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ISOLATION, SCREENING, PARTIAL PURIFICATION AND CHARACTERIZATION OF PROTEASE FROM HALOPHILIC BACTERIA ISOLATED FROM INDONESIAN FERMENTED FOOD

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ABSTRACT

The protease producing bacteria were screened from Indonesian traditional fermented food, *tauco* and *terasi, and* 4 halophilic protease producers were isolated. Among these isolates, halophilic bacterial isolate TANN 4 was recorded as the best protease producer. Extracellular protease from isolate TANN 4 was partially purified using ammonium sulfate precipitation. The protease was partially purified with final yield of 72.87 % and 25.41 fold purity. This moderate thermoactive and alkaliphilic protease showed a pH optimum of 8.0 and temperature optimum was 50 °C. The enzyme was also active at salt concentrations ranging from 1 to 15 % (w/v), with optimum activity at 1 % NaCl (w/v). Ethylenediaminetetraacetic acid (EDTA) completely inhibited the enzyme activity suggesting that it was a metalloprotease. Among metal ions, the Ca^{2+}, K^{+} and Mg^{2+} ions enhanced the activity of enzyme. The K_M and Vmax values exhibited by partially purified protease were 0.0649 mM and 216.45 U mg^{-1} using casein as substrate. The molecular weight was estimated to be 19.8 kDa on SDS PAGE. The enzyme was also fairly stable in Triton X-100, SDS, 1 % commercial detergents (OMO and Ariel) and 25 % methanol and it was capable of hydrolyzing casein, hemoglobin and bovine serum albumin (BSA). These characteristics make this halophilic bacterial extracellular metalloprotease seem to be potentially useful for biotechnological and industrial applications. Automated ribotyping analysis revealed that 3 isolates (TANN 4, TR 2 and TR 4) resembled *Halobacillus trueperi* that exhibited 71, 68 and 69 % similarity respectively, and isolate (TR 1) resembled *Virgibacillus pantothenticus* with 64 % similarity.

Keywords: Metalloprotease, Indonesian fermented food, Halophilic bacteria

1. INTRODUCTION

Most of commercial enzymes are extracellular enzymes such as proteolytic enzymes. Diverse groups of microorganisms, including fungi, yeasts and bacteria synthesize these enzymes. [Dias et al. 2008]. Of the industrial enzymes, 75% are hydrolytic enzymes. Proteases are hydrolytic enzymes which catalyze the total hydrolysis of proteins, play role in degradation of proteins into smaller peptides and amino acids [Rao et al. 1998]. The use of proteases has increased significantly in various industrial processes including detergents production, leather products, pharmaceuticals, as meat tenderizers, protein hydrolysates, food products, and in the waste processing industry [Joo and Chang 2005]. These enzymes account for nearly 60% of the total worldwide enzyme production. Among the various proteases-those produced by microorganisms (microbial proteases) play an important role in biotechnological processes accounting for approximately 59% of the total enzyme [Chu, 2007].

Halophilic enzymes require salt for their activity, stability, and solubility. Stabilities of these enzymes under extreme high salt conditions is speculated due to the presence of higher acidic amino acid residues than the nonhalophilic homologues. Their stability and activity at low water levels make it scientifically potential for industrial and biotechnological applications [Madern D et al. 2000]. Nawab et al. (2016) reported that a halotolerant protease producing from salt mines of Karak, Pakistan identified as *Bacillus subtilis* strain BLK-1.5 produce halophilic protease with a molecular mass of 38 kDa. The proteolytic activities were screened on nutrient agar plates supplemented with 5 % (w/v) NaCl and 1 % (w/v) skim

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milk. The enzyme active was partially purified with 70 % ammonium sulfate and its maximum activity was observed at pH 10.0, 50 °C and 2 % (w/v) NaCl.

Halophiles and halotolerants can be found in hypersaline brines in arid, coastal, salt lake, sea mud, deep layer of oceans, artificial solar salterns and even in fermented salty foods [DasSarma and Arora 2001].

In this study the protease-producing strains were isolated from *tauco* (saline paste made from preserved fermented yellow soybeans) and *terasi* (traditional salty fermented seafood product which is made from fish and/or shrimp in the form of a paste). The proteolytic activity was measured and the one with the highest proteolytic activity was characterized and purified using ammonium sulfate precipitation and dialysis.

2. MATERIALS AND METHODS

2.1 Test Samples

The protease-producing strains were isolated from salty food samples of *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), which were collected from traditional market in Bengkulu, Indonesia and immediately brought to Turkey on September of 2015.

2.2. Screening of Bacterial Strains for Production of Protease

2.2.1. Enrichment and screening for protease-producing halophilic strains from Indonesian traditional fermented food samples of tauco and terasi

For the enrichment of halophilic protease-producing strains, 90 mL of the liquid 18% MGM (Modified Growth Medium) was inoculated with 10 g of each sample. The inoculated flasks were incubated at 37 °C for 1 month at 150 rpm on a rotary shaker incubator. A number of serial dilutions (10^{-1}-10^{-5}) from each inoculated culture were spread onto the surface of 18% MGM contained 1 % of skim milk agar plates and then incubated in sealed plastics at 37 °C for 2 weeks. The protease-producing halophilic strains detected by the formation of a clear zone surrounding the colonies indicated that the skim milk had been degraded. The single colonies were isolated and after culturing on 18% MGM they were transferred into 15 % glycerol for storing at -85 °C [Elbanna et al. 2015; Mutlu 2006].

2.3. Production of halophilic protease and effect of fermentation period on protease production

Protease-producing isolates from food samples were firstly aerobically cultured at 37 °C 150 rpm in 10 ml of starter medium containing 18% MGM. After 6 hours, the bacteria reached its log phase and were transferred into fermentation medium in 250 ml conical flasks containing 40 ml of 18% MGM supplemented with 1% skim milk at 37 °C by 150 rpm agitation for 3 days [Elbanna et al. 2015; Fitriani 2013]. The effect of fermentation period on protease production was conducted for a period of 96 hours of incubation at 37 °C with 150 rpm and the protease activity was measured for every 24 hours [Elbanna at al. 2015].

2.4. Enzyme Isolation and Assay of Protease Activity

After 72 hours cultivation the culture broth was centrifuged at 5.000 rpm for 30 minutes at 4 °C. The crude enzymes were stored at 4 °C. The proteolytic were assayed using modified Amano's method [Fitriani 2013]. Briefly, 625 µL 0.6% casein solution was pipetted into a test tube and was kept in an incubator for 10 minutess 37 ºC. Then 125 µL enzyme solution was added into test tube and shaken thoroughly and then incubated for 10 minutes 37 ºC. At the end of incubation 625 µL TCA (Trichloroacetic Acid) was pipetted and mixed. The mixture was centrifuged at 10000 rpm for 5 minutes

at 4 °C. Then 300 μL of the solution was pipetted into a new test tube and 750 μL of Na2CO3 0.55 M solution and 150 μL 1:2-folds Folin's reagent were added. After thoroughly mixed, the reaction should stand at 37 ºC for 10 minutess in water bath. Finally the reaction solution was pipetted into a cuvette and the absorbance of the mixture solution was measured at 660 nm (Absorbance of samples -A1). As a blank, 300 μL of 0.1 M HCl solution was used (Absorbance of blank-A4), meanwhile tyrosine solution (1 mg/ml) was used as standard (Absorbance of standards-A3).

2.5. Protein Determination using Modified Lowry Assay

Firstly, 20 μl of each standard using bovine serum albumin (BSA) and protease samples replicate was pipetted into labelled test tubes. Then 180 μl of 1X phosphate buffer saline (PBS) and 2 ml Reagent Lowry was added to each test tube. The mixture was mixed and incubated at room temperature for exactly 10 minutess. After 10 minutess of incubation period, 200 μl of 1:2-folds Folin's reagent was added, mixed well by vortex. Then incubated at room temperature for 30 minutes. For the blank, distilled water was used. The absorbance was measured at 750 nm with spectrophotometer. The standard curve was prepared by plotting the average Blank-corrected 750 nm value for each BSA standard vs. its concentration in μl/mg [Lowry et al. 1951].

2.6. Ammonium Sulfate Precipitation and Dialysis

The proteins that present in the crude fraction were precipitated by using ammonium sulfate at 85% saturation level by adding the solid ammonium sulfate pinch by pinch to the cell-free supernatant and stirred for 1 hour then left overnight at 4 °C. After overnight incubation the precipitate was centrifuged at 7500 rpm for 50 minutes and dissolved in Tris-Cl buffer (pH.8.0; 50 mM). The precipitate was collected and dialyzed against the same buffer at 4 °C for overnight [Maruthiah 2013].

2.7. Characterization of Partially Purified Halophilic Protease

2.7.1. Effect of temperature, pH and salt concentration (NaCl) on halophilic protease activity

Effect of temperature for enzyme activity was determined at various temperatures (25, 30, 35, 40, 45, 45, 50, 55, 60 °C) using Amano's method. pH values ranging from 6.0 to 10.0, in 0.05 M Na-phosphate (pH 6.0-8.0); 0.05 M Tris-HCl (9.0); 0.05 M bicarbonate-carbonate (pH 10.0) buffers, was tested at optimum temperature. 0, 1, 5, 10, 15 % (w/v) NaCl at optimum pH and temperature was tested to determine the effect of salt concentration [Elbanna at al. 2015].

2.7.2. Effect of metal ions on halophilic protease activity

The assay mixture was supplemented with 2 mM of $CaCl_2$, $MgCl_2$, $ZnCl_2$, KCl, $FeCl_2$ and $CoCl_2$ at room temperature for 1 hour to show the effect of metal ions on the partially purified enzyme [Elbanna at al. 2015].

2.7.3. Effect of inhibitors and surfactant on halophilic protease activity

Phenylmethyl sulfonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) with final concentrations of 1 mM and 5 mM and SDS and Triton X-100 with final concentrations of 1.0% and 5.0% were added at room temperature for 30 minutes to test the effect of inhibitors and surfactants on enzyme activity [Elbanna at al. 2015].

2.7.4. Halophilic protease stability in commercial detergents

The purified enzyme was incubated with detergent solutions (OMO and Ariel) with final concentration of 1% at 40 °C for 60 minutes for determination of stabilisation of the halophilic enzyme [Elbanna at al. 2015].

2.7.5. Determination of K_M and Vmax of halophilic protease

Different concentrations of casein substrate 0.2-1.0% was used to determine the K_M and Vmax values of the pure enzyme by using Lineweaver–Burk plot [Elbanna at al. 2015].

2.7.6. Effect of organic solvents on protease stability

The partially purified enzyme was incubated with organic solvents (isopropanol, ethanol, methanol, butanol, and acetone) with final concentration of 25 % at 40 °C for 1 hour with shaking [Ibrahim et al. 2015].

2.7.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [1970]. The samples were heated at 95 °C for 5 minutes, then the molecular mass of the protease was determined using 5 % (w/v) stacking and 12 % (w/v) resolving gels (Acrylamide/Bis). A ready to use molecular marker (Color Burst Electrophoresis Marker (SIGMA C1992) 8,000 - 220,000 Da)was used as a standard. The gel was run through stacking gel at 60 V for 30 minutes and for the resolving gel at 100 V. Finally, the gel was washed with distilled water and the proteins were stained with EzBlue Gel Staining Reagent (SIGMA) for 45 - 60 minutes. Destaining the gel was carried out by washing with distilled water for 2 hours.

2.7.8. Substrate specificity of partially purified protease

The substrates studied were casein, bovine serum albumin (BSA), collagen, gelatin and hemoglobin. Partially purified protease (0.5 ml) was added to 2.0 ml of 1% substrate. The mixture then was incubated at 50 °C for 30 minutes, the reaction then was stopped by adding 2.5 ml of 10 % TCA. The mixture was incubated for 15 minutes and then centrifuged at 10,000 rpm for 10 min at 4 °C. Finally, the precipitate was removed. The mixture solution was measured at 280 nm and 260 nm. One unit of protease activity was defined as the amount of enzyme required to release 1 μg of tyrosine per milliliter per minute under the specified assay conditions [Olajuyigbe and Falade, 2014].

2.8. Identification of protease-producing halophilic strains by automatic ribotyping (DuPont™ RiboPrinter® System)

The halophilic strains were characterized by automated ribotyping using *Eco* RI. The automated ribotyping was performed by using a robotized instrument (RiboPrinter® System Microbial Characterization System, Qualicon, DuPont™, Wilmington, DE, USA) and the RiboPrinterTM System Data Analysis Program [Güven et al. 2010].

3. RESULTS

3.1. Screening for Protease-Producing Halophilic Strains and Protease Activity

Indonesian traditional fermented salty sauce food, tauco and terasi, were chosen as the sources of halophilic protease producers and 4 halophilic strains were successfully isolated. The isolates were

picked up by screening on 18% MGM agar plates supplemented with 1% skim milk for detection of proteolytic activity. The protease- producing isolates namely, TANN 4, TR 1, TR 2, and TR 4 gave clear zones around the bacterial colonies (Figure 1a). Among these isolates, the data in Figure 1b showed that isolate TANN 4 was found to have the highest specific activity with 11.85 U mg-1. Based on this fact, isolate TANN 4 was selected for further purification and characterization.

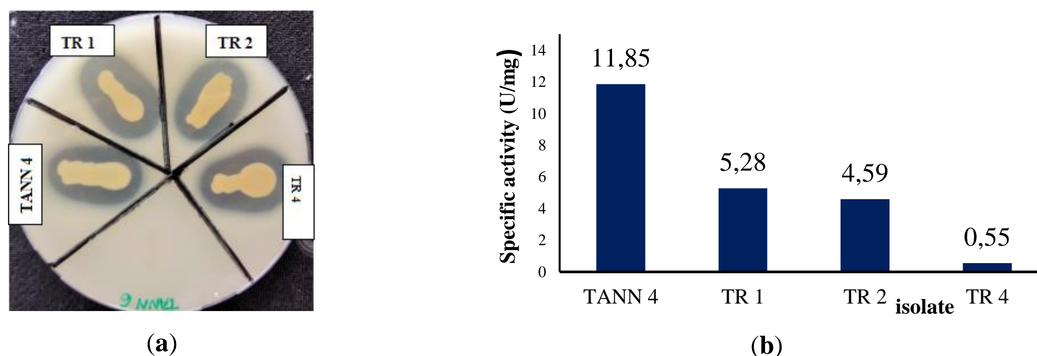


Figure 1. (a) Halophilic protease producing isolates on 18% MGM agar plates supplemented with 1% skim milk; (b) Specific Activity of Halophilic Protease from Different Isolates

3.2. Effect of Fermentation Period on Halophilic Protease Production

The best fermentation period was determined by monitoring the enzyme activity of isolate TANN 4 during 4 days. The enzyme activity of isolate TANN 4 reached its maximum after 3 days on 18% MGM medium supplemented with 1% skim milk at pH 7.5 with specific activity of 10.41 U mg^{-1}. Meanwhile, minimal activity was detected after 1 day (Figure 2), then decreased after 4th day.

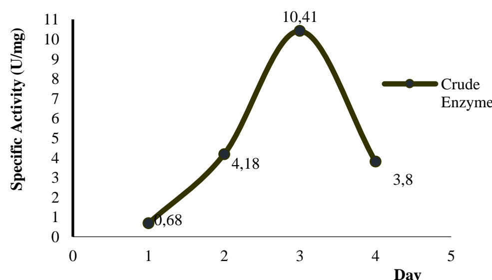


Figure 2. Effect of Fermentation Period on Halophilic Protease Production

3.3. Partial Purification of Halophilic Protease from Isolate TANN 4

After ammonium sulfate and dialysis step, as presented in the purification table (Table 1), the enzyme was purified up to 25.41 fold-enrichment with 72.82 % recovery from total crude enzyme. The specific activity of the partially purified enzyme (Table 1) also increased from 11.85 to 301.14 U mg^{-1}.

Table 1. Properties of the halophilic protease from isolate TANN 4

Fraction	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	*Yield* (%)	Purification (Fold)
Cell-Free Supernatant (Crude)	22.04	1.86	48.00	1 057.92	89.28	11.85	100.00	1.00
Dialysate 85%	192.73	0.64	4.00	770.92	2.56	301.14	72.87	25.41

3.4. Characterization of Halophilic Protease from Isolate TANN 4

Biochemical properties such as temperature and pH profile of the crude and the partially purified (dialysate 85%) extracellular metalloprotease from halophilic bacterial isolate TANN 4 revealed a moderate thermoactive and alkalophilic character. The partially purified fraction was active at a temperature range of 25–60 °C and the maximum activity of the protease was detected at 50 °C (Figure 3a). The partially purified protease showed activity over a broad range of sodium chloride concentrations and pH values. The results showed that the enzyme was active in a wide pH range from 6.0 to 10.0. The highest activity was found at pH 8.0 (Figure 3b) and the enzyme was active at salt concentrations ranging from 1 to 15 % (w/v) that showed maximal activity at 1 % (w/v) / 0.1 M NaCl (Figure 4).

The enzyme inhibition studies showed that the partially purified protease was completely inhibited by the metalloprotease inhibitor Ethylenediaminetetraacetic acid (EDTA) and not inhibited by the serine-protease inhibitor Phenylmethylsulfonyl fluoride (PMSF), suggesting that the partially purified protease from isolate TANN 4 is a halophilic extracellular metalloprotease (Figure 4).

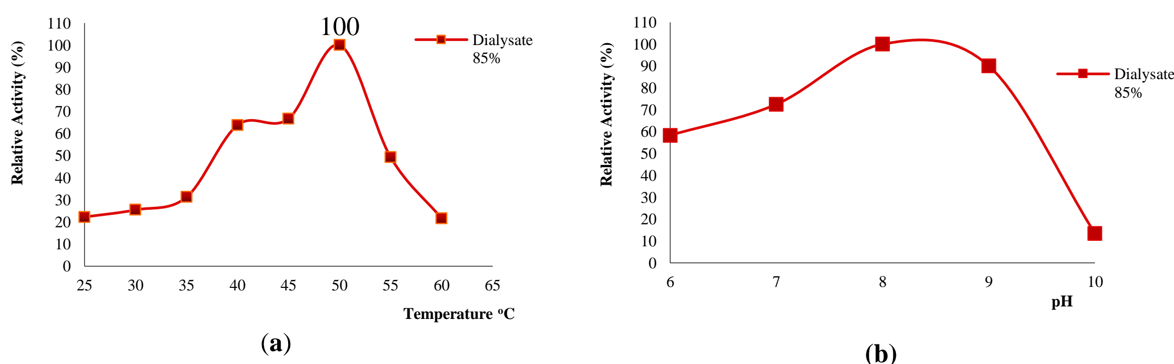


Figure 3. Effect of temperature (a) and pH (b) on relative activity of protease.

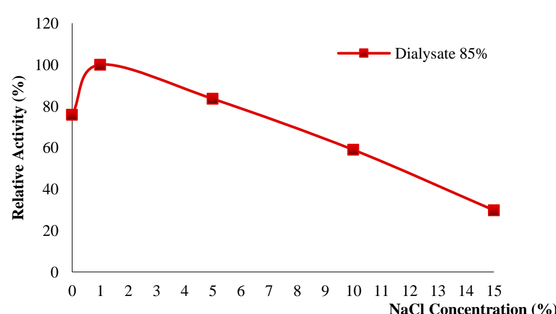
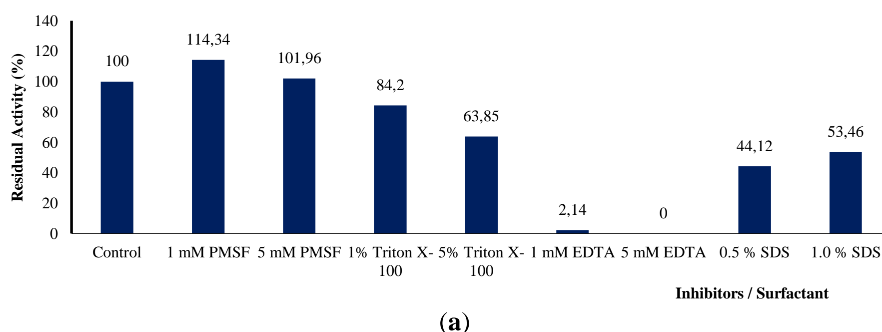


Figure 4. Effect of the NaCl concentration (The maximum activity in each figure was considered as 100 % and the other data were calculated as relative to it)

Ions Ca^{2+}, K^{+} and Mg^{2+} showed no inhibition or enhanced the enzyme activity. In contrast, Co^{2+} and Zn^{2+} caused severe inhibition of the enzyme activity, meanwhile Fe^{3+} slightly inhibited the enzyme activity with residual activity of 66.32 % compared to the control (Figure 5).



(a)

Figure 5. Effect of inhibitors and surfactants on partially purified protease activity.

The partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 was stable and active by addition of 1 % detergents (Ariel and Omo) with 53.61 of residual activity and 42.78 % of original activity respectively (Figure 6). Under similar conditions, the commercial detergent stability of partially purified protease from isolate TANN 4 was higher than that reported for purified protease from *Bacillus* sp. strain NPST-AK15 [Ibrahim et al. 2015], which retained 45.9 and of 36.2 % of its activity in the presence of the detergents.

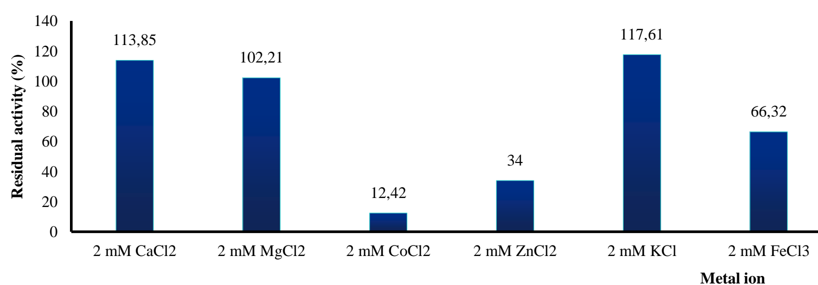
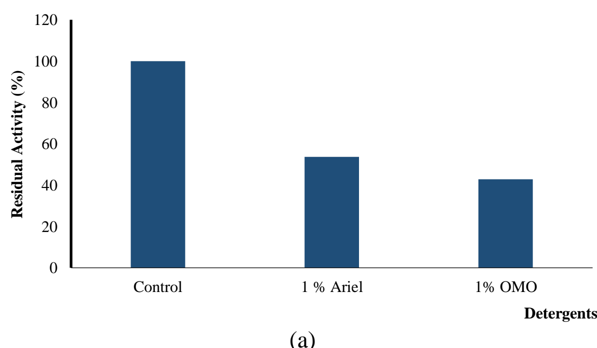


Figure 6. Effect of metal ions on partially purified protease activity (The enzyme activity of control in each figure was considered as 100 % and the other data were calculated as relative to it).

The partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 was partially active and stable in the presence of 25 % (v/v) methanol and less stable in the presence of 25 % (v/v) ethanol, propanol, and acetone (Figure 7).



(a)

Figure 7. Stability of partially purified protease in commercial detergents Areie and Omo (The enzyme activity of control in each figure was considered as 100 % and the other data were calculated as relative to it).

The proteolytic activity of partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 was increased up to 1.0 % (w/v) by casein. The K_M and Vmax values which calculated using a Lineweaver–Burk plot were 0.0649 mM and 216.45 U mg^{-1} (Figure 8), respectively.

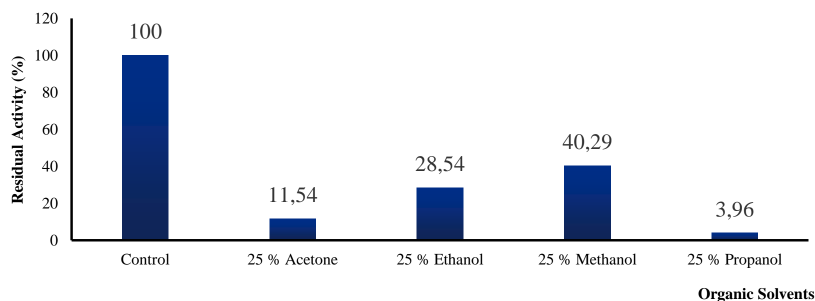


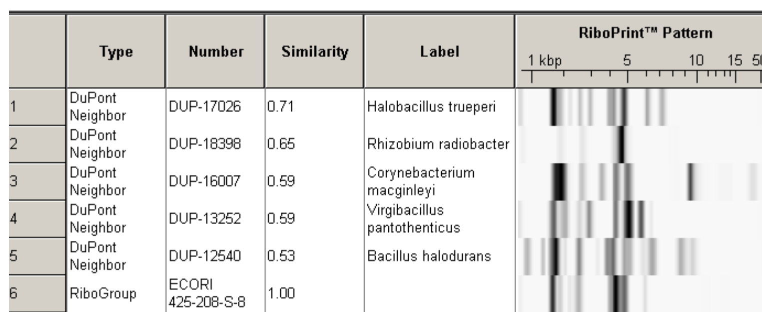
Figure 8. Effect of organic solvents on protease stability (The enzyme activity of control in each figure was considered as 100 % and the other data were calculated as relative to it).

[Substrate]	[Protein concentration mg/ml]	Relative Activity (%)
Casein (Control)	0.1227	100
BSA	0.0181	14.75
Hemoglobin	0.1533	124.94
Collagen	Unhydrolyzed	
Gelatin	Unhydrolyzed	

The partially purified protease from isolate TANN 4 hydrolyzed casein, bovine serum albumin, and hemoglobin which casein was used as control (100 % activity). Hemoglobin was perfectly hydrolyzed with relative activity of 124.94 % and BSA was poorly hydrolyzed with relative activity of 14.75 % (Table 2), respectively. Meanwhile, collagen and gelatin were unhydrolyzed by the enzyme.

3.5. Identification of Protease-producing Halophilic Strains by Automatic Ribotyping (DuPont™ RiboPrinter® System)

Gram-staining analysis revealed that isolate TANN 4 is a gram-positive, coccobacillus bacterium. The rRNA based phylogenetic analysis, automated ribotyping, revealed that isolate TANN 4 *resembled Halobacillus trueperi* by 71 % similarity (Figure 9). In addition, protease positive isolates TR 2 and TR 4 also resembled *Halobacillus trueperi* that exhibited 68 and 69 % similarities (Table 3). On the other hand, automated ribotyping (RiboPrinter® System Microbial Characterization System, DuPont™) revealed that isolate TR 1 resembled *Virgibacillus pantothenticus* which exhibited 64 % similarity.



	Type	Number	Similarity	Label	RiboPrint™ Pattern 1 kbp 5 10 15 50
1	DuPont Neighbor	DUP-17026	0.71	Halobacillus trueperi	
2	DuPont Neighbor	DUP-18398	0.65	Rhizobium radiobacter	
3	DuPont Neighbor	DUP-16007	0.59	Corynebacterium macginleyi	
4	DuPont Neighbor	DUP-13252	0.59	Virgibacillus pantothenticus	
5	DuPont Neighbor	DUP-12540	0.53	Bacillus halodurans	
6	RiboGroup	ECORI 425-208-S-8	1.00		

(a)

Figure 9. Automated ribotyping result for isolate TANN 4.

Table 3. Automated ribotyping results

Isolate	Agar Media	Gram Staining	Source	Automated Ribotyping Result	similarity
TANN 4	18%MGM	Positive	Tauco	*Halobacillus trueperi*	0.71
TR 1	18%MGM	Positive	Terasi	*Virgibacillus pantothenticus*	0.64
TR 2	18%MGM	Positive	Terasi	*Halobacillus trueperi*	0.68
TR 4	18%MGM	Positive	Terasi	*Halobacillus trueperi*	0.69

4. DISCUSSION

Terasi is described as a traditional Indenosian salty fermented seafood or shrimp paste with a typically characteristic aroma of cheese and ammonia. Earlier study reported that terasi was composed of 16.8 % NaCl, 25.4 % protein, 6.1 % fat, 1.9 % carbohydrates, 29.1 % ash (including salt) with moisture content of 37.4 % and pH 7.5 [Surono and Hosono 1994]. They also reported that the dominant bacteria isolated from terasi were identified as halophilic *Bacillus* sp. and enzyme activities were detected including esterase and protease. Proteolytic activities were detected in terasi sample due to during its fermentation process, protease are responsible for the hydrolysis of the protein that belong to fish and shrimp into small peptides and amino acids. This hydrolysis is responsible for cheesy odor in terasi [Christanti A.D 2006]. Meanwhile, *tauco* is defined as the product of mold, bacteria, and yeast fermentation of yellow soybeans, which is on its fermentation process 25 to 30% salt based upon the weight of soybeans is added. During *tauco* fermentation process especially during the initial mold growth, the pH rises as soybean protein is hydrolyzed to peptides, peptones, and free amino acids [Steinkraus 1995]. Dewi's study [Dewi W.K 2006] also reported that bacterial isolate W-1 that was isolated from black tauco showed a fibrinolytic protease activity that effectively hydrolyzed casein and fibrinogen.

Many researches have been done on the properties of the enzymes from halophilic and halotolerant bacteria and their industrial applications [2010c]. In this investigation, a novel extracellular protease was partially purified from the culture supernatant of halophilic bacteria isolate TANN 4. Biochemical properties of the crude and the partially purified (dialysate 85%) extracellular metalloprotease from halophilic bacteria isolate TANN 4 such as temperature and pH profile revealed a moderate thermoactive and alkalophilic character. Similar results were reported for *Bacillus cereus* WQ9-2 [Xu et al. 2010], *Salinivibrio* sp. Strain MS-7 [Shahbazi M and Karbalaei-Heidari 2012] and *Bacillus* sp. APCMST-RS7 [Maruthiah T et al. 2015]. Some studies also reported moderate thermoactive and alkalophilic proteases with a broad pH and salinity activities. Karbalaei-Heidari et al. have reported a serine metalloproteases of the bacterium *Halobacillus karajensis* strain MA-2 showed maximum activity at at 50 °C, pH 9.0 and 0.5 M NaCl [Karbalaei-Heidari et al. 2009b]. Another metalloprotease from *Salinivibrio* sp. strain AF-2004 was also observed to be stable and active at broad pH profile (5.0–10.0) with an optimum of 8.5 for casein hydrolysis and showed its maximum activity at temperature of 55 °C and salinity of 0–0.5 M NaCl [Karbalaei-Heidari et al. 2007a].

The enzyme inhibition studies showed that the partially purified protease from isolate TANN 4 was completely inhibited by the metalloprotease inhibitor Ethylenediaminetetraacetic acid (EDTA) and not inhibited by the serine-protease inhibitor Phenylmethylsulfonyl fluoride (PMSF), suggesting that the partially purified protease from the isolate is a halophilic extracellular metalloprotease (Figure 7). BEMP (Bacterial extracellular metalloproteases) are endoproteases which coordinate with other extracellular proteases to degrade the proteins outside the cell. Metalloproteases contain Zn^{2+}, while a few contain Mg^{2+}, Ni^{2}, or Cu^{2+} in their active centers. These metal ions serves as a nucleophile in catalysis that activated a water molecule. Some examples of BEMPs have been reported from earlier studies. Thermolysin like-metalloproteases (TLPs) are group of metalloprotease that were isolated from *Bacillus thermoproteolyticus* and *Alicyclobacillus acidocaldarius*. Many of metalloproteases have been applied in industries. Examples are Thermoase PC10F (Amano Enzyme Inc., Japan), Neutrase (Novo Nordisk, Denmark), Protin PC10F (Amano Enzyme Inc., Japan), and the highly stable TLP-ase variant

Boilysin (Groningen, The Netherlands). In food industries, thermolysin and vimelysin from *Vibrio* sp. T1800 has been used for the synthesis of artificial sweetener aspartame. Some BEMPs are also used to hydrolyze food proteins to produce flavor-enhancing peptides, like neutrase which has been used in sausages fermentation processes and in bread manufacturing, brewing and leather processing industry [Wu and Chen 2011]. The enzyme is also stable in the presence of nonionic surfactant 1 and 5 % Triton X-100, retaining 84.20 and 63.85 % of its original activity.

Metalloproteases contain Zn^{2+}, while a few contain Mg^{2+}, Ni^{2+} or Cu^{2+} in their active centers [Wu and Chen 2011]. Based on its metal ions effect profile, metalloprotease from isolate TANN 4 might be contain Mg^{2+} in its active site. The results are relatively similar to those reported for purified *Bacillus* sp. strain NPST-AK15 protease [Ibrahim et al. 2015]. Metal ion Ca^{2+} reported also caused an increase in the proteolytic activity in serine metalloprotease from *Salinivibrio* sp. strain AF-2004 [Karbalaei-Heidari et al. 2007a].

The partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 was partially active and stable in the presence of 25 % (v/v) methanol and less stable in the presence of 25 % (v/v) ethanol, propanol, and acetone (Figure 9). These results indicate that the protease from isolate TANN 4 is not suitable for some biotechnological applications involving organic solvents in their processes, such as synthesis of peptide and ester under nonaqueous conditions [Maruthiah et al. 2015]. On the other hand, the organic solvent tolerance metalloproteases are reported from *Bacillus cereus* WQ9-2 [Xu et al. 2010] and *Salinivibrio* sp. strain AF-2004 [Karbalaei-Heidari et al. 2007a], which were highly stable in several organic solvents like hexane, methanol, ethanol, toluene, propanol, and benzene.

The proteolytic activity of partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 was increased up to 1.0 % (w/v) casein. The K_M and Vmax values were 0.0649 mM and 216.45 U mg^{-1}, respectively. These result is comparable to purified metalloprotease from *Salinivibrio* sp. strain AF-2004 [Karbalaei-Heidari et al. 2007a]. Higher K_m and Vmax values in the literature also were reported from purified halophilic serine protease produced by *Halobacterium* sp. strain HP25 [Elbanna et al.2015]. Lower K_M indicates that the enzyme requires only a small amount of substrate to become saturated.

Most bacterial extracellular metalloproteases contain one catalytic domain and their molecular weight ranges from 20 to 35 kDa [Wu and Chen 2011]. In this study, the partially purified extracellular metalloprotease of isolate TANN 4 showed a protein band on SDS-PAGE with estimated molecular weight of 19.8 kDa (Figure 12). The molecular mass of the partially purified halophilic protease of isolate TANN 4 is significantly lower than other halophilic metalloprotease indicating that it is a novel metalloprotease. For example, molecular weight of the metalloprotease from *Salinivibrio* sp. strain AF-2004 was 31 kDa [Karbalaei-Heidari et al. 2007a], *Halobacillus karajensis* strain MA-2 was 36 kDa [Karbalaei-Heidari et al. 2009b], *Bacillus cereus* WQ9-2 was 37 kDa [Xu et al. 2010] and *Salinivibrio* sp. Strain MS-7 was 21 kDa [Shahbazi M and Karbalaei-Heidari 2012].

The partially purified protease from isolate TANN 4 hydrolyzed casein, bovine serum albumin, and hemoglobin. Meanwhile, collagen and gelatin were unhydrolyzed by the enzyme. Earlier study reported that a serine metalloprotease from *Bacillus brevis* MWB-01 was also capable to hydrolyze BSA and egg albumin, while gelatin and collagen were poorly hydrolyzed [Olajuyigbe and Falade, 2014].

The rRNA-based phylogenetic analysis, automated ribotyping (RiboPrinter® System Microbial Characterization System, DuPont™) revealed that isolate TANN 4 *resembled Halobacillus trueperi* by 71 % similarity index. In addition, protease positive isolate TR 2 and TR 4 also resembled *Halobacillus trueperi* by 68 and 69 % similarities, respectively (Table 3). On the other hand, automated ribotyping (RiboPrinter® System Microbial Characterization System, DuPont™) revealed that isolate TR 1

resembled *Virgibacillus pantothenticus* which exhibited 64 % similarity. *Virgibacillus* described as a genus of aerobic endospore-forming bacteria. Their cells are motile, Gram-positive rods which often form chains [Heyndrickx et al. 1998]. These automated ribotyping results actually did not reach the 85 % similarity threshold with reference riboprint patterns, so further identification such as phylogenetic analysis based on 16S rRNA gene sequences are needed. Earlier studies reported that a serine protease was isolated from *Halobacillus* sp. SR5-3 resembled *Halobacillus trueperi* with 97.1% similarity [Namwong et al. 2006]. *Halobacillus trueperi* SS1 and SS3, isolated from soil sediment in India, were also reported to exhibit salt dependent extracellular amylase and protease activities [Gupta et al. 2015]. Additionally, Taprig et al (2013) revealed that a total of 7 isolates based on 16S rRNA gene sequences analysis were identified as *Halobacillus* sp. and *Virgibacillus* sp exhibited protease activities.

5. CONCLUSION

Among four of halophilic protease producing isolates, halophilic bacterial Isolate TANN 4 isolated from tauco (fermented yellow soybeans) has been found to exhibit the highest extracellular metalloprotease activity and the bacterium was identified as *Halobacillus trueperi*. The partially purified enzyme from isolate TANN 4 showed a moderate thermoactive and alkalophilic character and it was active at salt concentrations ranging from 1 to 15 % (w/v). The enzyme was fairly stable in several commercial detergents, surfactants and organic solvents and has been found to be able to hydrolyze casein, hemoglobin and BSA which are the typical characteristics of enzymes for biotechnological and industrial applications. Therefore, further protein purification, bacterial identification and media optimization are needed to improve the biotechnological potential of the enzyme.

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