987).

Optimization of protease production

Protease production of strain TKNR13-3 which showed highest protease specific activity (4.23 units/mg protein) was optimized by cultivation in JCM No. 377 medium containing 10% (w/v) NaCl and incubated at 37°C (200 rpm). The influence of several factors including incubation time, medium composition, NaCl concentration, initial pH and incubation temperature on protease production in cell-free supernatant were studied. Bacterial growth and specific activity of protease were monitored daily. The strain TKNR13-3 showed a maximum protease specific activity (5.2 units/mg protein) after 3 days of incubation, while the growth was maximum (A600 nm of 1.6) after 2 days (Figure 3A). When the strain TKNR13-3 was cultivated in JCM No.377 medium which was modified by using the following nutrients; soybean, skim

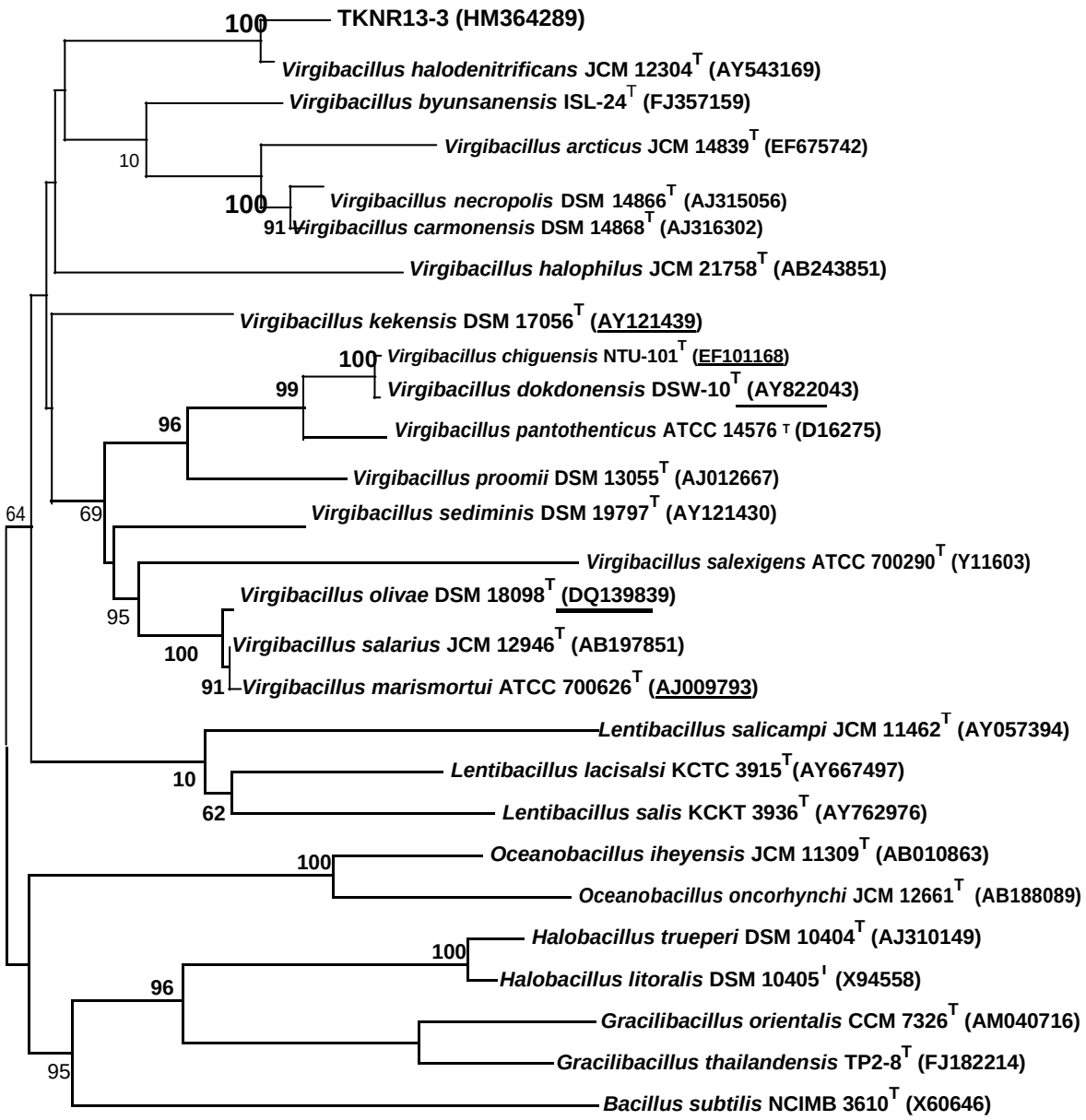

Figure 2. Neighbour-joining tree showing the phylogenetic position of strain TKNR13-3,*Virgibacillus* species, and related taxa based on 16S rRNA gene sequences. Bar, 0.005 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications.

milk, casein, yeast extract, gelatin, sodium caseinate at 0.5% (w/v) instead of casamino acids for 3 days. Maximum protease production (0.9 units/ml) and growth (A600 nm of 1.9) were obtained in the modified medium containing yeast extract (Figure 3B). An effect of yeast extract concentration in the modified JCM No.377 medium was further investigated. Maximum protease production (1.75 units/ml) was obtained at 2% (w/v) yeast extract (Figure 3C).

Effect of NaCl concentration on protease production of the strain TKNR13-3 grown in modified JCM No.377 medium containing 2% (w/v) yeast extract and various concentration of NaCl (0, 5, 10, 15 or 20%, w/v), after incubation for 3 days was determined. Maximum protease production was 7.7 units/mg protein in the medium containing 5% (w/v) NaCl (Figure 3D). The result indicated that the strain TKNR13-3 is a moderately halophilic bacterium. Strain TKNR13-3 was grown in the modified JCM No.377 medium containing 2% (w/v) yeast extract and 5% (w/v) NaCl which was adjusted to pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0 and incubated for 3 days. Optimal pH for protease production was 6.5 (Figure 3E).

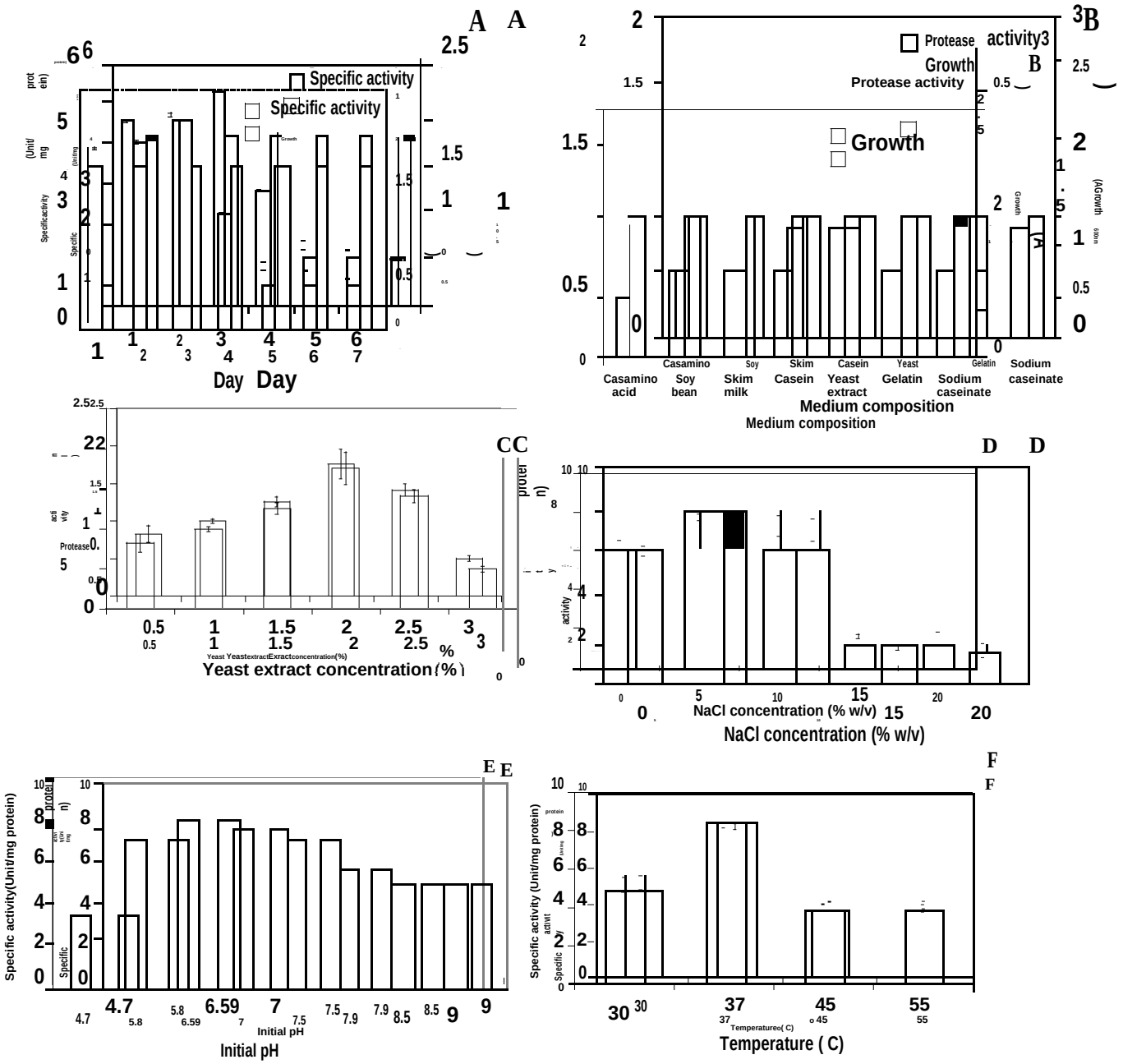

Figure 3. Effect of various factors on protease production of strain TKNR13-3. Incubation time (A), Nutrients (0.5% w/v) substituted for casamino acids in JCM No. 377 (B), Yeast extract concentration in modified JCM No. 377 (C), NaCl concentration (D), Initial pH (E) and Temperature (F).

As shown in Figure 3F, the strain TKNR13-3 grew in the modified JCM No.377 medium containing 2% (w/v) yeast extract, 5% (w/v) NaCl, pH 6.5 and incubated at 30, 37, 45 or 50°C for 3 days, the optimal temperature for protease production was 37°C.

Characterization of protease activity

Some characteristics of crude protease produced by strain TKNR13-3 were determined using Hammersten casein as substrate and in the presence of 10% (w/v)NaCl. Protease activity assay was carried out over the pH range of 5 to 10 at 37°C. Optimal pH for protease activity was pH 8. Protease activity (pH 8) was assayed at various temperatures. Optimal temperature for protease activity was 60°C. Protease activity was assayed at pH 8 and at 60°C in the presence of various concentration of NaCl. Optimal concentration of NaCl for protease activity was 5% (w/v) (Figure 4). Strain TKNR13-3 produced protease at 4.23 units/mg protein and the enzyme was most active at pH 8, 60°C and in the presence of 5% (w/v) NaCl. However, Chamroensaksri et al. (2008) reported that *V. marismortui* NB2-1, a moderately halophilic

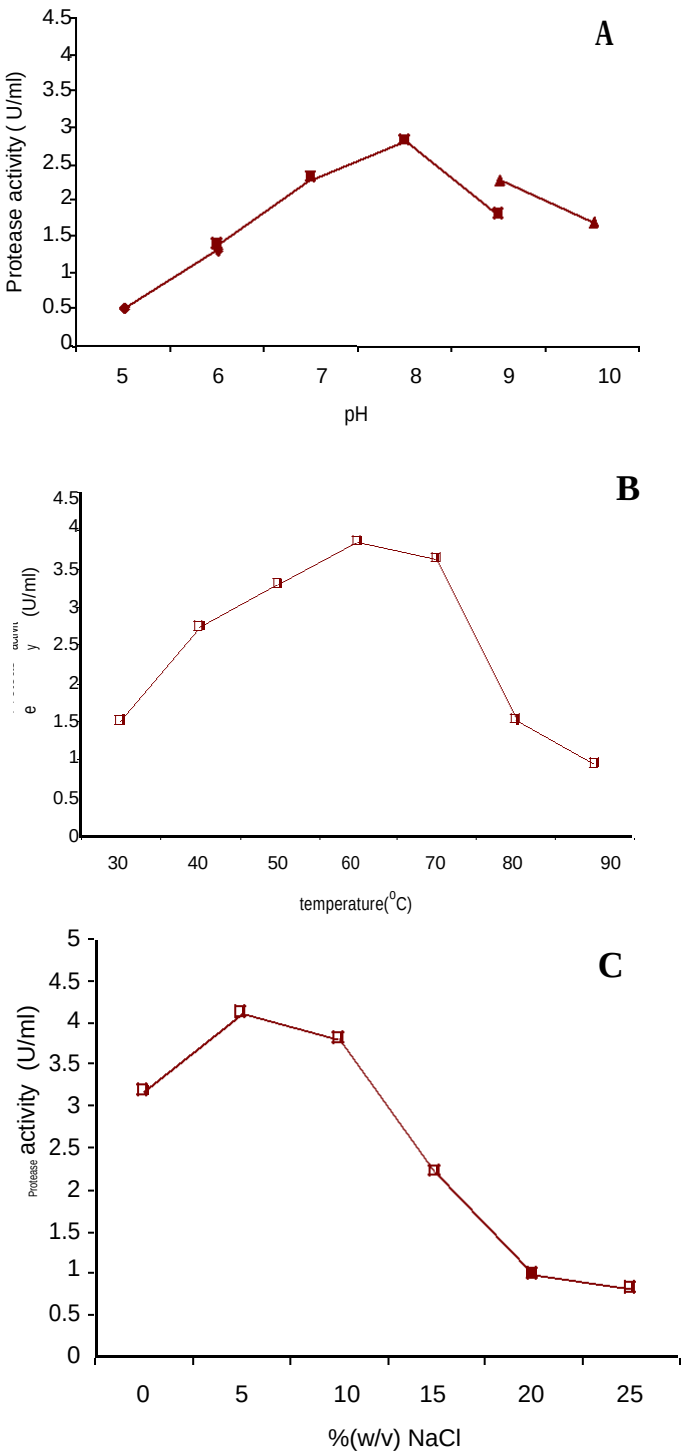

Figure 4. Effect of pH (A), temperature (B) and NaCl concentrations (C) on protease activity of strain TKNR13-3.

bacterium, isolated from *pla-ra* produced almost the same amount of protease (4.38 units/mg protein) as strain TKNR13-3 but has the optimal pH for activity in alkaline pH (pH 10) in the presence of 5% (w/v) NaCl. As shown in Figure 4, the protease of strain TKNR13-3 worked well at higher temperature (60-70°C) than the previous protease of *V. marismortui* reported by Chamroensaksri et al. (2008).

Crude protease incubated with various kind of protease inhibitors including 1mM PMSF, 1mM EDTA, 1 µM E-64, 1 µM pepstatin A, 10 µM chymostatin, 10 µM leupeptin, 10 µM TLCK, 10 µM TPCK, 10 µM phosphoramidon and 0.1 mg/ml trypsin from soybean at 30°C for 30 min was assayed for the residual activity. Protease activity was measured in reaction mixture containing 10% (w/v) NaCl, pH 7.5 and at 37°C using Hammerstain casein as substrate. The enzyme activity was strongly inhibited by chymostatin leupeptin and PMSF by 77.62, 74.86 and 50.71%, respectively. The results suggested that the protease from strain TKNR13-3 belonged to serine type protease as reported in *Virgibacillus* sp. SK33 (Sinsuwan et al., 2010) and in *Virgibacillus marismortui* NB2-1 (Chamroensaksri et al., 2008). Due to the highest inhibition of chymostatin, it was assumed to be chymotrypsin-type (Table 1).

The crude extracellular protease of strain TKNR13-3 was identified by separation on gel electrophoresis followed by staining for proteolytic activity. Activity bands of protease were shown as clear zones on a dark background. Appearance of activity bands observed indicated the presence of proteases with the estimated molecular mass of 12, 21, 29, 39 and 49 kDa (Figure 5). Extracellular protease of strain TKNR13-3 was relatively small in comparison with the extracellular protease previously characterized from other moderately halophilic bacteria: 38 kDa from *Pseudoalteromonas* sp. CP76 (Sánchez-Porro et al., 2003), 38 kDa from *Salinivibrio costicola* (Lama et al., 2005), 17-35 kDa from *Virgibacillus marismortui* NB2-1 (Chamroensaksri et al., 2008). 19-44 kDa from *Virgibacillus* sp. SK37 (Sinsuwan et al., 2007; Phrommao et al., 2010) and 19 kDa and 33.7 kDa from Virgibacillus sp. SK33 (Sinsuwan et al., 2010). However, some others were 120 kDa from *Pseudomonas* sp. A-14 (Duong et al. 1981) and 100, 42 and 17 kDa from *Halobacillus thailandensis* (Chaiyanan et al., 1999).

Proteases have many industrial applications, especially in industrial processes of laundry and dishwashing detergents manufacturing, seafood industrial waste treatment, and deproteinization of crustacean waste for chitin preparation. This present study reports on the identification and protease optimization of *V. halodenitrificans* TKNR13-3. Thus, slightly or moderately thermophilic property of this enzyme will be useful for food fermentation including many industrial purposes.

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Table 1. Effect of various inhibitors on protease activity of strain TKNR13-3.

Inhibitors	% Inhibition	Specificity of inhibitor
Control (without inhibitor)	0	
1mM PMSF	50.71	Serine protease
10 μM Leupeptin	74.86	Serine, cysteine protease
10 μM Chymostatin	77.62	Chymotrypsin-like serine
10 μM TLCK	25.52	Trypsin
10 μM TPCK	10.7	Chymotrysin
0.1 mg/ml Soybean trypsin	10.93	Serine protease, trypsin
1mM EDTA	20.72	Metalloprotease
10μM Phosphoramidon	5.63	Metalloprotease
10μM E-64	1.52	Papain, cysteine protease
1μM Pepstatin A	0.48	Aspartic protease

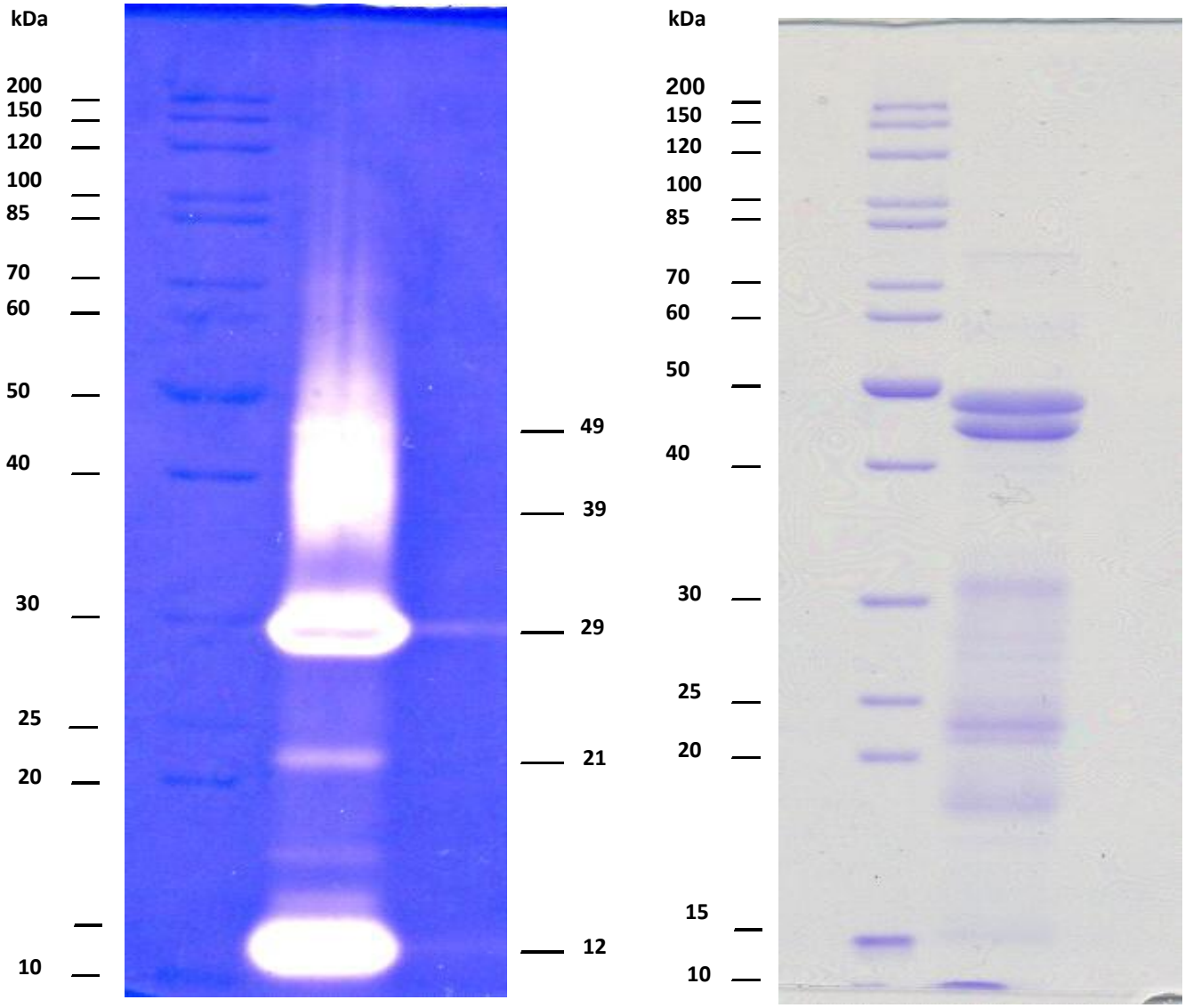

Figure 5. Zymogram of crude proteae of strain TKNR13-3. Proteolytic activity stained gel (A), SDS-PAGE (B). MW marker, lane 1; crude protease, lane 2.

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