ISOLATION, SCREENING, PARTIAL PURIFICATION AND CHARACTERIZATION OF HALOPHILIC PROTEASE FROM DIFFERENT SAMPLES

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ABSTRACT

ISOLATION, SCREENING, PARTIAL PURIFICATION AND CHARACTERIZATION OF HALOPHILIC PROTEASE FROM DIFFERENT SAMPLES

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Anadolu University, Graduate School of Sciences, June, 2016

Supervisor: Prof. Dr. Kıymet GÜVEN

In this research the protease producing bacteria were screened from Indonesian traditional fermented food and saline soil sample from Indonesia. The purpose of this research was to partially purify and characterize microbial protease from halophilic bacteria. During the study, 4 halophilic protease producers were isolated from *tauco* and *terasi*. Among these isolates, halophilic bacteria isolate TANN 4 was recorded as the best protease producer. The isolate TANN 4 was grown in 18 % MGM (Modified Growth Medium) agar and broth containing 1% skim milk. Extracellular protease from isolate TANN 4 was partially purified using amonium sulfate precipitation and dialysis. The protease was partially purified with final yield of 72.87 % and also with 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 also was 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 V_{max} 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 also fairly stable in Triton X-100 (1 and 5 %), SDS (0.1 and 0.5 %), 1 % commercial detergents (OMO and Ariel) and 25 % methanol. In addition, this enzyme was capable of hydrolyzing casein, hemoglobin and bovine serum albumin (BSA). Automated ribotyping analysis revealed that 3 isolate (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. To improve this study, further protein purification, bacterial identification and media optimization are needed.

Keywords: Protease 1, Halophilic microorganisms 2, Purification 3, Characterization 4, Indonesian fermented foods 5.

ÖZET

FARKLI ÖRNEKLERDEN HALOFİLİK PROTEAZ TARANMASI, İZOLASYONU, SAFLAŞTIRILMASI VE KARAKTERİZASYONU

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Danışman: Prof. Dr. Kıymet GÜVEN

Bu çalışmada, Endonezya fermente tuzlu gıdaları ve tuzlu toprak örneklerinden proteaz üreten bakteriler taranmış ve izole edilmiştir. Bu çalışmanın amacı, halofilik bakterilerin izolasyonu ve daha sonra bunlardan halofilik proteazların taranması, saflaştırılması ve karakterize edilmesidir. Bu çalısmada, Endonezya geleneksel fermente tuzlu gıdaları olan tauco ve terasi'den 4 tane halofilik bakterileri izole edildi. Bu izolatların arasında en iyi proteaz üreten izolat olarak, tauco'dan izole edilen TANN 4 seçildi. Bu izolat % 1 süt tozu ile takviye edilmiş % 18 MGM (Modifiye Geliştirme Medium) broth ve agar besiyerinde geliştirildi. İzolat TANN 4'ten hücre dışı proteaz enzimi, $(NH_4)_2SO_4$ çöktürmesinden sonra dializ ile kısmi olarak saflaştırıldı. Proteaz enzimi kısmi olarak %72.87 verimle ham homojenata göre 25.41 kat saflaştırıldı. Bu termoaktif ve alkalifilik saflaştırılan proteaz enziminin optimum pH ve sıcaklığı sırasıyla 8.0 ve 50 °C olarak bulundu. Saflaştırılan enzim, %1 'den %15'e kadar olan genis bir tuz konsantrasyon aralığında aktif olarak belirlendi ve optimum tuz konsantrasyonu ise %1 (w/v) olarak bulundu. Enzim aktivitesi etilen diamin tetra asetik asit (EDTA) spesifik proteaz inhibitörüyle tamamen inhibe olduğu için enzim grubu metalloproteaz olduğu belirlendi. Proteaz enziminin Ca^{2+}, K^+ ve Mg^{2+} gibi metal iyonlarıyla aktive olduğu belirlendi. Saflaştırılan enzimin K_m ve V_{mak} kinetik parametreleri kazein substratı kullanılarak sırasıyla 0.0649 mM ve 216.45 U mg^{-1} olarak bulundu. Saflaştırılan enzimin molekül ağırlığı sodyum dodesilsülfat poliakrilamit jel elektroforezi (SDS PAGE) ile yaklaşık 19.8 kDa olarak tayin edildi. Enzimin yüzey aktif maddeleri (% 0.1-0.5 SDS ve % 1-5 Triton X-100), %1 OMO ve Ariel deterjanlar ile ve ayrıca % 25 metanol gibi organik çözücülerle kararlılığını koruduğu tespit edildi. Ayrıca, saflastırılan proteaz enziminin en iyi aktiviteyi kazein, sıgır serum albumini (BSA) ve hemoglobin substratlarına karsı gösterdigi görülmüstür. Proteaz üretici izolatlarin identifikasyonu için ribotiplendirme analizi gerçekleştirilmiş ve bu örneklerden 3 (izolat TANN 4, TR 2 ve TR 4) tanesinin sırasıyla % 71, 68 ve 69 % benzerlik oranları ile *Halobacillus trueperi*) ve 1 tanesinin (izolat TR 1) ise % 64 oranı ile *Virgibacillus pantothenticus* oldukları tespit edilmiştir. Bu çalışmayı geliştirmek için daha ileri seviyede protein saflaştırma, bakteriyel tanımlama ve besiyerleri optimizasyonu gerekmektedir.

Anahtar Sözcükler: Proteaz 1, Halofilik mikroorganizmalar 2, Saflaştırma 3 Karakterizasyon 4, Endonezya fermente gıdaları 5.

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Sarah Fitriani

04/08/2016

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis is original work prepared by me; that I have behaved in accordance with the scientific ethical principles and rules throughout the stages of preparation, data collection, analysis and presentation of my work; that I have cited the sources of all data and information that could be obtained within the scope of this study, and included these sources in the references section; and that this study has been scanned for plagiarism with "scientific plagiarism detection program" used by Anadolu University, and that "it does not have any plagiarism" whatsoever. I also declare that, if a case contrary to my declaration is detected in my work at any time, I hereby express my consent to all the ethical and legal consequences that are involved.

(Signature)

Sarah Fitriani

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LIST OF SYMBOLS AND ABBREVIATIONS

β **:** Beta

ADP **:** Adenosine diphosphate

ATP **:** Adenosine triphosphate

°**C** **:** Degree Celsius

CoA **:** Coenzyme A

g **:** Gram; mg : milligram ; μg : micro gram

Da **:** Dalton, a unit of mass; kDa: kilodaltons

L **:** Liter; ml: milliliter; μl: microliter

M **:** Molar concentration, also called molarity**;** mM: millimolar

U **:** Enzyme unit, a unit for the amount of a particular enzyme

I. INTRODUCTIONS

Halophiles defined as salt-loving organisms that live in hypersaline environments. They can be found in all three domains *Archaea*, *Bacteria*, and *Eucarya* (Oren, 2003a, p. 23). Halophiles use two strategies to maintain their live in hypersaline environments. The first strategy is the accumulation of molar concentrations of KCl and that interfere with enzymatic activity. Another strategy is to synthesize and accumulate organic compatible solutes that do not get involved in enzymatic activity (Oren, 2008b, p. 6). Research nowadays has led to an increased interest in halophiles especially in their potencies and values for biotechnology. The biotechnological applications of halophiles for example in the production of some fermented foods, retinal proteins, compatible solutes, biosurfactans, bio polymers, β-carotene and also the current potency is the production of salt-tolerant enzymes. These salt-tolerant enzymes including amylase, proteases and nucleases (Oren, 2010c, p. 825).

Enzymes are proteins which have molecular weights ranging from about 12,000 to more than 1 million, play an important role in every biochemical process. For example in organized sequences, catalyze the hundreds of step wise reactions, conserve and transform chemical energy, and make biological macro molecules from simple precursors (Nelson and Cox, 2007, p. 184). 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, p. 2027). Proteases are one of the proteolytic enzymes. 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, p. 1263). These enzymes accounting for nearly 60% of the total worldwide enzyme production. Among the various proteases, proteases that produced by microorganisms (microbial proteases) play an important role in biotechnological processes accounting for approximately 59% of the total enzyme. Microorganisms serve as preferred enzymes source because of their rapid growth, the

limited space required for their cultivation and they can be genetically manipulated to produce new enzymes (Chu W.H, 2007, p. 241).

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, Ebel C, and Zaccai G, 2000, p. 91). Halophiles 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, p. 1). Current study reported that a halotolerant protease producing from salt mines of Karak, Pakistan and 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 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 (Nawab et al., 2016, p. 1). In this study the proteases were isolated from *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal, Indonesia. The proteolytic activity were measured and the one with the highest proteolytic activity was characterized and purified using ammonium sulfate precipitation and dialysis.

1.1. Statement of Problem

The broad applications of halophilic microbial proteases in biotechnology and industries has led to an increased interest in the study of these enzymes. The study area including optimization, characterization, and purification. Their special characteristic that requires salt for their activity, stability, and solubility leading to the differences culture conditions compared to the nonhalophilic one. Despite the fact that mentioned above, the study on halophilic enzymes especially haloprotease are needed, not only

interesting from scientific viewpoint, but also based on their potencies in industrial and biotechnological applications, owing to their stability and activity at low water levels.

1.2. Objectives of the Study

The objective of this study was to screen, isolate, characterize, and purify the halophilic protease from isolates obtained from *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal, Indonesia. Proteolytic activity were measured not only qualitatively but also quantitatively and the isolate with the highest proteolytic activity was identified, protease was characterized and purified using ammonium sulfate precipitation and dialysis.

1.3. Significance of the Study

The findings of this study will be used in the future halophiles research using *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Indonesian coastal since research on halophiles among Indonesian researchers are limited. This study also will be beneficial to other halophiles researchers since halophiles properties like their enzymes are useful in industries and biotechnology. It will also serve as future references for the next researchers on the subject of halophilic enzymes, especially halophilic proteases. Moreover, this study will be helpful as references to the industry that use halophilic proteases in their industrial processes.

1.4. Hypothesis

The halophilic microorganisms (isolates) from Indonesian traditional fermented salty foods and sediment soil from Bengkulu coastal will have proteolytic activity. The protease will have high proteolytic activity and can be purified using ammonium sulfate precipitation and dialysis. The purified protease can be characterized and will be beneficial in industries or biotechnology.

1.5. Scope and Limitations

This study was conducted at the Microbiology Research Laboratory, Department of Biology, Anadolu University, Eskişehir, Turkey from September of 2015 until June of 2016. The source for bacterial isolation are *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal, Indonesia.

Although the research has reached it aims, there were some unavoidable limitations. First, not every isolate has protease activity, actually only isolates that isolated from *terasi* and *tauco* exhibited protease activity. Second, in this study the bacterial isolates yielded protease enzyme, but the protease might be insignificant to be used in industrial processes. Third, because of the time limit (scholarship period), the purification step on this research was only conducted until partial purification including ammonium sulfate precipitation and dialysis which were not enough to get high yield and high purity of the protease enzyme, to some extent due to the time limit the zymography also cannot be conducted. Finally, the protease enzymes that were isolated from halophiles might be not have extremozymes characteristics, such as halotolerant or thermo tolerant properties.

1.6. Definition of Terms

For the purpose of clarification, the important terms used in this study have been defined. The following terms are:

Characterization. Refers to the use of methods or mathematical modelling to describe the characteristics of halophilic protease enzyme.

Enzyme Activity. Refers to the catalytic effect exerted by an enzyme, expressed as units per milligram (U/mg) of enzyme (specific activity) or molecules of substrate transformed per minute per molecule of enzyme (molecular activity).

Isolation. Refers to any procedure in which a given species of microorganism, present in particular sample is obtained in pure culture.

U (Unit). One unit is defined as the enzyme quantity which liberates Folin-positive

amino acids and peptides corresponding to 1 μg of Tyrosine per minute under the conditions that use in assay method of Protease "Amano" K.

Partial Purification. Defined as a series of protein (enzyme) purification realize by applying successively ammonium sulfate precipitation and dialysis.

Screening. Refers to examination to detect proteolytic activity from isolate.

Total Protein. Refers to the total level of protein in enzyme solution.

II. REVIEW OF RELATED LITERATURE AND STUDIES

2.1 Halophilic Microorganisms

2.1.1 Introduction to halophilic microorganisms

Halophiles defined as salt-loving organisms that live in hypersaline environments. They can be also described as microorganisms that show growth at salt concentrations higher than 1.7 M or 100 g l^{-1}. Halophiles are distributed in all three domains of life *Archaea, Bacteria*, and *Eucarya.* (Oren, 2003a, p. 23). Among halophilic microorganisms are a variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes. Halophiles can be generally classified as slightly, moderately or extremely halophilic, depending on their requirement for salt. Archaeal from *Halobacterium* species, cyanobacteria such as *Aphanothece halophytica*, and the green alga *Dunaliella salina* are some examples of extremely halophilic microorganisms (DasSarma and Arora, 2001, p. 1).

Table 2.1. *Classification of microorganisms according to their response to salt*

Category	Properties	Examples
Non-halophilic	Grows best in media containing less than 0.2 M salt	Most freshwater bacteria
Slight halophile	Grows best in media containing 0.2 - 0.5 M salt	Most marine bacteria
Moderate halophile	Grows best in media containing 0.5 - 2.5 M salt	*Salinivibrio costicola*
Borderline extreme halophile	Grows best in media containing 1.5 - 4.0 M salt	*Halorhodospira halophila*
Extreme halophile	Grows best in media containing 2.5 - 5.2 M salt	*Halobacterium salinarum*
Halotolerant	Non-halophile which can tolerate salt	*Staphylococcus aureus*

Source: *Kushner, 1978.*

2.1.2 Taxonomy of halophilic microorganisms: *Archaea, Bacteria,* and *Eucarya*

Halophiles are spread over in all three domains of life *Archaea, Bacteria*, and

Eucarya (Fig 2.1) (Oren, 2003a, p. 23). Among the non-halophilic relatives in phylogenetic tree, halophilic microorganisms also distributed (Oren, 2003a, p. 25). In bacterial domain, halophiles are distributed within the phyla *Proteobacteria*, *Cyanobacteria, Firmicutes*, *Spirochaetes*, *Actinobacteria* and *Bacteroidetes.* Within the Archaea halophiles are found in the the order *Methanococci* and *Halobacteriales*, family *Halobacteriaceae* (*Euryarchaeota*) which composed almost completely halophiles. In the eukaryotic domain halophiles are found in Plants, Fungi, Cilliates and Flagellates (Oren, 2008b, p. 3).

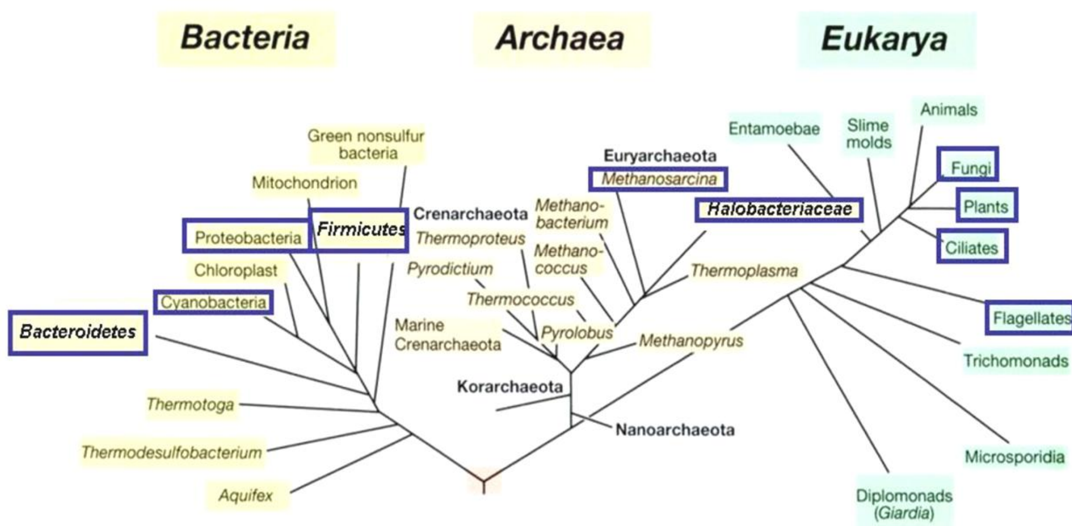


Figure 2.1. *The universal phylogenetic tree of life as based on small subunit rRNA gene sequences and the distribution of halophilic microorganisms within the tree*

Source: *Oren, 2008b, p. 3*

Halophilic and halotolerant *Bacteria* vary in their physiological properties. They are including aerobic and anaerobic chemoheterotrophs, photoautotrophic, photoheterotrophic species and chemolithotrophs (Oren, 2003a, p. 36). The anaerobic fermentative bacteria of the order *Halanaerobiales* (*Firmicutes*) consist of halophile only (Oren, 2008b, p. 3). Photosynthetic *Bacteria* from the genus *Halospirulina* with the species *Halospirulina tapeticola* represents the halophilic from cyanobacterial species. The *Halorhodospira* - *Ectothiorhodospira* group are the example of halophiles from anoxygenic photosynthetic sulfur bacteria. The family *Halomonadaceae* contains a

large number of aerobic moderate halophiles and only few non-halophilic representatives. The phylum *Firmicutes* classified as obligatory anaerobic halophilic Bacteria (Oren, 2003a, p. 38).

In the domain Archaea, halophilic microorganisms spread in three families: the *Halobacteriaceae*, the *Methanospirillaceae*, and the *Methanosarcinaceae*. All of these Archaea belong to the phylum *Euryarchaeota.* The order *Halobacteriales* with family, the *Halobacteriaceae* almost exclusively consists of halophiles. The halophiles spread over the family *Halobacteriaceae* including the genus *Natrialba, Natronorubrum, Natronobacterium, Natronococcus, Halococcus, Halobacterium, Natromonas, Haloarcula, Haloferax, Halobaculum, and Halorubrum* (Oren, 2003a, p. 27).

The green algae from the genus *Dunaliella* are the most studied eukaryotic halophiles. *Dunaliella* are the main producers in many hypersaline environments (Oren, 2008b, p. 3). Species from this genus including *Dunaliella salina*, *Dunaliella bardawil* and the smaller green algae *Dunaliella viridis* and *Dunaliella parva.* Within the diatoms the halotolerant strains that had been found were two unidentified *Amphora* species*, Amphora* cf. *subaculiuscula, Nitzschia fusiformis,* and *Entomoneis* sp. The Fungi also contains a number of halophiles. They are the meristematic fungus *Trimmatostroma salinum* which is isolated from a saltern pond at the Adriatic coast and the black yeast *Hortaea werneckii.* Among the protozoa from the study that conducted in in Hutt Lagoon, Australia, ten zooflagellates and four sarcodines were studied in brines with above 150 g l^{-1} salt. Ciliates found include the bacteriophagous *Trachelocerca conifer, Metacystis truncata, Chilophrya utahensis, Rhodopalophrya salina, Uronema marinum, Condlylostoma* sp*.,* and *Palmarella salina* (Oren, 2003a, p. 57).

2.2. Hypersaline Environments

Salt lakes, salterns, and other high-salt ecosystems are describe as hypersaline environments. Hypersaline environments classified into two groups: thalassohaline and athalassohaline. Thalassohaline environments come from the evaporation of seawater and their ionic composition reflects the seawater. The dominant cation is Na^+ meanwhile

the dominant anion are Cl^- and SO_4^{2-}. The pH of thalassohaline environments is neutral to slightly alkaline. Example for thalassohaline environment are the man-made solar saltern evaporation, crystallizer ponds, and Great Salt Lake. The Great Salt Lake is a thalassohaline desert lake located in the Great Basin of North America (Oren, 2003a, p. 393).

Another one is athalassohaline environments. Contrary to thalassohaline environments, athalassohaline environments have an ionic composition that differs from that of seawater. The dominant cation are Ca^{2+} and Mg^{2+} also contain high concentrations of carbonate/bicarbonate. The pH of athalassohaline environments may reach extremely alkaline of pH 10-11. Example for athalassohaline environment are some alkaline soda lakes and the Dead Sea. The Dead Sea is a hypersaline terminal desert lake, located on the border between Israel and Jordan. The Dead Sea describe as a slightly acidic lake with divalent cations Ca^{2+} and Mg^{2+} dominate the ionic composition (Oren, 2003a, p. 393).

2.3. Physiology of Halophilic Microorganisms

2.3.1. Physiology of the halophilic A*rchaea*

2.3.1.1. *Nutritional demands of aerobic halophilic Archaea*

Members of the *Halobacteriaceae* require high concentrations of yeast extract or other rich sources of nutrients in their growth medium or using single carbon sources with ammonia as nitrogen source. *Halobacterium salinarum*, the most studied archaeal halophile species needs complex nutrition for their growth. Grey and Fitt's study (as cited in Oren, 2003a, p. 126) found that defined media designed for the growth of different isolates may contain between 10 and 21 amino acids, also supplemented with vitamins, up to 5 different nucleosides, and glycerol. Rodriguez-Valera et al's study (as cited in Oren, 2003a, p. 126) found that *Haloferax mediterranei*, grows on simple compounds such as succinate, acetate, and others as single carbon and energy source. According to Kauri et al's study (as cited in Oren, 2003a, p. 126) a synthetic medium for the growth of *Haloferax volcanii* contains glycerol and succinate as carbon and

energy sources, thiamine and biotin as stimulatory vitamins, and inorganic salts give a good result for it growth. Species from genus *Haloferax* and *Haloarcula* utilize simple sugars such as glucose and sucrose for their growth (Oren, 2003a, p. 126)

2.3.1.2. *Membrane transport systems for nutrients*

Halophilic Archaea use amino acids as carbon and energy source. Different amino acid transport systems of *Halobacterium salinarum* have been characterized. MacDonald and Lanyi's study (as cited in Oren, 2003a, p. 127) found that most of the amino acid transport systems depend on Na^+ ions, for example leucine transport was facilitated by symport with Na^+ ion. According to Kevbrina et al's study (as cited in Oren, 2003a, p. 127) transport systems for acetate and propionate in the alkaliphilic *Natronococcus occultus* occcur in two systems. One of them is a high affinity system, driven by the Na^+ gradient over the membrane. Zoratti and Lanyi's study (as cited in Oren, 2003a, p. 127) found that transport of phosphate in *Halobacterium salinarum* cells occur via active transport and depends on the cellular ATP level. Another halophilic archaeal *Desulfonatronovibrio hydrogenovorans* uses electro neutral symport with Na^+ ions to transport sulfate into cells (Oren, 2003a, p. 127).

2.3.1.3. *Chemotaxis and transducer proteins*

According to Storch et al's study (as cited in Oren, 2003a, p. 127) chemotaxis occur in *Halobacterium salinarum* which this archaea shows a strong chemotactic behavior toward leucine, isoleucine, valine, methionine, cysteine, arginine and several peptides. A transducer protein the myoglobin-like, heme-containing protein (HemAT-Hs) has been identified in *Halobacterium salinarum* and functions as an oxygen-sensing transducer for the aerotaxis reaction (Oren, 2003a, p. 127).

2.3.1.4. *Dissimilatory biochemical pathways*

Most of *Halobacteriaceae* are aerobic heterotrophs. The degradation of carbon sources is based on the tricarboxylic acid cycle and a respiratory electron transport

involving a chain of cytochromes even with the combination of glyoxylate cycle and reactions of the Embden-Meyerhof pathway or modified Entner-Doudoroff pathway. Aitken and Brown's study (as cited in Oren, 2003a, p. 128) found that all enzymes of the tricarboxylic acid cycle are present in *Halobacterium salinarum.* Organisms such *Halococcus saccharolyticus*, *Haloferax volcanii* and *Halorubrum saccharovorum* able to use acetate as growth substrate. Example the *Halococcus saccharolyticus* contained activity of ADP-forming acetyl-CoA synthase (ADP-ACS; E.C. 6.2.1.13) when they are grown on glucose (Oren, 2003a, p. 128). Kevbrina and Plakunov's study (as cited in Oren, 2003a, p. 128) stated that *Natronococcus occultus* activate acetate via acetylphosphate via acetate kinase and acetyl-CoA synthetase.

2.3.1.5. *Life at temperature extremes*

Haloterrigena thermotolerans member of *Halobacteriaceae* can grows at high temperature optimally at 50 °C with maximum temperature 60 °C. Another example is *Haloferax mediterranei* can grow up to 54-55 °C (Oren, 2003a, p. 142). Meanwhile *Halorubrum lacusprofundi* from Deep Lake, Antarctica can tolerate low temperatures. At 4 °C the species grows at about 10% the optimal rate, which is achieved at 30-35 °C (Oren, 2003a, p. 142).

2.3.1.6. *Reactions to the presence of reactive oxygen species*

The *Halobacteriaceae* use catalase and superoxide dismutase to cope with the presence of reactive oxygen species such as peroxides and superoxide radicals. For example according to Brown-Peterson and Salin's study (as cited in Oren, 2003a, p. 142) when *Halobacterium salinarum* was suspended in low salt concentrations (1 or 1.25 M NaCl) for 12 hours, a nearly 100-fold increase in catalase activity was observed compared with cells kept at high NaCl concentrations and suggested that the solubility of oxygen increases with decreasing salinity. The genes for the manganese-containing superoxide dismutase detected on *Halobacterium salinarum* and *Haloferax volcanii* (Oren, 2003a, p. 143).

2.3.2. Physiology of halophilic bacteria

2.3.2.1. *Nutritional demands of the aerobic heterotrophic bacteria*

The *Halomonadaceae* require simple nutrition and most species can grow in mineral media supplemented with single carbon sources such as sugars, amino acids, and organic acids. *Halomonas halodenitrificans* require addition of thiamine for their growth at defined medium and for anaerobic growth methionine addition is required. (Oren, 2003a, p. 146). According to Flannery and Kennedy's study (as cited in Oren, 2003a, p. 146) *Salinivibrio costicola* require defined media contain glucose supplemented with a number of amino acids (cysteine, glutamate, arginine, valine and isoleucine).

2.3.2.2. *Membrane transport systems for nutrient accumulation*

In *Salinivibrio costicola* transport of α-aminoisobutyrate is driven by the membrane potential, but it require Na^+ for optimal rates. Transport is competitively inhibited by glycine, alanine, and methionine. In *Halomonas elongata* α-aminoisobutyrate transported via sodium gradient. Other example are the transport of amino acids (aspartate, arginine, alanine) through the membranes of Gram-negative (*Salinivibrio costicola, Halomonas halodenitrificans*) and Gram-positive aerobic halophiles (*Nesterenkonia halobia, Planococcus citreus*) also via sodium gradient (Oren, 2003a, p. 148).

2.3.2.4. *Life at temperature and pressure extremes*

According to McMeekin and Franzmann's study (as cited in Oren, 2003a, p. 148) *Halomonas subglaciescola* is an Antarctic isolate and it can tolerate low temperature with a theoretical minimum temperature of-3.3 °C, an optimum of 23.4 °C, and a maximum of 32.3 °C, respectively, for the strain ACAM 12. Formation of heat shock proteins was examined in *Chromohalobacter marismortui* with the higher the salt concentration in which the cells were grown, the higher the upper temperature limit at which heat shock proteins could be synthesized. The pressure tolerant bacterial

halophilic is *Nesterenkonia rosea* (*Micrococcus roseus*) that can survive at a pressure of 139 MPa (equivalent to 1,372 atmospheres) increased with the salinity at which the cells were grown (Oren, 2003a, p. 148)

2.4. Adaptation to Hypersaline Environments

Halophilic microorganisms use two different strategies to balance their cytoplasm osmotically with their medium. The first strategy is the accumulation of molar concentrations of KCl (high-salt-in strategy) and that interfere with enzymatic activity. Another strategy is to synthesize and accumulate organic compatible solutes that do not get involved in enzymatic activity. The first strategy requires extensive adaptation of the intracellular enzymatic to the presence of salt. The 'high-salt-in strategy' microorganisms generally cannot survive in low salt media. This strategy used by extremely halophilic Archaea of the family *Halobacteriaceae* and species such as *Halobacterium salinarum* and *Haloarcula marismortu.* Not only halophilic Archaea, bacterial halophilic such as *Halanaerobiales* (*Firmicutes*) also use KCl rather than organic solutes to osmotically balance their cytoplasm (Fig 2.2) (Oren, 2008b, p. 6).

The second strategy is the biosynthesis and/or accumulation of organic osmotic solutes or ‘low-salt-in strategy’ and that not interfere with enzymatic activity. Microorganisms that use this strategy need few adaptations of the cells’ proteome and can often adapt to a surprisingly broad salt concentration range. Most of compatible solutes are amino acids and amino acid derivatives, sugars, sugar alcohols and most of them are uncharged or zwitterion. In domain Archaea compatible solutes was found in halophilic methanogens such as the *Methanohalophilus* species also sulfotrehalose has been found only in a few alkaliphilic members of the *Halobacteriaceae* (Fig 2.3). Within domain Bacteria large diversity of organic solutes are detected. For example the biosynthesis of glycine betaine has been detected in heterotrophic bacteria *Actinopolyspora halophila* (*Actinobacteria*) and methanogenic archaeon *Methanohalophilus portucalensis.* Another organic solute is ectoine that synthesized by many aerobic heterotrophic bacteria (Oren, 2008b, p. 9).

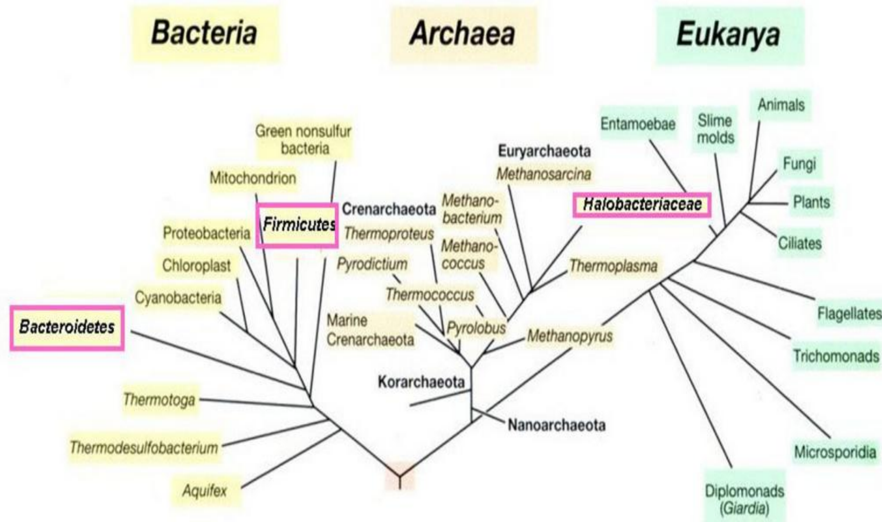


Figure 2.2. *Distribution within the phylogenetic tree of microorganisms accumulating KCl as their sole or main osmotic solute (High-salt-in strategy)*

Source: *Oren, 2008b, p. 7*

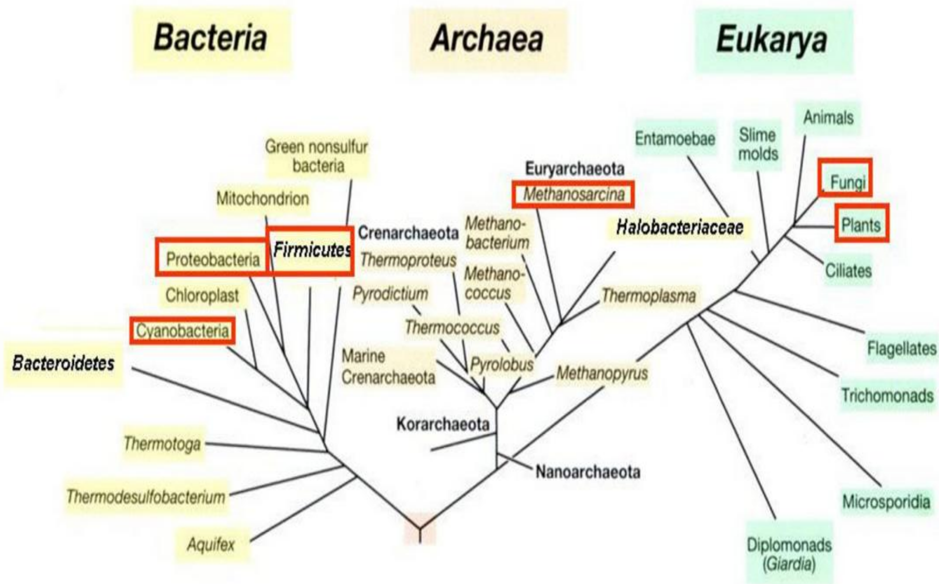


Figure 2.3. *Distribution within the phylogenetic tree of microorganisms accumulating organic solutes to provide osmotic balance (Low-salt-in strategy)*

Source: *Oren, 2008b, p. 9*

2.5. Media and Conditions for the Growth of Halophilic Microorganisms

Hypersaline media can be classified into complex media that include organic components for which exact chemical constituent are not known and defined media where all the exact chemical formulae are known. Yeast extract, peptone, tryptone, and casamino acids are the most popular organic ingredients that used for hypersaline media. The dominant salt is nearly always NaCl with other salts addition to reflect seawater condition since the source of microorganisms usually from the marine solar salterns or other thalassohaline environments. Extreme halophiles often require high of magnesium level for their growth. General consideration when working with hypersaline cultures is that microbial growth in media of high salinity is often slow, so the culture often maintain for weeks rather than days (Schneegurt, 2012, p. 37).

2.5.1. Media composition

Media for halophilic generally selected based on salinity. Media for halophilic archaea typically contain higher salinities than media for halotolerant bacteria with salinities above 20 % are suggested for halophilic archaea (Schneegurt, 2012, p. 40). The recipe for several hypersaline media created for *Haloarchaea* are given in table 2.2, meanwhile media for moderates halophiles and halotolerant bacteria is given in table 2.3.

Table 2.2. *Compositions of common extreme halophile media*

Component	**Medium composition (g L-1)**					
	A	B	C	D	E	F
NaCl	250	125	234	250	220	200
KCl	2		6	2	5	4
K2SO4		5				
KNO3					1	
K2HPO4						0.5
(NH4)2SO4						1
MgSO4.7H2O	20		29		10	20
MgCl2.6H2O		50	19.5	20		
CaCl2.6H2O		0.12	1.1	0.2	0.2	
NaBr			0.8			

Component	Medium composition (g L-1)					
	A	B	C	D	E	F
NaHCO3			0.2			
FeCl2	0.023					
Na-citrate	3				3	
Casamino acids	7.5					
Yeast Extract	10	5	5	5	1	
Tryptone/peptone		5		5	5	
Glycerol						2.5
Pyruvate						2.5

Source: *Schneegurt, 2012, p. 40*

Table 2.3. *Compositions of common moderate halophile media*

Component	Medium composition (g L-1)				
	A	B	C	D	E
NaCl	80	98	29-174	29-174	80
KCl		2		0.02	1
MgSO4.7H2O	20	1	0.1	2	20
CaCl2.6H2O		0.36	1.1	0.2	0.2
NaBr			0.23		
NaHCO3			0.06		
FeCl3.6H2O			0.001		
Na-citrate	3				3
Casamino acids	7.5				7.5
Yeast Extract	1	10			1
Tryptone/peptone	5	5			1
(NH4)2SO4			2		
Fe(NH4)2(SO4)2.6H2O	0.05				0.005
K2HPO4	0.5		3.12		7.5
KH2PO4			0.28	0.01	
NH4Cl				2	
Glucose		1	10	10	
Glutamate				2	
Trace minerals		yes			

Source: *Schneegurt, 2012, p. 40*

2.5.2. Concentrated salt water (sw) stock solution - 30% (w/v)

The formulation of concentrated salt water are based on the Rodriguez-Valera et al (Rodriguez-Valera et al., 1980, p. 535) and Torreblanca et al's study (Torreblanca et al.,

1986, p. 90). These formulation contain salts that reflect of the seawater. The complete formulation is given in table 2.4.

Table 2.4. *Composition of Concentrated Sea Water Stock Solution (30%)*

Salt	g per 1 Liter	g per 5 Liter	g per 10 Liter
NaCl	240	1200	2400
MgCl2.6H2O	30	150	300
MgSO4.7H2O	35	175	350
KCl	7	35	70
1 M Tris.Cl pH 7.5	5 ml	15 ml	50 ml
*NaBr	0.8	4	8
*NaHCO3	0.1	1.0	2.0

**This is not include in normal media, but it can be considered*

Source: *Rodriguez-Valera et al., 1980, p. 535; Torreblanca et al., 1986, p. 90*

2.5.3. Modified growth medium (MGM) for halophiles

Modified Growth Medium (MGM) was formulated by Dr. Mike Dyall-Smith based on Rodriguez-Valera et al's study (Rodriguez-Valera et al., 1980, p.535). This formulation gave the faster growth for *Haloferax* species. For halobacteria 23% (SW) MGM gave a good results, but for *Haloferax* spp. will grow faster on 18% MGM, and *Halobacterium* spp.on 25% MGM. The composition of Modified Growth Medium (MGM) are given in table 2.5 (Dyall-Smith, 2009, p. 14)

Table 2.5.*Composition of Modified Growth Medium (g l^{-1})*

Component	12 % MGM	18% MGM	23%MGM	25% MGM
Salt Water (30% Stock)	400	600	767	833
Pure Water	567	367	200	134
Peptone (Oxoid)	5	5	5	5
Yeast Extract	1	1	1	1

Source: *Dyall-Smith, 2009, p. 14*

2.5.4. Environmental conditions

Most of halophiles grow best at room temperature. Generally laboratories use 37 °C or higher (>40 °C) (Torreblanca et al. 1986, p. 90). The growth at 30 °C have been reported for halophilic and halotolerant bacteria. For psychrotrophic and psychrophilic organisms, they are maintained at 5–10 °C and also can grow at or below –5 °C. A large number of halophilic and halotolerant microorganisms are isolated at neutral pH, growing best in media with pHs from 6.8 to 7.5 but some of them classified as alkaliphilic which grow best at pHs of 8–10. Most of study of the halotolerant and halophilic microorganisms are conducted under aerobic condition although anaerobes are known (Schneegurt, 2012, p. 48).

2.6. Protease

2.6.1. Introduction to Protease

Protease are hydrolytic enzymes which catalyze the total hydrolysis of proteins, play role in degradation of proteins into smaller peptides and amino acids. Of the industrial enzymes, 75% are hydrolytic enzymes (Rao et al., 1998, p. 598). The industrial enzymes market was valued at USD 4.2 billion in 2014 and is projected to grow at 7.0% from 2015 to 2020. The key players in industrial enzymes market are BASF SE (Germany), E.I du Pont de Nemours and Company (U.S), Associated British Foods plc (UK), Koninklijke DSM N.V (Netherlands), and Novozymes A/S (Germany). (Rohan, 2016). Proteases account for about 60% of the total worldwide sale of enzymes (Fig.2.4).

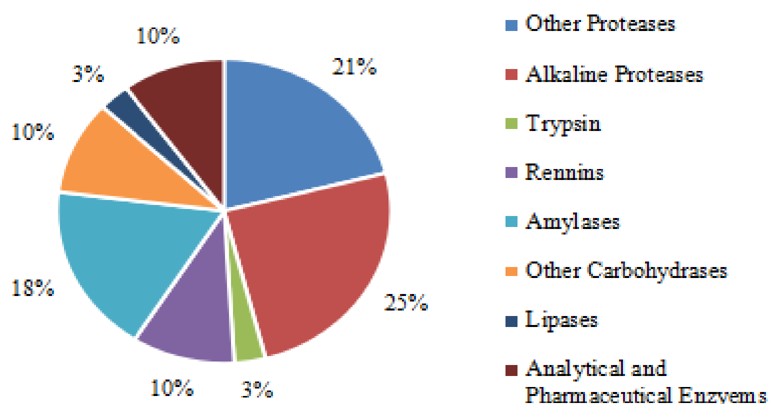


Figure 2.4. *Distribution of enzyme sales* **Source:** *Rao et al., 1998, p. 599*

2.6.2. Sources of proteases

Proteases can be isolated from plants, animals, and microbial sources. Among those producers microbial proteases are one of the most important industrial enzymes (Alvarez et al., 2006, p. 625).

2.6.2.1. *Plant proteases*

Some examples of plant proteases are papain, bromelain, keratinases, and ficin. The production of plant proteases are depending on several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Papain are extracted from the latex of *Carica papaya* fruits. Traditionally papaya fruit is used as tenderizer for the red meat. Papain is active between pH 5 and 9 and is stable up to 80 or 90°C and widely used in industry. Another plant protease is bromelain. Bromelain is extracted from the stem and juice of pineapples (*Ananas comosus*). The major producer of the enzyme is Great Food Biochem, Bangkok, Thailand. Bromelain classified as cysteine protease and is active from pH 5 to 9. Keratinases are plant proteases which degrade hair. This enzyme is used in the production of essential amino acids such as lysine (Rao et al., 1998, p. 599).

2.6.2.2. *Animal proteases*

Pancreatic trypsin, chymotrypsin, pepsin and rennins are the well-known animal proteases. Trypsin (Relative molecular mass (Mr) 23,300) classified as serine protease hydrolyzes peptide bonds in which the carboxyl groups. Trypsin is used in the preparation of bacterial media and in some medical applications. Chymotrypsin (Mr 23,800) is extracted from animal pancreas. Chymotrypsin cleavage peptide bonds in which the carboxyl groups are provided by one of the three aromatic amino acids such as phenylalanine, tyrosine, or tryptophan. Chymotrypsin is generally used in the de-allergenizing of milk protein hydrolysates. Another animal protease is pepsin. Pepsin (Mr 34,500) categorized as an acidic protease that can be extracted from the stomachs of almost all vertebrates. Pepsin is inactivated above pH 6.0 and catalyzes the

hydrolysis of peptide bonds between two hydrophobic amino acids. Rennin is a pepsin-like protease that can be extracted from the stomachs of all nursing mammals. It is widely used in the dairy industry to separate milk into solid curds for cheese making and liquid whey (Rao et al., 1998, p. 600).

2.6.2.3. *Microbial proteases*

Microbial proteases play an important role in biotechnological processes accounting for approximately 60% of the total enzyme. Microorganisms serve as preferred enzymes source because of their rapid growth, the limited space required for their cultivation and they can be genetically manipulated to produce new enzymes (Chu, 2007, p. 241). Bacteria from the genus *Bacillus* produce most of commercial protease including neutral and alkaline proteases. Neutral proteases are active in pH around 5-8 and relatively low thermotolerance. A large number of the neutral proteases classified as metalloprotease that require divalent metal ions for their activity, meanwhile others are serine proteinases. Bacterial alkaline proteases are active in alkaline pH, around pH 9-10, and they have broad substrate specificity. They are suitable for application in the detergent industry because of their thermotolerance characteristic, with the optimal temperature is around 60 °C (Rao et al., 1998, p. 600).

2.6.3. Classification of proteases

Proteases are classified in subgroup 4 of group 3 (hydrolases) that catalyze the hydrolysis of a chemical bond based on Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB). Generally, proteases are classified into three major criteria: type of reaction catalyzed, chemical nature of the catalytic site, and evolutionary relationship with reference to structure. Exopeptidase and endopeptidase are protease two major group classified according to their site of action (Table 2.6). Exopeptidases cleave the peptide bond proximal to the amino or carboxy ends of the substrate, while endopeptidases cleave peptide bonds distant from the ends of the substrate. According to the functional group present at the active site,

proteases are classified into four prominent groups. They are serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Rao et al., 1998, p. 601).

Table 2.6. *Classification of proteases*

Protease	Mode of Action$^{\alpha}$	Enzyme Commission (EC) number
Exopeptidase		
Aminopeptidase	●-↓○-○-○-○---	3.4.11
Dipeptidyl peptidase	●-●-↓-○-○-○---	3.4.14
Tripeptidyl peptidase	●-●-●-↓-○-○---	3.4.14
Carboxypeptidase	---○-○-○-○-○-↓●	3.4.16-3.4.
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl dipeptidase	---○-○-○-○-↓●-●	3.4.15
Dipeptidases	●-↓●	3..4.15
Omega peptidases	*-●↓○-○---	3.4.19
Endopeptidases	-○-○-○-↓○-○-○-	3.4.21-3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown catalytic mechanism		3.4.99

Source*: Rao et al., 1998, p. 601*

2.6.3.1. *Exopeptidases*

Exopeptidases act near the terminal of polypeptide chains. Exopeptidases classified into aminopeptidases and carboxypeptidases based on their site of action at the N or C terminus. Aminopeptidases are known to remove the N-terminal of the polypeptide chain. Aminopeptidases produce by microorganisms including bacteria and fungi. The other are carboxypeptidases which is act at C terminals of the polypeptide chain. Carboxypeptidases can be classified into three major groups, serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases, based on the amino acid residues at the active site of the enzymes (Rao et al., 1998, p. 601).

2.6.3.2. *Endopeptidases*

Endopeptidases act at the peptide bonds in the inner regions of the polypeptide chain. Based on their catalytic mechanism endopeptidases are classified into four subgroups, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases. Serine proteases are distinguished by the presence of a serine group in their active site. These enzymes are generally active at neutral and alkaline pH and they have broad substrate specificities. Serine proteases are produced by viruses, bacteria, and eukaryotes. Serine proteases grouped into 20 families, which have been further subdivided into six clans. Serine proteases are identified by their irreversible inhibition by 3,4-dichloroisocoumarin(3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK) (Rao et al., 1998, p. 602).

Aspartic acid proteases are the endopeptidases that distinguished by the presence of aspartic acid residues on their catalytic activity. These enzymes divided into three families, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3). Aspartic acid proteases show maximal activity at low pH (pH 3 to 4). These enzymes are also sensitive to diazoketone compounds such as diazoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP) in the presence of copper ions (Rao et al., 1998, p. 602).

Metalloproteases require a divalent metal ion for their activity. Examples are enzymes such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria with about 30 families of metalloproteases have been recognized, of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 (M3) contains both endo- and exopeptidases. Metalloproteases divided into four groups based on the specificity of their action, neutral, alkaline, Myxobacter I, and Myxobacter II. The alkaline proteases possess a very broad specificity meanwhile neutral proteases show specificity for hydrophobic amino acids. Metalloproteases are inhibited by chelating agents such as EDTA

(Ethylenediaminetetraacetic acid). Thermolysin is a neutral protease. Thermolysin that is produced by *B. stearothermophilus* is a single peptide without disulfide bridges with the molecular mass of 34 kDa. Thermolysin have been characterized as very stable protease, with a half-life of 1 h at 80°C (Rao et al., 1998, p. 602).

2.7. Applications of Proteases

Proteases are generally applied in the detergent and food industries. But nowadays proteases are also used in leather treatment and in several bioremediation processes. In the pharmaceutical industry proteases are used for preparation of medicines such as ointments for debridement of wounds. Proteases that are used in the food and detergent industries are used as crude preparations, meanwhile those that are used in medicine are produced with extensive purification (Rao et al., 1998, p. 606).

2.7.1. Detergent industries

Application of proteases in detergents accounts for approximately 25% of the total worldwide sales of enzymes. The first enzymatic detergent, “Burnus,” contained of sodium carbonate and a crude pancreatic extract. The first bacterial enzyme detergent based was introduced in 1956 under the trade name BIO-40. Novo Industry A/S an enzyme producer introduced alcalase, which was isolated from *Bacillus licheniformis* with trade name BIOTEX. Protease works on laundry industries by removing a large variety of stains due to food, blood, and other body secretions. Currently, all detergent proteases that used in the market are serine proteases produced by *Bacillus* strains (Rao et al., 1998, p. 606).

2.7.2. Leather industries

The process on leather industries are including soaking, bating, dehairing and tanning. In conventional methods of leather processing, hazardous chemicals such as sodium sulfide are used, this chemical create problems of pollution and effluent

disposal. Application of enzymes as alternatives to chemicals can improve leather quality and reduce environmental pollution (Rao et al., 1998, p. 606).

2.7.3. Food industries

Proteases have been used since ancient periods to process their food such as in cheese making, baking, preparation of soya hydrolysates, and meat tenderization. However, the major application of proteases in the food industry is in the manufacture of cheese. Proteases are used as milk coagulator. These enzymes are animal rennet, microbial milk coagulants, and genetically engineered chymosin. Animal and microbial milk-coagulating proteases are belong to acid aspartate proteases with the molecular weights between 30,000 to 40,000.. In cheese making, the function of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate *para*-κ-casein and macro peptides. The proteases should be produced by GRAS (genetically regarded as safe)-cleared microbes such as *Mucor michei, Bacillus subtilis*, and *Endothia parasitica* (Rao et al., 1998, p. 607).

Wheat flour contains an insoluble protein called gluten, which determines the properties of the bakery doughs. Some proteases such as endo and exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten and improve the extensibility and strength of the dough. Proteases also have been used from ancient times to prepare soy products. Examples are the alkaline and neutral fungal proteases which are play an important role in the soy sauce making. For example the application of alcalase on soy proteins at pH 8 results in soluble hydrolysates with high solubility, good protein yield, and low bitterness. The protein hydrolysates are generally used in protein-fortified soft drinks and in the formulation of dietetic products (Rao et al., 1998, p. 607).

Aspartame is an artificial sweetener consists of dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine. The use of aspartame as an artificial sweetener has been approved by the Food and Drug Administration (FDA). The enzymatic synthesis of aspartame is preferred than the chemical one to cut production cost. Proteases also catalyze the reverse reaction under certain kinetically controlled

conditions not only to hydrolyze substance. For example an immobilized preparation of thermolysin from *Bacillus thermoprotyolyticus* is used for the enzymatic synthesis of aspartame. Example of some major industrial producers of aspartame are Toya Soda (Japan) and DSM (The Netherlands) (Rao et al., 1998, p. 607).

2.7.4. Pharmaceutical industry

Some application of proteases in pharmaceutical industries including the oral administration of proteases from *Aspergillus oryzae* (Luizym and Nortase) that are used in enzyme deficiency syndromes. Another example is *Clostridial* collagenase or subtilisin which is used in combination with broad-spectrum antibiotics for the treatment of burns and wounds. Protease also used in leukemia therapy, an asparginase isolated from *E.coli* is used to eliminate asparagine from the bloodstream (Rao et al., 1998, p. 607).

2.8. Screening, Isolation, Production, and Purification of Proteases

2.8.1. Production of protease

Optimization of fermentation media for the growth and production is important in protease production. Media for protease production generally contain carbohydrate sources like starch, ground barley, or lactose, and nitrogen sources like soybean meal, casein, yeast extract or corn steep liquor. Peptides and proteins induce protease synthesis while, free amino acid often represses protease production. Protease production has been conducted by submerged fermentation (SmF) as well as solid-state fermentation (SSF) processes (Sandhya et al., 2005, p. 167).

2.8.1.1. *Factors affecting protease production*

Microbial protease is generally classified as extracellular enzyme. Their growth are influenced by nutritional and physicochemical factors, such as temperature, pH, nitrogen and carbon sources, inorganic salts, agitation, and dissolved oxygen concentration. A huge number of microorganisms classified as protease producers but the application of them are limited only to those which yield substantial amounts of

enzyme with desired properties and are GRAS (genetically regarded as safe)-cleared microbes. Many researchers have reported that the conditions which favor maximum growth will result in highest enzyme production not always true. Efficiency is one of the most important things in research. Modeling and optimization in methods are the tools to reach this efficiency without affecting the economics (Kasana et al., 2011)

2.8.1.2. *Submerged fermentation*

Submerged fermentation (SmF) is the fermentation process which biomass is completely surrounded in the liquid culture medium by stirred or no stirred. For example, the proteases of *Mucor michei, Endothia parasitica*, and all species of *Bacillus* are produced in submerged fermentation. The medium components are usually supplemented with 10–15% dry substance and high protein content, also carbohydrate that may be fed including glucose, lactose, sucrose, or starch hydrolysate (Sandhya et al., 2005, p. 168).

2.8.1.3 *Solid-state fermentation* (SSF)

Solid-State fermentation is a metabolite manufacturing process used in the food, pharmaceutical, cosmetic, fuel and textile industries. These metabolites generated by microorganisms grown on a solid support selected for this purpose. This technology for the culture of microorganisms is an alternative to liquid or submerged fermentation. In SSF processes we can use wastes or agro industrial substrates such as defatted soybean cake, wheat bran, and rice bran. Nowadays the production of different types of proteases (acid, neutral, and alkaline) through the SSF route have been increased (Sandhya et al., 2005, p. 168).

2.8.2. Detection and measurement of protease activity

Protease activity in crude, partially purified or purified proteases can be measured by qualitatively and quantitatively methods. The amount of protease that produced by microorganisms can be measured by determining the rate of disappearance (hydrolysis) of the substrate, the protein or the rate of production (appearance) of product, amino acids (Kasana et al., 2011, p. 263).

2.8.2.1. *Screening for protease producing isolates on solid media*

Protease producing isolates are observed directly by changes in the appearance of the substrate which are supplemented into solid media on petri dishes. Generally skim milk agar, casein agar, gelatin agar, fibrin agar, and elastin agar are used for protease screening. The positive protease producing isolate is indicated by the formation of clear zone on the solid media which is directly visible. The screening of microorganisms for proteases on skim milk agar is simple and does not require flooding with any reagents because on positive protease producing isolate the clear zones are visible and spread around the bacterial colonies (Fig 2.5) (Kasana et al., 2011, p . 263).

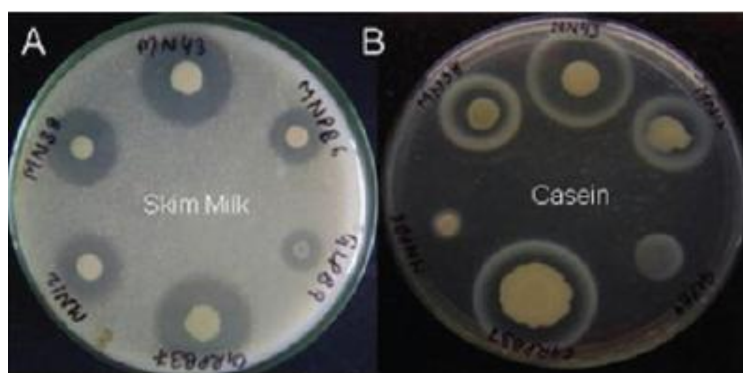


Figure 2.5. *Screening of bacteria belonging to Gram + and Gram − groups for protease production on skim milk (A) and casein (B)*

Source: *Kasana et al., 2011, p. 263*

2.8.2.2. *Screening for halophilic proteases*

Selection of niche/site is one of the important criteria for isolation of microorganisms with desired traits. For example an extremely halophilic archaeon *Halogeometricum* sp. was isolated and screened for protease activity on gelatin agar containing: 2% (w/v) agar, 1% (w/v) gelatin in 50 mmol glycine-NaOH buffer of pH 10 with 20% NaCl. It means the probability of getting a microorganism with halophilic protease is more in sample from hypersaline area as compared to sample from normal environment. Also the choice for screening medium (substrate) and method used is generally determined by the type of enzyme/microorganism, as some of the microorganisms hydrolyze one substrate, but another enzyme/microorganism may have broad specificity (Kasana et al., 2011, p . 265).

2.8.2.3. ***Spectrophotometric assay***

Several methods to assay protease activity are conducted by using various substrates and the most common among them are casein and azocasein. For example in Anson's method which is based on the principle of precipitation of undigested protein by trichloroacetic acid and degradation of the protein releases small, trichloroacetic acid (TCA)-soluble peptides. The activity is measured with the phenol reagent which releases a blue color when reacts with tyrosine and tryptophan (Fig 2.7) (Kasana et al., 2011, p. 265). Same principle are used in Amano's method. In this method one unit of enzyme is defined as the enzyme quantity which liberates Folin-positive amino acids and peptide corresponding to 1 μ of tyrosine per minute under the condition on Amano's method. Also in this method they used TCA and casein as substrate. The protease activity are measured at 660 nm by monitoring the release of tyrosine (Amano Inc, 2013, p. 77).

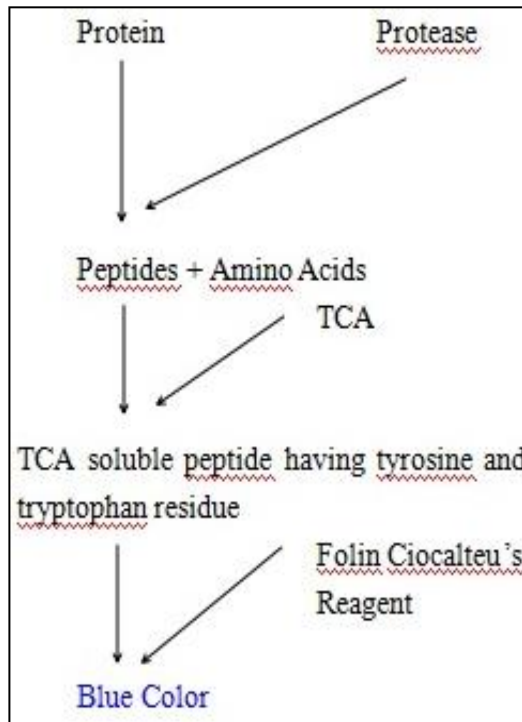


Figure 2.6. *Flow diagram for spectrophotometric protease assay*

Source: *Kasana et al., 2011, p. 265*

2.8.3. Recovery and partial purification of proteases

Product recovery during bio processing is needed to separate cell biomass and insoluble nutrient ingredients from the supernatant (crude enzyme). Partial purification is achieved by precipitation using ammonium sulfate and dialysis.

2.8.3.1. *Ammonium sulfate precipitation*

Protein precipitates are aggregates of protein molecules which is collected by centrifugation. The solubility of protein depends on the distribution of hydrophilic and hydrophobic residues, which is the result of polar interactions with the aqueous solvent and ionic interactions. The addition organic solvents on the certain temperature can cause selective precipitation. Precipitation of protein can be achieved when the charge on a protein is near zero, by minimize the electrostatic repulsion and making the molecules to attract each other. Precipitation with ammonium sulfate is the early step in some protein purifications. Precipitation with ammonium sulfate is used to reduce the large volume of extract to a volume suitable for subsequent purification steps (Rosenberg, 2005, p. 143).

The principle of precipitation with ammonium sulfate is the dehydration in the microenvironment of the protein molecule. In this precipitation, the water molecules are bound to the sulfate ion (SO_4^{-2}), so the amount of water that bound interact with the protein molecules are reduced, resulting in the precipitation of the protein at a particular concentration of $(NH_4)_2SO_4$. A saturated solution in water is ~4M with a density of 1.235 g/ml compared to a protein aggregate density in this solution of ~1.29 g/ml, this difference makes it possible to pellet out the aggregates by centrifugation. Operating the precipitation in low temperature will increase stability and decrease solubility and the best to operate precipitation is at a neutral pH between 6–7.5. The most important step in precipitation with ammonium sulfate is deciding the percentage saturation to try. The following percentages usually used by the researcher: 0–25%, 25–4.0%, 40–60%, 60–80%, and the 80% supernatant in order to find suitable percentage (Table 2.7) (Rosenberg, 2005, p. 144).

Table 2.7. *The appropriate amount of solid ammonium sulfate according to the starting volume, initial, and final concentration*

Initial concentration of ammonium sulfate, % saturation	Final concentration of ammonium sulfate, % saturation																
	10	20	25	30	33	35	40	45	50	55	60	65	70	75	80	90	100
	Grams solid ammonium sulfate to be added to 1 L of solution																
0	56	114	144	176	196	209	243	277	313	351	390	430	472	516	561	662	767
10		57	86	118	137	150	183	216	251	288	326	365	406	449	494	592	694
20			29	59	78	91	123	155	189	225	262	300	340	382	424	520	619
25				30	49	61	93	125	158	193	230	267	307	348	390	485	583
30					19	30	62	94	127	162	198	235	273	314	356	449	546
33						12	43	74	107	142	177	214	252	292	333	426	522
35							31	63	94	129	164	200	238	278	319	411	506
40								31	63	97	132	168	205	245	285	375	469
45									32	65	99	134	171	210	250	339	431
50										33	66	101	137	176	214	302	392
55											33	67	103	141	179	264	353
60												34	69	105	143	227	314
65													34	70	107	190	275
70														35	72	153	237
75															36	115	198
80																77	157
90																	79

Source: *Rosenberg, 2005, p. 144*

2.8.3.2. *Dialysis*

Dialysis is described as a separation process that use the osmotic forces between two liquids or a liquid and a solid as the principle. In protein partial purification dialysis is used to remove excess low molecular weight solutes and simultaneously equilibrating the sample in the new buffer. The dialysis tubing that used is D9777 Sigma dialysis tubing cellulose membrane, a semi-permeable membrane, made from cellulose acetate, with the dimensions and nominal molecular weight cut-offs (NMWC) (M.W 12.000), so this membrane will retain most proteins of molecular weight 12.000 or greater. In dialysis process the dialysate solution should be changed at least once, so the solutions can be concentrated against a high molecular weight solid hydroscopic substance. The procedure for dialysis including wetting the tubing with water, tie a knot in one end or dialysis clips can be used. The next step is loading the sample into the tubing. Water will enter the tubing due to the high concentrations of salt in the sample, resulting in a volume increase during the early stages of dialysis. The last step is put the loaded dialysis tubing in a beaker containing the dialysis buffer and a magnetic stirrer and dialyzed against the specific buffer at 4 ºC overnight (Rosenberg, 2005, p. 141).

2.9. Characterization of Protease

The protease enzyme can be characterized by testing the protease based on the effect of its activity on different environmental parameters such as pH, temperature, alkalinity, acidity, substrates, and its stability on the addition of metal ions and organic solvents. The molecular weight of protease can be measured by SDS-PAGE and its substrate specifity can be detected by zymography.

2.9.1. Effect of different kinetic parameters on protease activity and its stability

The pH and temperature are critical parameters to be characterized on protease activity, since a protein has their own minimum, optimal, maximum pH and temperature for their activity (Kasana et al., 2011, p. 271). Effect of pH can be studied by diluting the substrate in different buffers of varying pH, meanwhile effect of temperature can be studied by assaying the protease on different range of temperature. Effect of substrate can be studied using different substrates to determine the specificity of enzymes under optimized assay conditions. The metal ions and organic solvents can increase or inhibit the proteolytic activity. The catalytic activity of enzymes is dependent on the native structure, so any slight variations may result in significant changes in the enzyme activity (Sandhya et al., 2005, p. 175).

2.9.2. Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is an important tool for analyzing and characterizing macromolecules that is used to investigate molecular weight, purity of proteins, posttranslational modifications, subunit structure, enzyme activity, protein processing, and amino acid sequence. This electrophoresis system uses acrylamide gel which is able to separate proteins by size. For the preparation of the gel, acrylamide is mixed with bisacrylamide that forms a cross-linked network. The polymerization between acrylamide and bisacrylamide is initiated by ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) accelerates the polymerization. The ideal percentage gel should be selected based on the targeted protein. The pore sizes on the

gel are inversely proportional to the concentration of acrylamide (Rosenberg, 2005, p. 63).

In polyacrylamide gel electrophoresis (PAGE) protein sample are treated with heat for 2–5min in sample buffer containing sodium dodecylsulfate (SDS), an anionic detergent. SDS denatures the proteins and binding also confers a negative charge on all of the proteins. Thiol reducer, β-mercaptoethanol (2-ME) is used in electrophoresis to reduce disulfide bonds on protein that contain a number of Cys residues (Fig 2.8). It makes the sample is run under reducing conditions. Recently, β-mercaptoethanol was replaced with dithiothreitol (DTT) and nowadays tributylphosphine is replacing DTT. During electrophoresis, regeneration of disulfide bonds can occur. The alkylating agent, iodoacetamide is used to solve this problem. Bromophenol blue, a color marker is used in polyacrylamide gel electrophoresis. This dye have low molecular weight and migrates at the front or leading edge of the electrophoretic run. For increasing the density of the sample, glycerol or sucrose is added in the sample buffer (Rosenberg, 2005, p. 65).

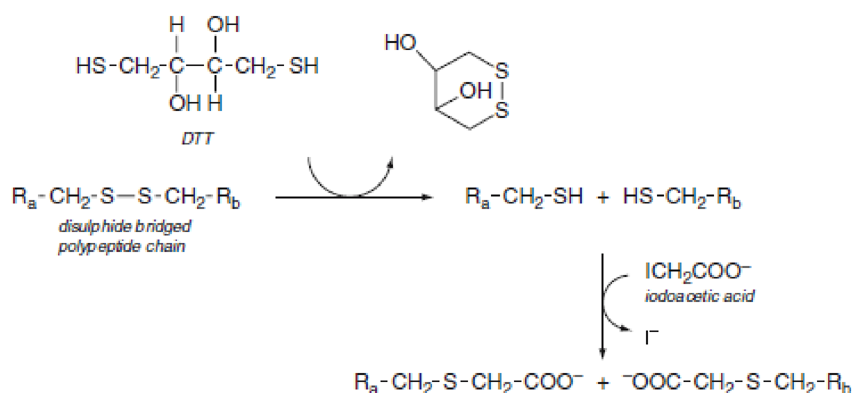


Figure 2.7. *Reduction and carboxymethylation of proteins containing disulfide bridges*

Source: *Rosenberg, 2005, p. 65*

2.9.3. Zymography

Zymography technique is used to measure proteolytic activity. Zymography is an electrophoretic technique which is based on the degradation of substrate by protease on discontinuous polyacrylamide gels that containing enzyme substrate. The procedure of zymography includes the removal of SDS from the gel by washing the gel in 2.5%

Triton X-100. This step allows enzymes to renature and to degrade the protein substrate. The clear bands of lysis against a blue background are detected after the gel is stained with Coomassie blue indicates the proteolytic activity (Sandhya et al., 2005, p. 169)

2.10. Source of Samples

2.10.1 Terasi - Indonesian shrimp paste

"Terasi" describe as traditional salty fermented seafood product which is made from fish and/or shrimp in the form of a paste. It has dark brown, gray or red color and has a specific taste and strong aroma (Fig 2.9). Indonesian people use "terasi" as a spice in their dishes. In Indonesia, "terasi" is produced near the seashore, the fish or shrimp are salted by being sun dried for 2 days, minced and allow to ferment for 2 days using "terasi" starter (Kobayashi et al., 2003, p. 279). Surono and Hosono's study (Surono and Hosono, 1994, p. 1167) found that "terasi" starter was composed of *Bacillus brevis, Bacillus pumilus, Bacillus megaterium, Bacillus coagulans, Bacillus subtilis,* and *Micrococcus kristinae* in the proportion of 39.1 %, 26.1 %, 8.7%, 8.7%, 8.7%, and 8.7%, respectively, all the strains were isolated under aerobic conditions on agar plates in the presence of 10% NaCl. The presence of species *Bacillus* and *Micrococcus* which is classified as halophilic bacteria could originate from the fish or the salt used in the fermentation process, since the previous study stated that solar salt obtained from Southeast Asian countries has an average of 2.7 x 10^4 bacteria/g of salt (Surono and Hosono, 1994, p. 1167). According to Kobayshi et al's study, species of *Tetragenococcus* were also detected in "terasi" sample. The species of *Tetragenococcus* are widely known for its presence in various fermented foods containing high salt (Kobayashi et al., 2003, p. 281).

"Terasi" was consisted of 25.4% protein, 6.1 % fat, 1.9% carbohydrate, 29.1 % ash (including salt), and 16.8% sodium chloride with pH of 7.5 Surono and Hosono (1994) also reported that enzyme activity was also detected in "terasi" sample. The isolates from "terasi" sample showed esterase, lipase and proteolytic activity by using Apizym kit (Bio Merieux S.A., France). The esterase, lipase hydrolyze the fat that produce the

low-molecular-weight fatty acids that are responsible for the cheesy odor in “terasi” (Surono and Hosono, 1994, p. 1167).

Figure 2.8. *The dark brown "terasi"*

Source: *https://www.flickr.com/photos/fotoosvanrobin/5713459496 (Accessed date: 31.03.16)*

2.10.2. Indonesian tauco

Indonesian “tauco” (taoco) is a saline paste made from preserved fermented yellow soybeans which has meat like flavor and the consistency of porridge (Fig 2.10). “Tauco” is related to Philippine *taosi* and to Japanese *miso* which is the product of mold, bacteria, and yeast fermentation of soybeans. In Indonesia “tauco” mainly consumed in Western Java and used as spice and preparation of certain soups. The ingredients that are used in “tauco” production including yellow soybeans, tapioca, rice, or maize flour, salt and brown palm sugar. The procedure in “tauco” production including washing the yellow soybeans. After cleaning, the soybeans are soaked for 12 hours then boiled and cooled. The next steps are dehulling, washing, and boiling again the soybeans. After they get soft, drained and cooled the soybeans, they are placed on bamboo trays or baskets and covered with other trays, polyethylene sheets, banana leaves, or gunny sacking. This conditions, make the beans undergo a spontaneous overgrowth by molds in the environment or by adding the ragi tempe starter. Incubation for 3 to 6 days yields a koji, dried in the sun, partially pulverized, and covered with brine. Fermentation then continues for 21 to 30 days outdoors. For final step, palm sugar is added. The mash then is boiled, resulting in tauco. After cooling, it can be partially dried in the sun for 15 days before bottling or packing in polyethylene bags (Steinkraus, 1995, p. 556).

A soaking time of 15 hours appears to be optimum in "tauco" production. The longer the mold fermentation, the higher the pH, probably due to increased proteolysis. Traditionally in "tauco" making 25 to 30% salt based upon the weight of soybeans is added, but a 20% w/v salt brine is optimum for best-quality tauco. The optimum temperature for "tauco" production is around 37 to 42°C. The total production time of "tauco" varied between 24 to 36 days, with 3 to 6 days for koji preparation and 21 to 30 days for brine fermentation. Winarno (as cited in Steinkraus, 1995, p. 559) found that the essential microorganisms that found in "tauco" are *Aspergillus oryzae, Rhizopus oligosporus, R. oryzae, Lactobacillus delbrueckii, Hansenula* sp., and *Zygosaccharomyces soyae.* In addition, Ohhira et al., (1990) detected *Lactobacillus coryniformis* subsp. c*oryniformis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* in their "tauco" sample. Furthermore during the initial mold growth, the pH rises as protein is hydrolyzed to peptides, peptones, and free amino acids. Total soluble solids increase, soluble nitrogen increases, and lipids are hydrolyzed to free fatty acids. Lactic, acetic, succinic, and phosphoric acids are produced (Steinkraus, 1995, p. 559).

Figure 2.9. *Yellow soybeans tauco*

Source: *http://www.justtryandtaste.com/2011/12/bandeng-masak-tauco.html (Accessed date: 31.03.16)*

2.10.3. Indonesian salted fish

Salted fish is fish cured with dry salt and thus preserved for later eating. Drying and/or salting, either with dry salt or with brine, was the only widely available method of preserving fish until the 19th century. Like other salt-cured meats, it provides preserved animal protein even in the absence of electrically powered refrigeration. In Indonesia, salted fish are produced by fisherman around the country. Extreme

conditions on salted fish that contain high levels of NaCl up to 20% (Standard Industrial Indonesia) still allows a group halophilic microorganisms esp. bacteria and some types of bacteria tolerant of salt, for example the genera *Micrococcus, Pseudomonas,* and *Flavobacterium*. (Andriyani 2005)

Figure 2.10. *Variation of salted fish*
Source: *www.teropongbisnis.com/teropong-usaha/usaha-kecil-menengah/gurihnya-peluang-bisnis-ikan-asin/ (Accessed date: 25.06.16)*

2.10.4. Sediment soil from Bengkulu coastal, Indonesia

Bengkulu City is a city on the west coast of Sumatra in Indonesia. The sediment samples were taken in Long Beach, an Indian Ocean coastal in Bengkulu city.

2.11. Studies on Halophilic Proteases

A halotolerant bacteria which was isolated from saltern pond sediment (Tuticorin) identified as *Bacillus licheniformis* (TD4) produced protease with specific activity 141.46 U/mg on certain production medium contain C-N sources and 1 M NaCl (Sughanti et al., 2013, p. 47). Another halotolerant bacteria identified as *Bacillus aquimaris* produced an extracellular protease with activity 630 U/ml on basal Zobell medium. This bacteria produced protease with maximum activity 796 U/ml on basal medium with 1 M NaCl addition. The optimum pH and temperature for production of this enzyme were pH 7.5 and 37 °C, respectively (Shivanand and Jayaraman, 2009, p. 1088). A haloalkaliphilic bacteria (isolate Vel) which is related to *Bacillus pseudofirmus* based on 16S r RNA gene sequencing produced a serine alkaline protease. The enzyme

was purified 10-fold with Phenyl Sepharose 6 Fast Flow column. The molecular mass of this enzyme based on SDS-PAGE estimated to be 29 000 Da. This enzyme has optimum pH around 10-11. This protease enzyme was activated with Ca^{2+} (1 mM) and Cu^{2+} (5 mM), meanwhile SDS (0.1%) and Triton X-100 (0.1%) slightly enhanced the enzyme activity (Gupta et al., 2005, p. 103).

A haloalkaliphilic strain 18AG, related to *Salinivibrio costicola* subsp. *costicola* produced an extracellular protease which showed an optimum activity at pH 8.0 and 60 °C in the presence of 2.0% NaCl and 2.0 mM $CaCl_2$, while in the absence of $CaCl_2$ the optimum temperature was 50 °C. The estimated molecular mass of the enzyme by zymography was 38 kDa. Phenylmethyl sulfonylfluoride (PMSF) strongly inhibited the activity of this enzyme (Lama et al., 2005, p. 478). A *Salinivibrio* sp. strain AF-2004 categorized as moderate halophile was produced an extracellular alkaline protease. The isolate secreted maximum protease activity in the medium containing 1% (w/v) NaCl at 60 °C. The optimum pH for enzyme activity was 8.5. The activity of this enzyme was inhibited by EDTA (Amoozegar et al., 2007, p. 369).

A halotolerant alkaliphilic bacteria *Bacillus* sp. strain NPST-AK15 produced an alkaline protease. The protease was purified by ammonium sulfate precipitation with 80% saturation, anion-exchange and gel permeation chromatography. The optimum activity of this enzyme was obtained at 60 °C and pH 10.5, meanwhile the molecular mass estimated to be 32 kDa. The K_m, V_{max} and k_{cat} values for the enzyme were 2.5 mg ml^{-1}, 42.5 μM min^{-1} mg^{-1}, and 392.46 × 103 min^{-1}, respectively. The purified enzyme showed stability in several organic solvents. In addition the purified enzyme showed high stability in several surfactants (Triton X-100 and Tween 80) and commercial detergents such as OMO, Arial, Persil, and Bonux (Ibrahim et al., 2015, p. 961). *Halobacterium* sp. strain HP25 was isolated from salt samples that collected from Emisal salt company at Lake Qarun, Fayoum, Egypt produced a haloalkalithermophilic protease. The purified enzyme have optimum activity at pH 8.0 and temperature of 60 °C. The estimated molecular mass of this enzyme was 21 kDa. The purified enzyme showed a specific activity of 6350 U mg^{-1}. The K_M and V_{max} values of the purified

halophilic protease with casein as a substrate were 523 μg ml^{-1} and 2500 μg min^{-1} ml^{-1}, respectively. The purified enzyme was stable on several organic solvents and laundry detergents such methanol, propanol, butanol, hexane, Persil and Ariel (Elbanna et al., 2015, p. 763).

An extracellular haloalkaline serine protease was produced by a moderately halophilic bacterium, *Bacillus iranensis* strain X5B. The molecular mass of the enzyme estimated to be 48-50 kDa. This enzyme was strongly inhibited by PMSF, indicated that it was serine protease. The optimum pH, temperature, and NaCl concentration of this enzyme were 9.5, 35 °C and 0.98 M, respectively. The activity of this enzyme was increased by the addition of Ca^{2+}. The specific activity of the purified protease was 425.23 μmol of tyrosine/min per mg of protein using casein as a substrate (Ghafoori et al., 2016, p. 115).

According to Nolasco et al's study (Nolasco et al., 2002, p, 202) an extracellular serine-protease class was produced by *Haloferax mediterranei*. The enzyme has optimum activity at 3 M NaCl, pH 8.0 and temperature 50 °C with estimated molecular mass around 26 500 Da. A serine metalloprotease was produced by moderately halophilic bacterium *Salinivibrio* sp. strain AF-2004. The estimated molecular mass of the enzyme was 31 kDa by SDS-PAGE. The specific activity of purified enzyme was 116.8 μmol of tyrosine/min per mg protein on casein. The optimum pH, temperature, and NaCl concentration of the enzyme were 8.5, 55 °C, and 0-0.5 M NaCl. The activity of this enzyme was strongly inhibited by phenylmethyl sulfonylfluoride (PMSF) and EDTA, indicating that the enzyme was a serine metalloprotease (Karbalaei-Heidari et al., 2007a, p. 237).

A moderately halophilic bacterium *Halobacillus karajensis* strain MA-2 was cultured on basal medium. This bacterium produced a serine metalloprotease since its enzyme activity was strongly inhibited by PMSF and EDTA. The purified haloalkaline protease was purified 24-fold by acetone precipitation and Q-Sepharose ion exchange chromatography. The estimated molecular mass of this enzyme was 36 kDa. The

optimum pH, temperature, and NaCl concentration for its activity were 9.0, 50°C and 0.5 M NaCl (Karbalaei-Heidari et al., 2009b, p. 21).

A trypsin-like serine protease was produced by an extremely halophilic archaeon *Haloferax lucentensis* VKMM 007, which was isolated from a solar saltern. The estimated molecular mass of the enzyme was 57.8 kDa. The enzyme was purified by a combination of ultrafiltration, bacitracin–Sepharose affinity chromatography and Sephadex G-100 gel filtration. The optimum pH, temperature, and NaCl concentration for its activity were 8.0, 60 °C, and 4.3 M NaCl. The enzyme also showed an important feature to be used in industry, which was stable on various polar and non-polar solvents, surfactants and reducing agents (Manikandan et al., 2009, p. 2247).

A moderately halophilic alkaline protease was produced by *Bacillus subtilis* AP-MSU 6 which was isolated from the intestine of marine fish. The enzyme was purified by ammonium sulfate precipitation at 85% saturation by DEAE-Sepharose Fast Flow ion exchange chromatography and Sephadex G-75. The molecular weight of purified protease was determined as 18.3 kDa. The optimum pH, temperature, and NaCl concentration for maximum protease activity were 9.0, 40 °C and 0.5 M NaCl. The activity of the enzyme was increased by Cu^{2+}, Hg^{2+}, Mn^{2+} and Ba^{2+}. The enzyme was also strongly inhibited by the presence of PMSF, indicating it was a serine protease (Maruthiah et al., 2013, p. 116).

2.12. Gram Staining

Gram staining is one of the most essential and the most important staining method in bacterial classification and identification. Bacteria that retain the stain have been called to be Gram-positive, those that are decolorized are called Gram-negative, and these characteristics were first introduced by H.C.J. Gram, a Danish physician, about a century ago. The principles of this staining are when bacterial cells were stained with crystal violet and treatment with iodine, a dye-iodine complex is formed within the cell. This complex is soluble in acetone (or alcohol) which is used as a decolorizer but insoluble in water. After decolorization, the dye iodine complex (blue/black in color) is

retained by the Gram positive group of bacteria but flows freely from the Gram negative group bacteria. Presumably, this is due to the former having a less permeable cell wall. Then, the counterstain, for example safranin, is added to the sample, staining it red. Safranin is lighter than crystal violet, it will not disrupt the purple color in Gram positive bacteria, but it will decolorized Gram negative bacteria with red color. This due to the differences in the thickness of a peptidoglycan layer in the cell membrane between Gram positive and Gram negative bacteria (Claus D, 1992, p. 451).

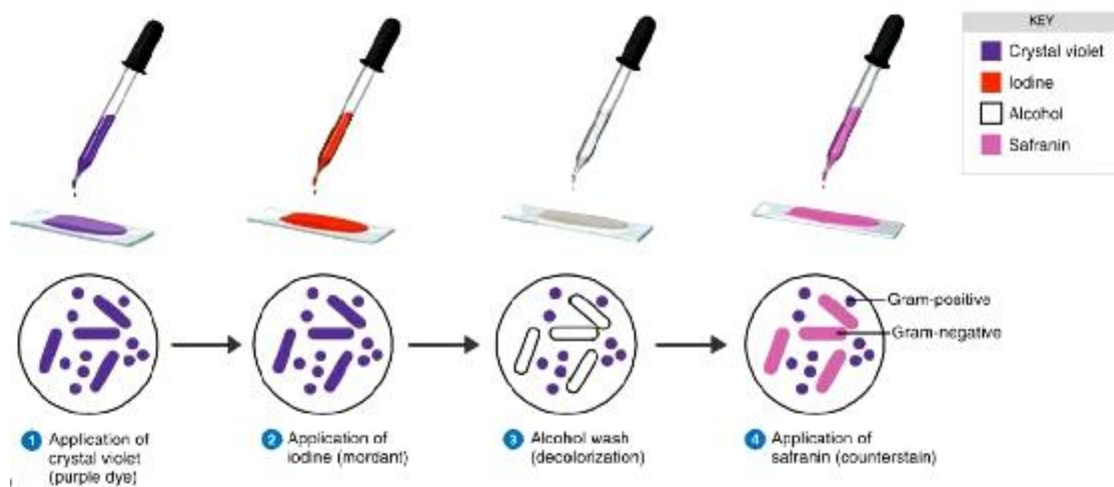


Figure 2.11. *Mechanism of Gram Staining*

Source: http://www.medicinehack.com/2012/02/gram-staining-procedure-mechanism.html (Accessed date: 15.06.16)

III. RESEARCH METHODOLOGY

3.1. Materials

3.1.1. Bacterial culture media

3.1.1.1. *18 % MGM (Modified Growth Medium)*

Concentrated Salt Water (SW) stock solution - 30% (w/v)............ 600 ml

Distilled water..367 ml

Pepton.. 5 g

Yeast Extract... 1 g

The pH was adjusted up to 7.5 with 1M Tris.Cl, pH 7.5, using 5 ml per Litre. The medium was sterilized by autoclaving at 121 °C for 15 minutes (Dyall-Smith, 2009, p. 14).

3.1.1.2. *18 % MGM (Modified Growth Medium) agar with 1% skim milk for protease screening*

Concentrated Salt Water (SW) stock solution - 30% (w/v)............. 600 ml

Distilled water.. 367 ml

Pepton.. 5 g

Yeast Extract... 1 g

Agar.. 15 g

Skim Milk Powder.. 10 g

The medium was prepared into two parts. First part contained all the components mentioned above except skim milk and it was sterilized by autoclaving at 121 °C for 15 minutes. For the second part, it contained only skim milk, which was sterilized using an intermittent sterilization method (heating to 100 °C for 15 min on three successive days) to minimize changes in skim milk properties. The pH was adjusted up to 7.5 with 1M

Tris.Cl, pH 7.5, using 5 ml per Liter (Dyall-Smith, 2009, p. 14; Elbanna, Ibrahim, and Revol-Junelles, 2015, p.764).

3.1.1.3. ***18 % MGM (Modified Growth Medium) as starter medium***

Concentrated Salt Water (SW) stock solution - 30% (w/v)............ 600 ml

Distilled water.. 367 ml

EZmix Hy-Case (Casein acid hydrolysate).................................... 5 g

Yeast Extract.. 1 g

The pH was adjusted up to 7.5 with 1M Tris.Cl, pH 7.5, using 5 ml per Liter. The medium was poured into culture tube (10 ml in each). The medium was sterilized by autoclaving at 121 °C for 15 minutes (Dyall-Smith, 2009, p. 14)

3.1.1.4. ***18 % MGM (Modified Growth Medium) with 1% skim milk as fermentation medium***

Concentrated Salt Water (SW) stock solution - 30% (w/v)............ 600 ml

Distilled water.. 367 ml

EZmix Hy-Case (Casein acid hydrolysate).................................... 5 g

Yeast Extract.. 1 g

Skim Milk Powder.. 10 g

The medium was prepared into two parts. First part contained all the components mentioned above except skim milk. For the first part, it was sterilized by autoclaving at 121 °C for 15 minutes. For the second part, it contained only skim milk, which was sterilized using an intermittent sterilization method (heating to 100 °C for 15 min on three successive days) to minimize changes in skim milk properties. The pH was adjusted up to 7.5 with 1M Tris.Cl, pH 7.5, using 5 ml per Liter (Dyall-Smith, 2009, p. 14; Elbanna, Ibrahim, and Revol-Junelles, 2015, p.764)

3.1.2. The composition of solutions

3.1.2.1. ***Concentrated salt water (sw) stock solution - 30% (w/v)***

NaCl.. 240 g

$MgCl_2.6H_2O$... 30 g

$MgSO_4.7H_2O$... 35 g

KCl.. 7 g

NaBr.. 0.8 g

$HNaCO_3$... 0.2 g

$CaCl_2$.. 0.5 g

The pH was adjusted up to 7.5 with 1M Tris.Cl, pH 7.5, using 5 ml per Litre. The medium was sterilized by autoclaving at 121 °C for 15 minutes (Dyall-Smith, 2009, p. 12)

3.1.2.2. ***Na-phosphate buffer 0.1 M***

Na_2HPO_4... 1.42 g (A)

NaH_2PO_4... 1.2 g (B)

The pH of final solution can be calculated using these calculation below:

pH 6.0 = 12.0 ml (A) + 88.0 ml (B)

pH 7.0 = 57.7 ml (A) + 42.3 ml (B)

pH 7.5 = 84.0 ml (A) + 16.0 ml (B)

pH 8.0 = 93.2 ml (A) + 6.80 ml (B)

Distilled water to make a total volume of 100 ml for each solution Na_2HPO_4 0.1 M and NaH_2PO_4 0.1 M. Sterilized buffer by autoclaving at 121 °C for 15 minutes. These buffer can be stored for up to 1 month in room temperature ("Buffer for Biochemical Reactions", 2012, p. 4)

3.1.2.3. ***Tyrosine standard (10 µg/ml)***

L-Tyrosine... 1 mg

0.1 M HCl solution.. 100 ml

L-tyrosine was dissolved with 100 ml 0.1 HCl solution (Amano Inc, 2013, p. 78).

3.1.2.4. ***Trichloroacetic acid solution -TCA (Amano's method)***

Weigh 18.0 g of Sodium Acetate and add 100 ml of 1 M Trichloro-acetic acid, 19 ml of Acetic Acid, and 800 ml of water. Then it was dissolved and pH was adjusted to 4.03 with 1 N Sodium Hydroxide (NaOH). Finally, it was filled up to 1 liter with water (Amano Inc, 2013, p. 78).

3.1.2.5. ***0.6% milk casein solution***

Weigh 0.6 gram casein (from bovine milk technical grade, Sigma) and add 50 ml of 0.1 M buffer phosphate pH 7.5. The solution was dissolved at 65°-70° C in water bath for 15 minutes. After cooling with tap water, pH was adjusted to 7.5 with 0.1N NaOH and filled up to 100 ml with water. (Expires after 6 days at 2-8 °C) (Amano Inc., 2013, p. 78).

3.1.2.6. ***3-folds Folin's reagent***

50 ml of Folin's Reagent was diluted with 100 ml of distilled water (Amano Inc, 2013, p. 78).

3.1.2.7. ***0.55 M*** Na_2CO_3

Na_2CO_3 (Anhydrous).. 17.48 g

Dilute in 300 ml of distilled water (Amano Inc, 2013, p. 78).

3.1.2.8. ***Tris-HCl buffer pH 8.0 0.05 M***

Trizma HCl (T5941 SIGMA)... 4.44 g

Trizma Base (T1503 SIGMA)... 2.65 g

The components were diluted with 1 Liter of distilled water (in room temperature 25 °C) ("Trizma Buffers", 1996).

3.1.2.9. *Tris-HCl buffer pH 9.0 0.05 M*

Trizma HCl (T5941 SIGMA)... 0.76 g

Trizma Base (T1503 SIGMA)... 5.47 g

The components were diluted with 1 Liter of distilled water (in room temperature 25 °C) ("Trizma Buffers", 1996).

3.1.2.10. *Bicarbonate-Carbonate buffer 0.1 M*

To create 100 ml of 0.1 M bicarbonate buffer solution pH 10.0 at room temperature, mix Sodium bicarbonate and sodium carbonate decahydrate, as given below.

Solution A 0.1 M $NaHCO_3$ .... 0.84 g

Solution B 0.1 M $Na_2CO_3.10\ (H_2O)$.. 2.86 g

Each composition was diluted with 100 ml distilled water. For pH 10.0 mix 40 ml solution A and 60 ml solution B at 20 °C ("Buffer for Biochemical Reactions", 2012, p. 4)

3.1.2.11. *Lowry Reagent solution*

Solution A (for 500 ml)

$NaOH$... 2.8598 g

Na_2CO_3... 14.308 g

Solution B (for 100 ml)

$CuSO_4.5H_2O$.. 1.4232 g

Solution C (for 1000 ml)

$NaKC_4H_4O_6.4H_2O$.. 2.0g

For fresh Lowry Reagent solution, the solution A, B, and C were mixed with a ratio of (98:1:1) (Dulekgurgen, 2004, p. 1).

3.1.2.12. *1 X phosphate buffer saline (PBS buffer) recipe*

NaCl.. 8.00 g

KCl... 0.20 g

Na_2HPO_4.. 1.44 g

KH_2PO_4.. 0.24 g

The following components above were dissolved in 800 ml distilled water. The pH was adjusted to 7.4 with HCl. Finally, the volume was adjusted to 1 L with additional distilled water. Sterilized by autoclaving at 121 °C for 15 minutes.

3.1.2.13. ***Metal ion solutions 0.05 M (50 mM)***

$CaCl_2$.. 0.0555 g

$MgCl_2$.. 0.0476 g

$CoCl_2$, hexahydrate.. 0.1189 g

$ZnCl_2$.. 0.0681 g

KCl.. 0.0372 g

$FeCl_3.6H_2O$.. 0.1351 g

The following components above were dissolved in 10 ml of distilled water. Stored at -20 °C.

3.1.2.14. ***0.05 M (50 mM) PMSF solution***

Phenylmethylsulfonyl fluoride (PMSF)... 0.0871 g

The PMSF was dissolved in 10 ml absolute ethanol (≥ 99.8%)

3.1.2.15. ***0.05 M (50 mM) EDTA solution***

Ethylenediaminetetraacetic acid (EDTA)....................................... 0.1461 g

The EDTA was dissolved at pH around 8.0.

3.1.2.16. 1*0% (w/v) commercial detergent solutions*

Ariel, OMO (powder)... 1 g

The detergents was dissolved in 10 ml distilled water.

3.1.2.17. ***Stock solutions and buffers for SDS PAGE***

3.1.2.17.1 ***Acrylamide/Bis (30% T, 2.67% C)***

Preweighed acrylamide/bis, 37.5:1 powder, DNase and RNase free, electrophoresis tested 100 g (FISHER BIOREAGENT).. 100 g

The mixture was dissolved in 244 ml of distilled water to make 333 ml solution.

3.1.2.17.2. ***10% (w/v) SDS***

SDS powder... 10 g

The SDS was dissolved in 90 ml water with gentle stirring and bring to 100 ml with distilled water.

3.1.2.17.3. ***1.5 M Tris-HCl, pH 8.8***

Tris base (18.15 g/100 ml)... 27.23 g

Distilled water.. 80 ml

The pH was adjusted to 8.8 with 6 N HCl. The total volume then was filled up to 150 ml with deionized water and the buffer was store at 4°C.

3.1.2.17.4. ***0.5 M Tris-HCl, pH 6.8***

Trizma Base (T1503 SIGMA)... 6 g

Distilled water.. 60 ml

The pH was adjusted to 6.8 with 6 N HCl. The total volume then was filled up to 100 ml with deionized water and the buffer was store at 4°C

3.1.2.17.5. ***10x Electrode (Running) buffer, pH 8.3 (1 L)***

Trizma Base (T1503 SIGMA)... 30.3 g

Glycine.. 144 g

SDS.. 10 g

The mixture was dissolved and bring total volume up to 1,000 ml with distilled water the buffer was store at 4°C.

3.1.2.17.6. ***10% (w/v) APS (fresh daily)***

Ammonium persulfate... 100 mg

Dissolved in 1 ml of distilled water.

3.1.2.17.7. ***Gel formulations (10 ml)***

Percent Gel	DD H_2O (ml)	30% Degassed Acrylamide/ Bis (ml)	Gel Buffer* (ml)	10 % (w/v) SDS (ml)
4 %	6.1	1.3	2.5	0.1
5 %	5.7	1.7	2.5	0.1
6 %	5.4	2.0	2.5	0.1
7 %	5.1	2.3	2.5	0.1
8 %	4.7	2.7	2.5	0.1
9 %	4.4	3.0	2.5	0.1
10 %	4.1	3.3	2.5	0.1
11 %	3.7	3.7	2.5	0.1
12 %	3.4	4.0	2.5	0.1
13 %	3.1	4.3	2.5	0.1
14 %	2.7	4.7	2.5	0.1
15 %	2.4	5.0	2.5	0.1
16 %	2.1	5.3	2.5	0.1
17 %	1.7	5.7	2.5	0.1

* Resolving Gel Buffer – 1.5 M Tris-HCl, pH 8.8

* Stacking Gel Buffer – 0.5 M Tris-HCl, pH 6.8

For 10 ml monomer solution:

Resolving gel: 50 µl 10% APS and 5 µl TEMED

Stacking gel: 50 µl 10% APS and 10 µl TEMED

The mixture was swirled gently to initiate polymerization.

3.1.3. Test samples

The bacterial strains were isolated from *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal, Indonesia, which were collected in Bengkulu, Indonesia on September of 2015.

3.2. Methods

3.2.1. Screening of bacterial strains for production of protease

3.2.1.1. *Enrichment and screening for protease-producing halophilic strains from Indonesian traditional fermented food samples and sediment soil*

The *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal samples were obtained from Bengkulu Indonesia. For the enrichment of halophilic protease-producing strains, 90 mL of the liquid modified 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 they were transferred into 15% glycerol for storing at -85°C (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.764; Mutlu, 2006, p. 92).

3.2.1.2. *Growth and production of protease*

Protease-producing isolates from food samples and sediment soil were firstly aerobically cultured at 37 °C 150 rpm in 10 ml of starter medium containing 18% MGM (Modified Growth Medium). 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 (Modified Growth Medium) supplemented with 1% skim milk. The fermentation conditions were maintained at 37 °C, 150 rpm agitation for 3 days (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.766; Fitriani, 2013, p.2).

3.2.1.3. *Effect of fermentation period on protease production*

The effect of fermentation period on protease production was conducted for a period of 96 hours of incubation at 37 °C 150 rpm and the protease activities were measured for every 24 hours (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.765).

3.2.1.4. *Enzyme isolation*

After 72 hours cultivation the culture broth was centrifuged at 5.000 rpm for 30 minutes at 4 °C to remove cells and insoluble materials (pellet) and the cell-free supernatant were used for the subsequent enzyme purification and characterization procedures as crude fraction. The crude enzymes were stored at 4 °C (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.766).

3.2.1.5. *Assay of protease activity (modified amano's method)*

For samples, 625 µL 0.6% casein solution was pipetted into a test tube and was kept in an incubator for 10 minutes 37 ºC. Then 125 µL enzyme solution was added into test tube and shaken thoroughly and then incubated for 10 minute 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 Na_2CO_3 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 minutes 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).

For controls, 625 µL of TCA (Trichloroacetic Acid) was pipetted into a test tube instead of 625 µL casein and the other procedures were as above. The absorbance of the

mixture solution was measured at 660 nm and it was recorded as A2 (Absorbance of controls).

As a blank, 300 µL of 0.1 M HCl solution was pipetted into a new test tube and 750 µL of Na_2CO_3 0.55 M solution and 150 µL 1:2-folds Folin's reagent was added. After thoroughly mixed, the reaction should stand at 37 ºC for 10 minutes 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 blank-A4).

For tyrosine standard, 300 µL of of tyrosine solution (1 mg/ml) was pipetted into a new test tube and 750 µL of Na_2CO_3 0.55 M solution and 150 µL 1:2-folds Folin's reagent was added. After thoroughly mixed, the reaction should stand at 37 ºC for 10 minutes 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 standards-A3). The potential producer (with the highest proteolytic activity) was taken for further characterization and partial purification studies (Amano Inc, 2013, p. 78).

Example of Calculation for Amano's Method:

$$Activity\,(U/ml) = \frac{A1 - A2}{A3 - A4}\, x 3 x \frac{1.375}{0.3}\, x \frac{1}{10}\, x \frac{Dm}{Volume.Enzyme}$$

A1 : Absorbance of Sample 3 : Tyrosine quantity per 0.3 mL of Tyrosin standard solution (µg)

A2 : Absorbance of Control A3 : Absorbance of Standard

A4 : Absorbance of Blank 10 : Reaction time

Dm : Dilution multiple of enzyme solution

1.375 : The final volume of the reaction mixture

0.3 : Volume of final filtrate

Vol.En : Volume enzyme (ml)

3.1.2.6. *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 minutes. After 10 minutes of incubation period, 200 µl of 1:2-folds Folin's reagent was added, mixed well by vortex. Then all the test tubes were covered and incubated at room temperature for 30 minutes. For the blank, water was used by changing the sample with 20 µl distilled water. 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. Finally, the standard curve was used to determine the protein concentration of the protease samples (Lowry et al., 1951, p. 265; Pierce Biotechnology Inc, 2002, p. 3)

3.2.2. Partial purification

3.2.2.1. *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. Finally, the dialyzed sample (dialysate 85%) was assayed for its protease activity and protein content. The sample was then stored at 4 °C for further characterization studies. (Ibrahim et al., 2015, p. 962; Maruthiah, 2013, p. 166).

3.2.3. Characterization of halophilic protease

3.2.3.1. *Effect of temperature on halophilic protease activity*

The optimum temperature for enzyme activity was determined by conducting the assay (both crude and dialysate 85%) at various temperatures from 25 to 60 °C with interval of 5 °C. The enzyme activity was assayed using Amano's method at 25, 30, 35, 40, 45, 45, 50, 55, 60 °C and pH 7.5 for 10 minutes (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.766; Fitriani, 2013, p. 3).

3.2.3.2. *Effect of pH on halophilic protease activity*

The optimum pH for enzyme activity was determined by conducting the assay using Amano's method at different pH values. The effect of pH on protease activity was examined by reacting the enzyme (both crude and dialysate 85%) with casein substrates with variation pH values ranging from 6.0 to 10.0, in the following buffer systems: 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) at optimum temperature (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.766; Fitriani, 2013, p. 3).

3.2.3.3. *Effect of salt concentration (NaCl) on halophilic protease activity*

To determine the optimum NaCl concentration for enzyme activity, the enzyme (both crude and dialysate 85%) was assayed at 0, 1, 5, 10, 15 % (w/v) NaCl at optimum pH and temperature using Amano's method (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.766).

3.2.3.4. *Effect of metal ions on halophilic protease activity*

To determine the effect of metal ions on the purified enzyme (dialysate 85%) the assay was carried out where the assay mixture was supplemented with 2 mM of $CaCl_2$, $MgCl_2$, $ZnCl_2$, KCl, and $CoCl_2$. The purified enzyme was incubated with metal ions solution with final concentration of 2 mM at room temperature for 1 hour. Then the

protease activity was measured using Amano's method (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.766).

3.2.3.5. *Effect of inhibitors and surfactant on halophilic protease activity*

The effect of inhibitors and surfactants on enzyme activity was carried out under standard assay conditions where the assay was supplemented with phenylmethyl sulfonylfluoride (PMSF) (1 mM and 5 mM), ethylenediaminetetraacetic acid (EDTA) (1 mM and 5 mM), SDS (0.5 % and 1.0 %) and Triton X-100 (1.0% and 5.0%). The purified enzyme was incubated with inhibitors and surfactan solution with final concentration of 1 mM and 5 mM on room temperature for 30 minutes. Then the protease activity was measured using Amano's method on pH, NaCl concentration, and temperature optimum (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.766).

3.2.3.6. *Halophilic protease stability in commercial detergents*

The halophilic protease stability in commercial detergents was carried out under standard assay conditions where the assay was supplemented with some commercial laundry detergents available in the local market including Ariel and Omo. One gram of each laundry detergent was prepared on 10 ml distilled water (10 % w/v) and boiled for 10 min to inactivate enzymes present in the detergent and then cooled on ice. The purified enzyme was incubated with detergent solutions with final concentration of 1% at 40 °C for 60 minutes. The protease activity was measured using Amano's method (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.767; Ibrahim et al., 2015, p. 964).

3.2.3.7. *Determination of K_M and V_{max} of halophilic protease*

The K_M and V_{max} values of the pure enzyme were determined by measurement of enzyme activity with various concentrations of casein substrate 0.2-1.0%. The protease activity was measured using Amano's method. The K_M and V_{max} value of the enzyme was calculated from a Lineweaver–Burk plot (Elbanna, Ibrahim, and Revol-Junelles, 2015, p.767).

3.2.3.8. *Effect of organic solvents on protease stability*

The effect of organic solvents on protease stability was carried out under standard assay conditions where the assay was supplemented with isopropanol, ethanol, methanol, butanol, and acetone. The partially purified enzyme was incubated with organic solvents with final concentration of 25 % at 40 °C for 1 hour with shaking. Then the protease activity was measured using Amano's method (Ibrahim et al., 2015, p. 964).

3.2.3.9. *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 after running 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. The bands and molecular mass of proteins was determined using Gel Analyzer software (Laemmli U.K., 1970, p. 681).

3.2.3.10. *Substrate specificity of partially purified protease*

Substrate specificity of partially purified enzyme from isolate TANN 4 was carried out by examining proteolytic activity on protein substrates. 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 minute and then centrifuged at 10,000 rpm for 10 min at 4 °C. Finally, the precipitate was removed. The reaction solution was

pipetted into a cuvette and the absorbance of 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 F.M and Falade A.M, 2014, p. 6).

3.2.4. Gram staining

The bacteria were grown on appropriate agar medium. One drop or a few loopful of water was added on the slide and then amount of colony was transferred from perti dishes to slide aseptically. The slide then was flooded with crystal violet for 1 minute and washed with tap water. Then, slide was flooded with fresh iodine solution for 1 minute and washed again with tap water. The slide then was decolorized with alcohol for 30 s and washed for 5 s with tap water again. The slide then was rinsed off with counter stain (safranin) for 45 s. The slide was washed for 5 s in tap water and was allowed to air-dry. Finally, the slides were examined under microscope with the oil immersion. Gram positive cells were appeared purple and Gram negative cells were pink (Claus D, 1992, p. 451).

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

A total of eight strains were characterized by automated ribotyping using *Eco* RI. Four out of eight isolates were protease positive. However, the protease negative isolates were also identified. The automated ribotyping was performed using a robotized instrument (RiboPrinter® System Microbial Characterization System, Qualicon, DuPont™, Wilmington, DE, USA) and the RiboPrinterTM System Data Analysis Program. The procedure used for processing each sample is described in detail by the manufacturer. The first step was growing the pure colonies then picking from the plate and heat treatment. Then, DNA was extracted from the bacterial cell lysate and cut into fragments by an *Eco*RI restriction enzyme. The DNA fragments were separated according to molecular size by gel electrophoresis and then transferred to a membrane.

After hybridization with a labeled DNA probe, a chemiluminescent agent was introduced. The emission of light from the hybridized fragments was then captured by a digitizing camera and stored as image data. Using proprietary algorithms, a RiboPrint™ pattern for each sample was extracted from the image data. This pattern was compared to other RiboPrint™ patterns stored in the system to characterize and identify the sample. A report that characterizes and identifies the bacterium and includes its RiboPrint™ pattern was automatically printed for the review. Sophisticated data analysis tools allow us to further process the information and share standardized data among network of users (Guven et al., 2010, p. 201; DuPont™, 2016, p.9)

IV. RESULTS AND FINDINGS

The purpose of this study was to screen, isolate, characterize, and purify the halophilic protease from *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal. Only the “tauco” and “terasi” samples yielded protease producing bacterial isolates. Therefore, the other isolates which were indicated as protease negative were not included into the study.

The proteolytic activities of the four strains were measured and the one with the highest proteolytic activity was characterized and purified using ammonium sulfate precipitation and dialysis. The isolates were identified by the RiboPrinter.

4.1. Screening for Protease-producing Halophilic Strains

Protease-producing halophilic strains were successfully isolated from “tauco” and “terasi” with a total of 4 isolates. The halophilic protease producers were picked up by screening on 18% MGM agar plates supplemented with 1% skim milk for detection of proteolytic activity. The protease- positive (producing) isolates namely, TANN 4, TR 1, TR 2, and TR 4 gave clear zones around the bacterial colonies (Fig 4.1). The protease activity was measured using Amano’s method. Among these isolates, the data in Figure 4.2 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.

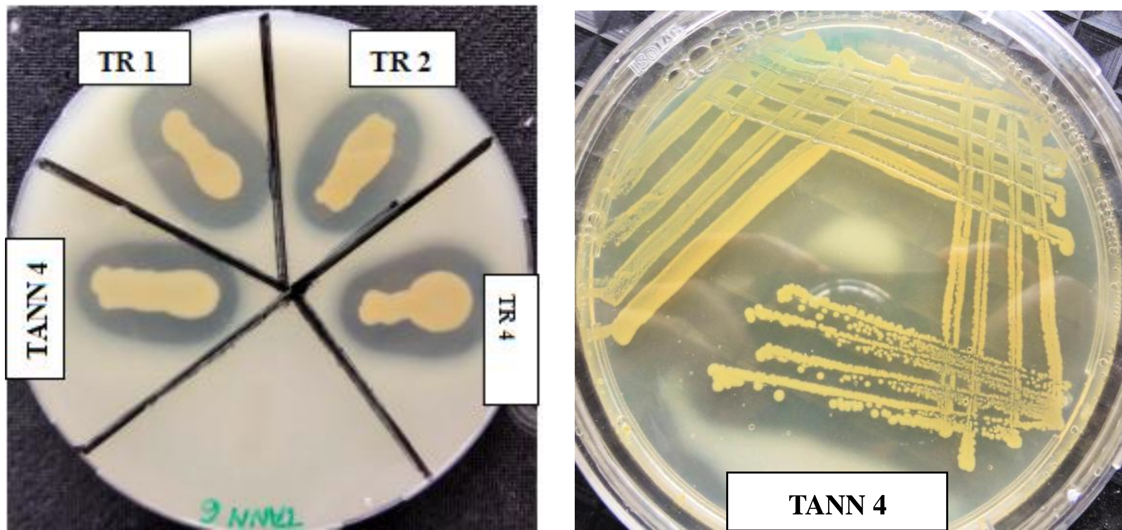


Figure 4.1 *Halophilic protease producing isolates on 18% MGM agar plates supplemented with 1% skim milk*

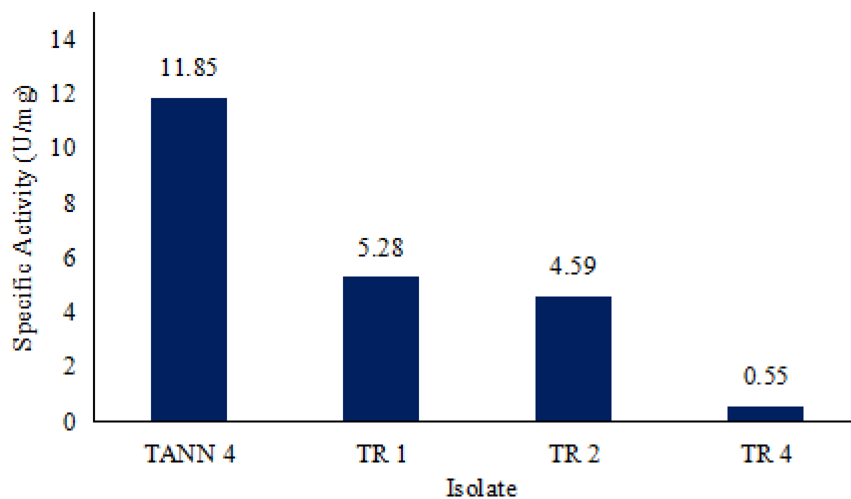


Figure 4.2 *Specific Activity of Halophilic Protease from Different Isolates*

4.2. Effect of Fermentation Period on Halophilic Protease Production

The best fermentation period was determined by monitoring the enzyme activity of isolate TANN 4 during for 4 days. The enzyme activity of isolate TANN 4 reached its maximum after 3 days (Fig 4.3) 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 (Fig. 4.3), then decreased after 4th day. So for all further experiments, fermentation period for 3 days was fixed.

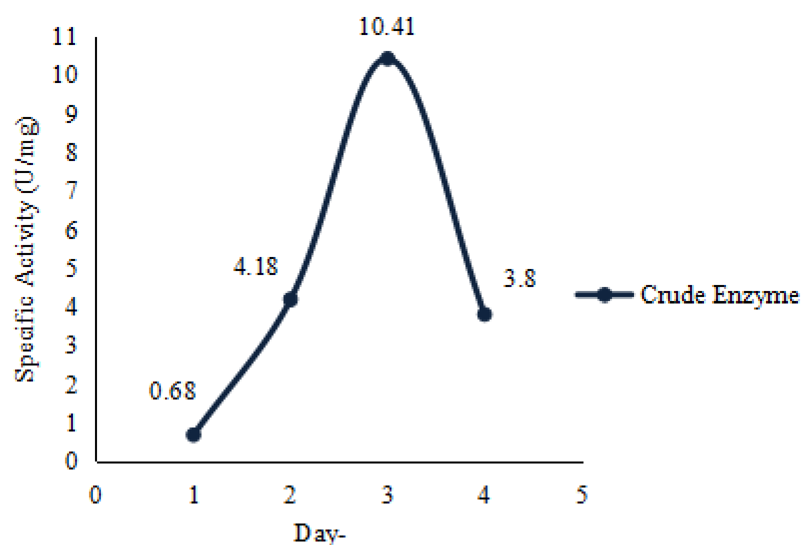


Figure 4.3 *Effect of Fermentation Period on Halophilic Protease Production*

4.3. Partial Purification of Halophilic Protease from Isolate TANN 4

The extracellular halophilic protease of isolate TANN 4 was partially purified from culture filtrate in two purification steps, ammonium sulfate precipitation and dialysis. The enzyme was purified with 85% ammonium sulfate and dialyzed against 50 mM Tris-HCl buffer pH 8.0. After ammonium sulfate and dialysis step, as presented in the purification table (Table 4.1), 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 4.1) also increased from 11.85 to 301.14 U mg^{-1}.

Table 4.1. *Purification table 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

4.4. Total Protein Analysis by Lowry's Method

The total of protein from the crude and partially purified enzyme was determined with Lowry protein assay. The protein concentration of the protease samples were determined using the BSA standard curve, with the linear regression $y = 0.095\ x$ (Fig 4.4). The assay is a colorimetric assay based on reduction of the Cupric Cu^{2+} to cuprous ions Cu^{+} in alkaline pH when reacting with peptide. The purpose of this method is to monitor the recovery of protein during purification. As presented in Table 4.2 total protein for crude enzymes were higher than the purified one, for example the total protein for crude enzyme from isolate TANN 4 on 3rd day was 1.86 mg/ml. After purification with ammonium sulfate at 85 % saturation the total protein was decreased into 0.64 mg/ml.

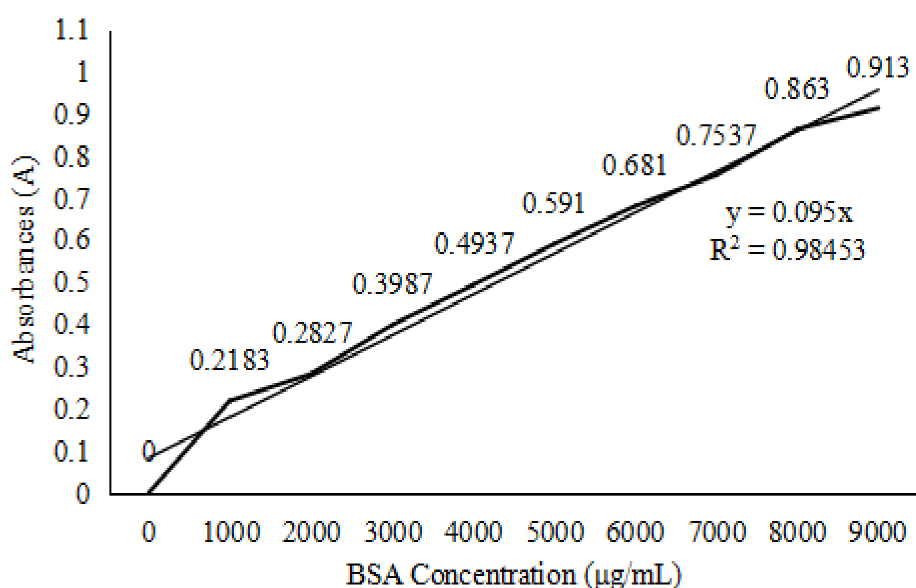


Figure 4.4 *Bovine Serum Albumin (BSA) Standard Curve for Lowry's Method*

Table 4.2. *Total protein estimation by Lowry's Method*

Fraction	Abs 1	Abs 2	Average	Blank	Corrected Absorbance	[Protein] (mg/ml)
Crude (Isolate TANN 4)	0.369	0.378	0.3735	0.197	0.1765	1.86
Crude (Isolate TR 1)	0.389	0.385	0.3870	0.197	0.1900	2.00
Crude (Isolate TR 2)	0.450	0.451	0.4505	0.197	0.2535	2.67
Crude (Isolate TR 4)	0.416	0.413	0.4145	0.197	0.2175	2.29
Crude (Isolate TANN 6)	0.352	0.367	0.3595	0.197	0.1625	1.17

Crude 1st Day TANN 4	0.474	0.485	0.4795	0.143	0.3365	3.54
Crude 2nd Day TANN 4	0.462	0.452	0.4570	0.143	0.3140	3.31
Crude 3rd Day TANN 4	0.369	0.378	0.3735	0.197	0.1765	1.86
Crude 4th Day TANN 4	0.447	0.444	0.4455	0.143	0.3025	3.15
Dialysate 85% TANN 4	0.212	0.196	0.2010	0.143	0.0610	0.64

4.5. Characterization of Halophilic Protease from Isolate TANN 4

4.5.1. Effect of the NaCl concentration on the crude and partially purified halophilic protease activity

The crude and partially purified (dialysate 85%) halophilic protease from isolate TANN 4 showed an optimum activity at 1 % (w/v) or 0.1 M NaCl, resulted in a specific activity of 14.29 U mg^{-1} for crude enzyme and 116.27 U mg^{-1} for partially purified enzyme (Fig 4.5). The crude enzyme retained about 30.73 %, 28.06 %, and 20.36 % of its activity at 5, 10, and 15 % (w/v) NaCl (Fig 4.6), respectively. Meanwhile, the partially purified enzyme retained about 83.53 %, 58.92 %, and 29.76 % of its activity at 5, 10, and 15 % (w/v) NaCl (Fig 4.6), respectively.

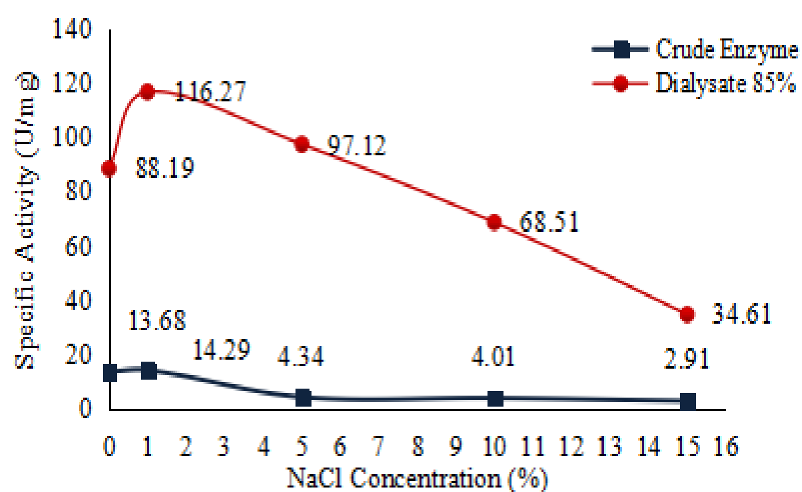


Figure 4.5 *Effect of the NaCl concentration on the crude and partially purified halophilic protease*

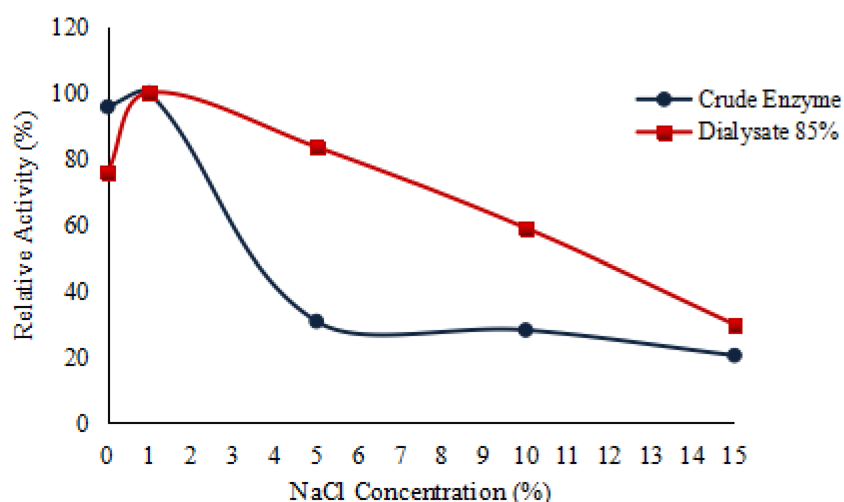


Figure 4.6 *Effect of the NaCl concentration (The maximum activity in each figure was considered as 100 % and the other data were calculated as relative to it)*

4.5.2. Effect of pH on the crude and partially purified halophilic protease activity

The pH profile of the crude and partially purified protease was estimated by measurement of the enzyme activity at pH values ranging from 6.0 to 10.0 at 1% (w/v) NaCl, 37 °C (default temperature for Amano's Method). The results showed that the enzyme (both crude and partially purified) was active in a wide pH range from 6.0 to 10.0, with maximum activity (100 %) at pH 8.0 (Fig 4.7) and resulted in a specific activity of 15.70 U mg^{-1} for crude enzyme and 128.66 U mg^{-1} for partially purified enzyme (Fig 4.8). The partially purified enzyme retained 90.07 % of its activity up to pH 9.0, which indicated that it is an alkaliphilic enzyme.

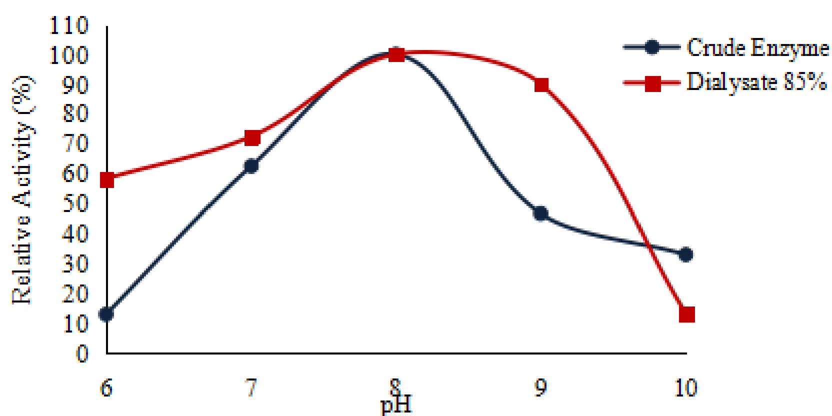


Figure 4.7. *Effect of pH (The maximum activity in each figure was considered as 100 % and the other data were calculated as relative to it)*

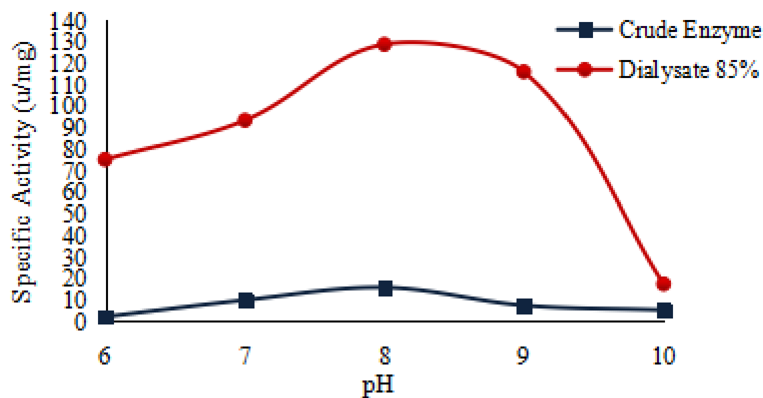


Figure 4.8. *Effect of pH on the crude and partially purified halophilic protease activity*

4.5.3. Effect of temperature on the crude and partially purified protease activity

The temperature profile of the crude and partially purified protease was estimated by measuring the enzyme activity at various temperatures ranging from 25 to 60 °C at 1% (w/v) NaCl, pH 8.0 (optimum pH). The crude enzyme was active at range of temperatures from 25 to 50 °C, exhibited maximum activity at 40 °C (Fig. 4.9) and resulted in a specific activity of 15.88 U mg^{-1} for partially purified enzyme (Fig. 4.10), meanwhile the partially purified enzyme was active at range of temperatures from 25 to 60 °C, exhibited optimum activity at 50 °C (Fig 4.9) and resulted in a specific activity of 56.49 U mg^{-1} for partially purified enzyme (Fig 4.10). For the partially purified enzyme the activity was decreased over the temperature of 50 °C, resulted in the relative enzyme activity from 100 % at 50 °C to 21.59 % at 60 °C (Fig 4.9). As a result, it was concluded that, the partially purified halophilic protease of isolate TANN 4 showed an optimum activity at 1 % (w/v) NaCl, 50 °C and pH 8.0.

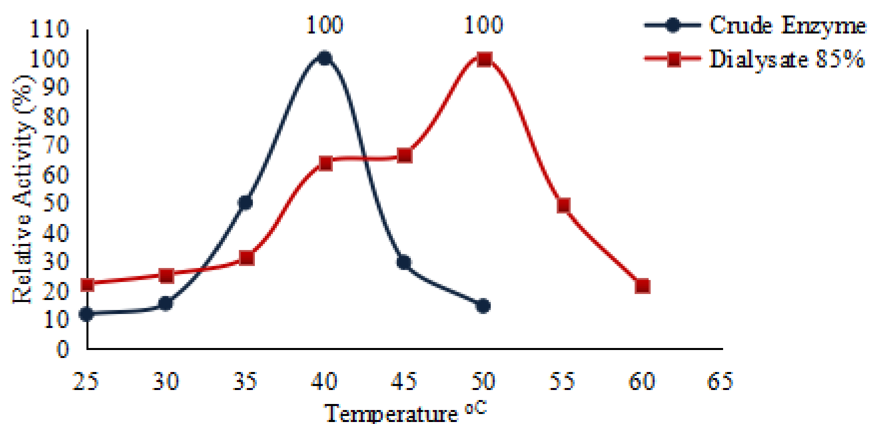


Figure 4.9. *Effect of temperature on relative activity of protease*

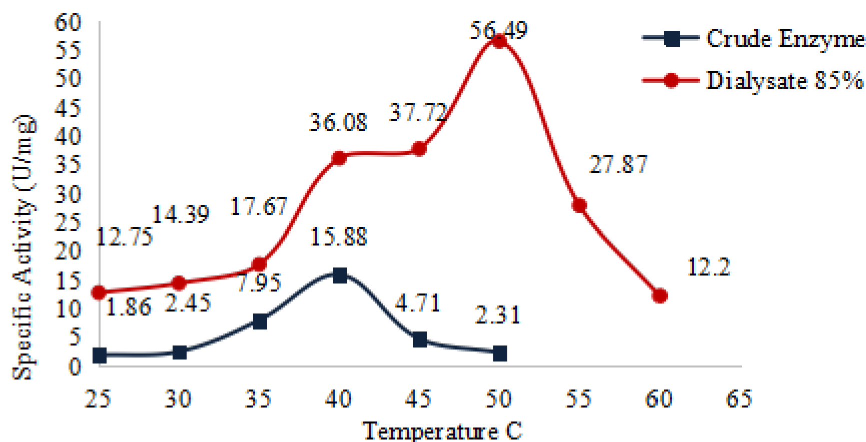


Figure 4.10. *Effect of temperature on protease activity*

4.5.4. Effect of metal ions on the partially purified halophilic protease activity

The effect of some metal ions (2 mM) on the activity of the partially purified isolate TANN 4 protease was investigated at optimal activity conditions (1 % (w/v) NaCl, 50 °C, pH 8.0) by the addition of the metal ions to the reaction mixtures. The study revealed that Ca^{2+} and K^{+} ions (2 mM) enhanced the protease activity up to 113.85 % and 117.61 % (Fig. 4.11) compared to the control (100 %), respectively. Meanwhile, Mg^{2+} ion slightly enhanced the enzyme activity with residual activities of 102.21 %. On the other hand, Co^{2+} and Zn^{2+} ions caused significant inhibition of the enzyme activity with residual activities of 12.42 and 34.00 % compared to the control and Fe^{3+} ion slightly inhibited the enzyme activity with residual activity of 66.32 % compared to the control, respectively (Fig. 4.11).

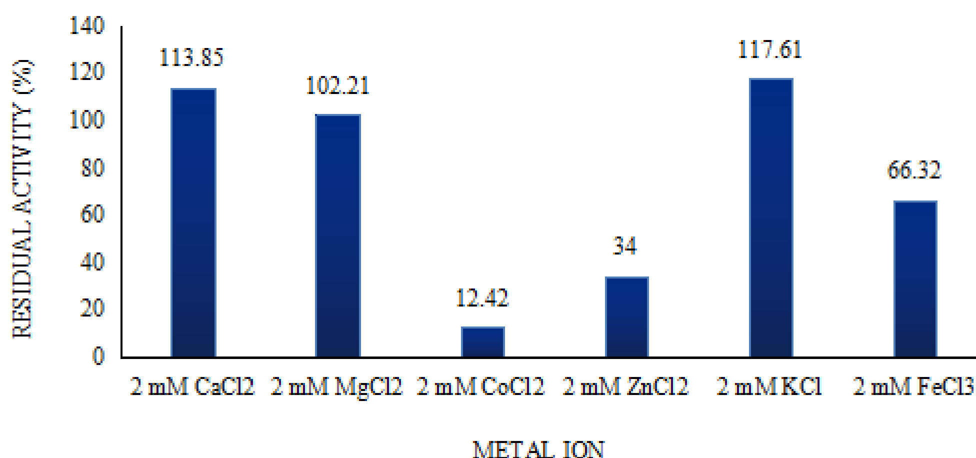


Figure 4.11. *Effect of metal ions on partially purified protease activity (The enzyme activity of control in each figure was considered as 100 %)*

4.5.5. Effect of inhibitors and surfactant on partially purified halophilic protease activity

The effects of various inhibitors and surfactants on the activity of the partially purified enzyme are shown in Figure 4.12. Protease activity was not inhibited by the serine-protease inhibitor Phenylmethylsulfonyl fluoride (PMSF), resulted in residual activity of 114.34 and 101.96 % of original activity in the presence of 1 and 5 mM of the PMSF, respectively, in comparison to control (no inhibitor), suggesting that the partially purified protease from isolate TANN 4 is not a serine protease. On the other hand, protease activity was completely inhibited by the metalloprotease inhibitor Ethylenediaminetetraacetic acid (EDTA), resulted in residual activity of 2.14 and 0 % of original activity in the presence of 1 and 5 mM of the EDTA, respectively, in comparison to control (no inhibitor), suggesting that the partially purified protease from isolate TANN 4 is a metalloprotease. The stability of partially purified enzyme toward surfactants such as nonionic surfactant SDS (sodium dodecyl sulfate) and Triton X-100 are shown in Figure 4.12. The enzyme was highly stable in the presence of 1 and 5 % Triton X-100, retaining 84.20 and 63.85 % of its original activity. Moreover, the enzyme was active and quite stable in the presence of 0.5 and 1.0 % SDS, retaining 44.12 and 53.46 % of its original activity.

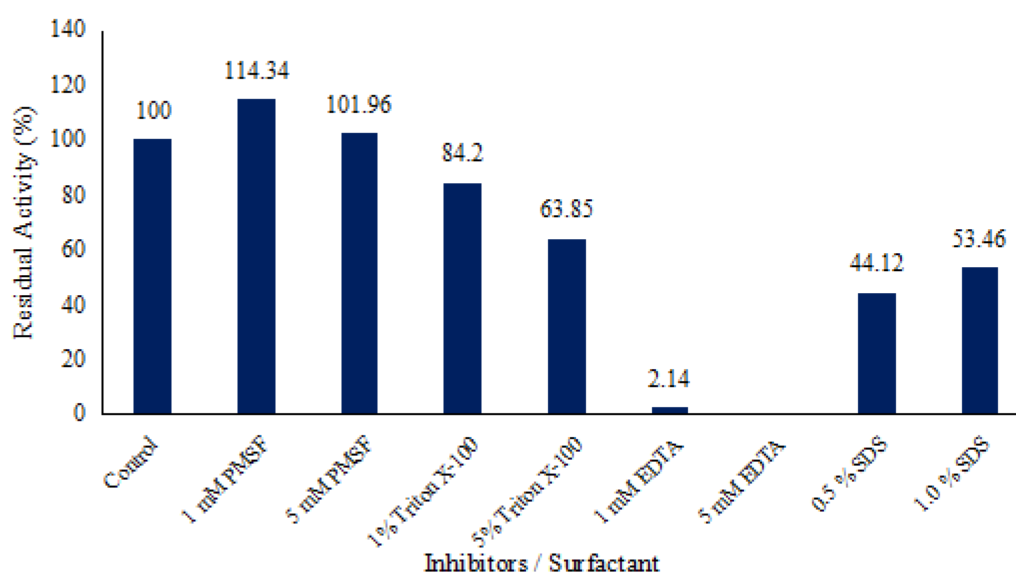


Figure 4.12. *Effect of inhibitors and surfactants on partially purified protease activity*

4.5.6. Halophilic protease stability in commercial detergents

The stability of partially purified protease with some commercial detergents were investigated by incubated the enzyme with commercial laundry detergents (Ariel and OMO) for 1 h at 40 °C. The study revealed that the enzyme was stable with the addition of 1 % detergents (Ariel and OMO), resulted in residual activity of 53.61 and 42.78 % of original activity respectively (Fig. 4.10), in comparison to control.

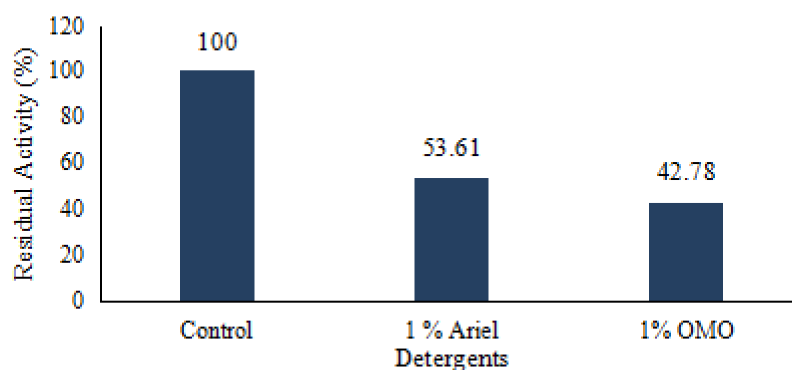


Figure 4.13 *Stability of partially purified protease in commercial detergents*

4.5.7. Effect of organic solvents on protease stability

The effects of various organic solvents on the stability of the partially purified protease from isolate TANN 4 are shown in Fig 4.14. The results revealed that partially purified protease from isolate TANN 4 was active and partially stable in 25 % (v/v) methanol. However, it showed less stability in the presence of 25 % (v/v) ethanol, propanol, and acetone with residual activities of 28.54, 3.96 and 11.54 %, respectively.

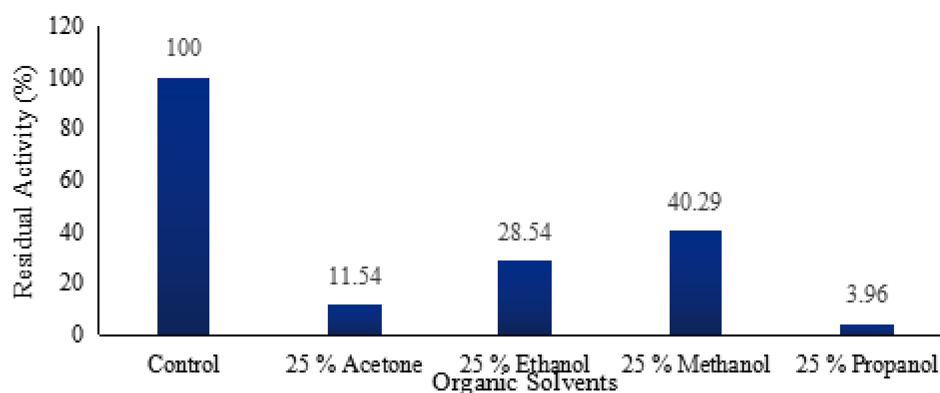


Figure 4.14 *Effect of organic solvents on protease stability*

4.5.8. Determination of K_M and V_{max} of partially purified halophilic protease

Based on the kinetic parameter assay the activity of purified protease from isolate TANN 4 was increased up to 0.8 % (w/v) casein (Table 4.3). The kinetic parameters of the partially purified enzyme K_M and V_{max} values were calculated using Lineweaver–Burk plot by plotting the substrate concentration versus specific activity (Fig. 4.10). As shown on the Lineweaver–Burk plot (Fig. 4.11) the K_M and V_{max} values were 0.0649 mM and 216.45 U mg^{-1}.

Table 4.3. *Calculation table for determination of K_M and V_{max}*

[Casein] (M)	1/[Casein] (M^{-1})	V(U mg^{-1})	1/V (U^{-1} mg)
(0.2 %) 8.47×10^{-5}	11,800.80	125.01	0.0079
(0.4 %) 1.69×10^{-4}	5,900.05	150.34	0.0066
(0.6 %) 2.54×10^{-4}	3,933.33	161.83	0.0061
(0.8 %) 3.39×10^{-4}	2,950.02	192.99	0.0052
(1.0 %) 4.24×10^{-4}	2,360.05	193.35	0.0051

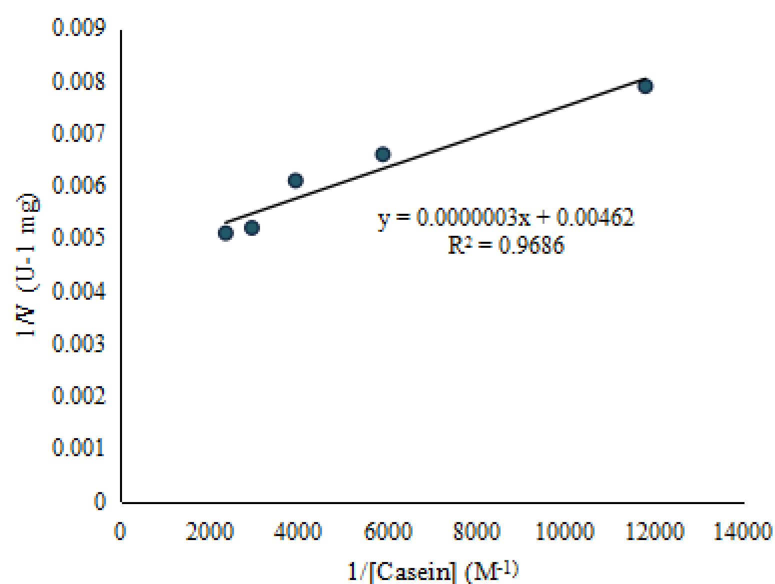


Figure 4.15 *Estimation of kinetic parameters of the partially purified isolate TANN 4 protease. The enzyme activity was measured at various casein concentrations (0.2 - 1.0 %) at pH 8.0, 1 % (w/v) NaCl and 50 °C. The Km and Vmax values were determined using linearized Lineweaver–Burk plot*

4.5.9 Determination of the molecular weight of partially purified protease isolate TANN 4

SDS-PAGE of the crude enzyme and partially purified enzyme was shown in Fig. 4.15; 4.16; 4.17 ; 4.18 after being analyzed using Gel Analyzer software. The partially purified protease showed a band that was fitted to a band from the crude fraction with estimated molecular mass of 19 890 Da.

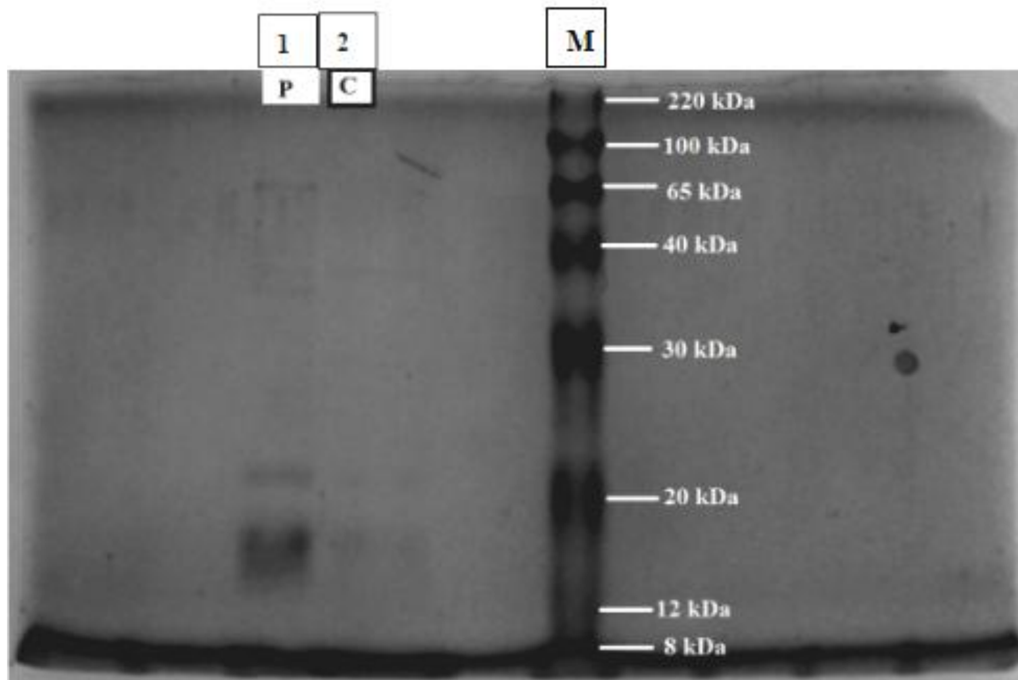
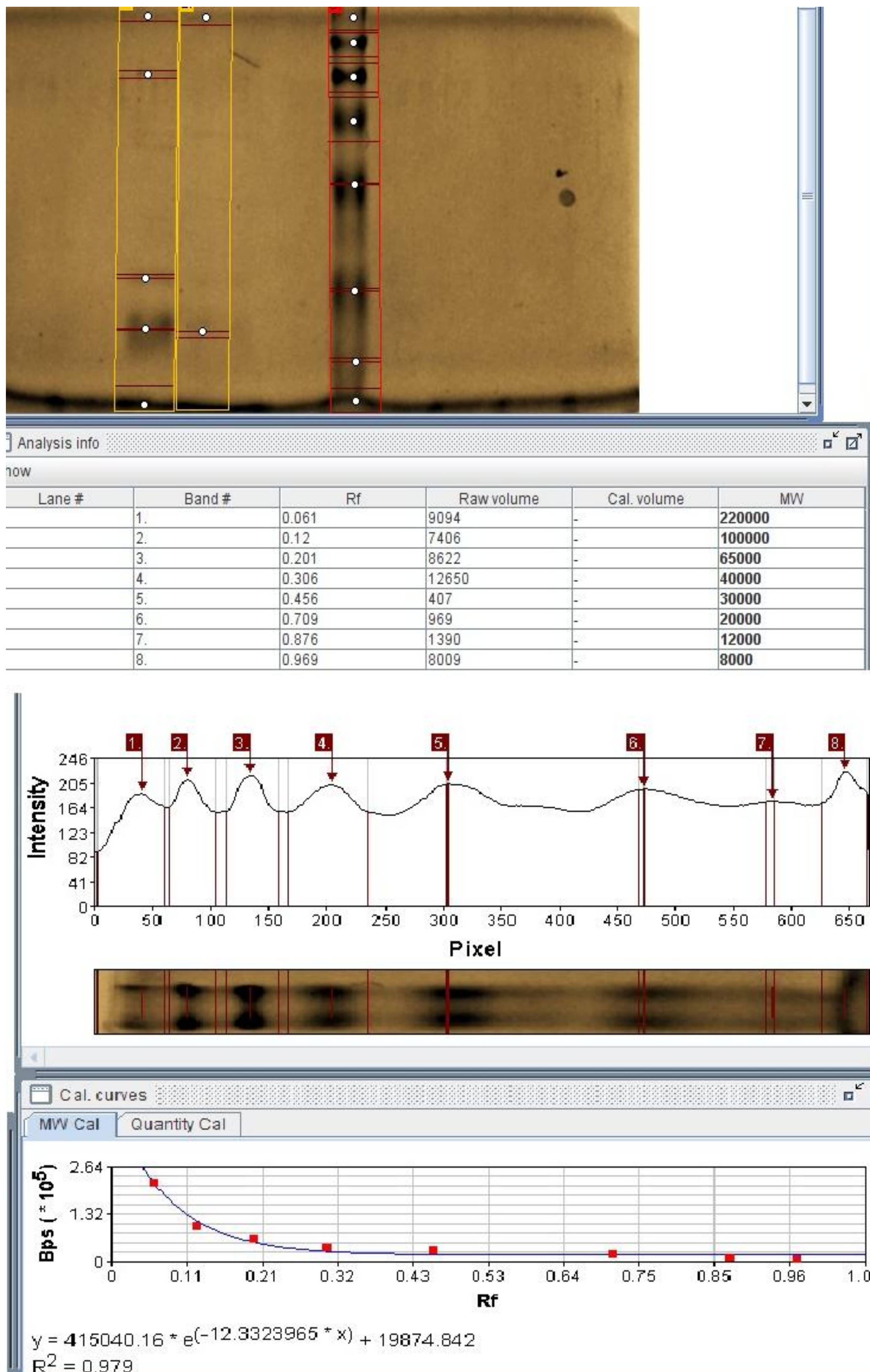


Figure 4.16. *SDS-PAGE analysis of the partially purified protease isolate TANN 4 on 12 % SDS–polyacrylamide. M Protein marker, lane 1 (P) Ammonium sulfate fraction, lane 2 (C) crude fraction*



Lane #	Band #	Rf	Raw volume	Cal. volume	MW
	1.	0.061	9094	-	220000
	2.	0.12	7406	-	100000
	3.	0.201	8622	-	65000
	4.	0.306	12650	-	40000
	5.	0.456	407	-	30000
	6.	0.709	969	-	20000
	7.	0.876	1390	-	12000
	8.	0.969	8009	-	8000

Figure 4.17 *Gel Analyzer Results for Marker Lanes*

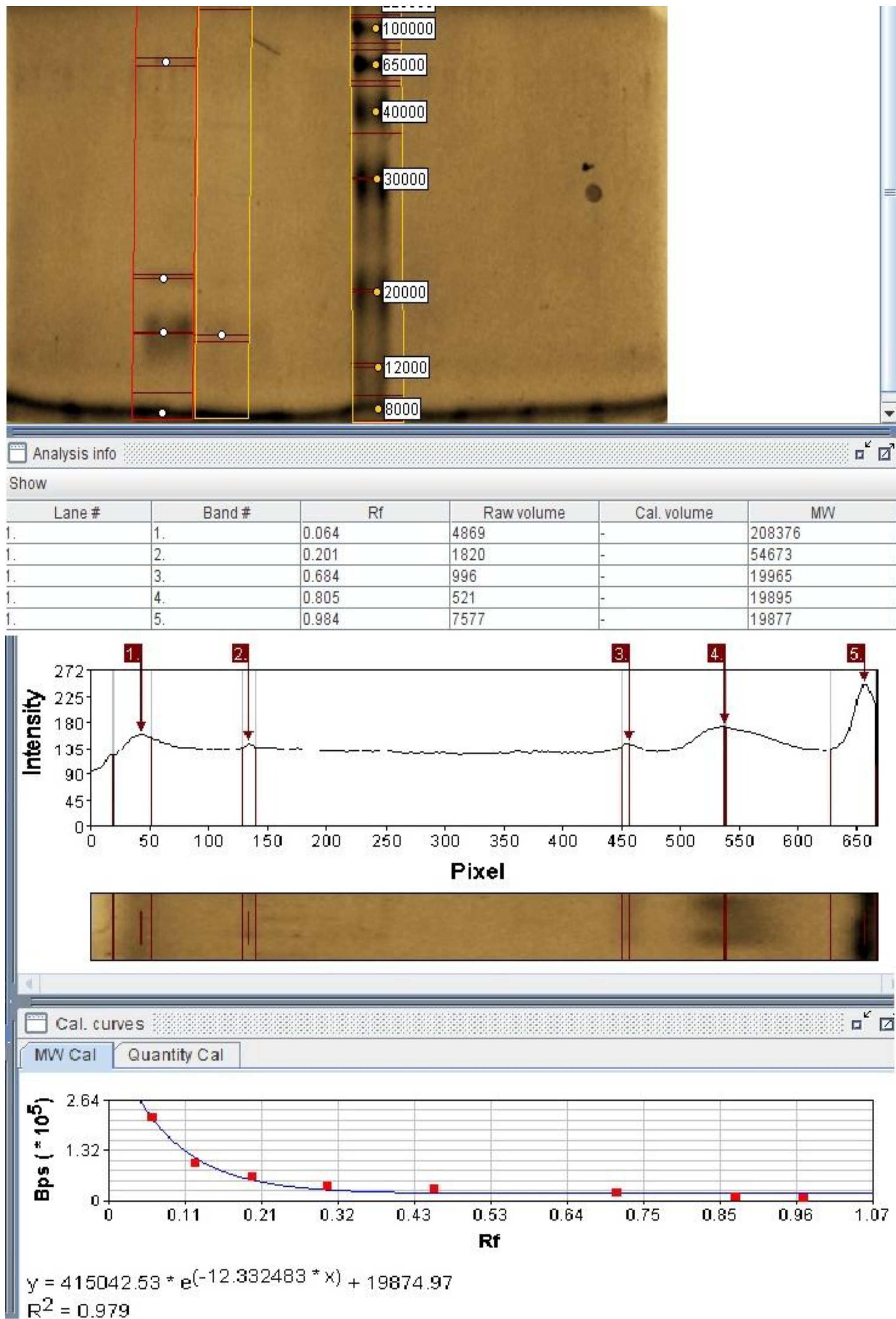
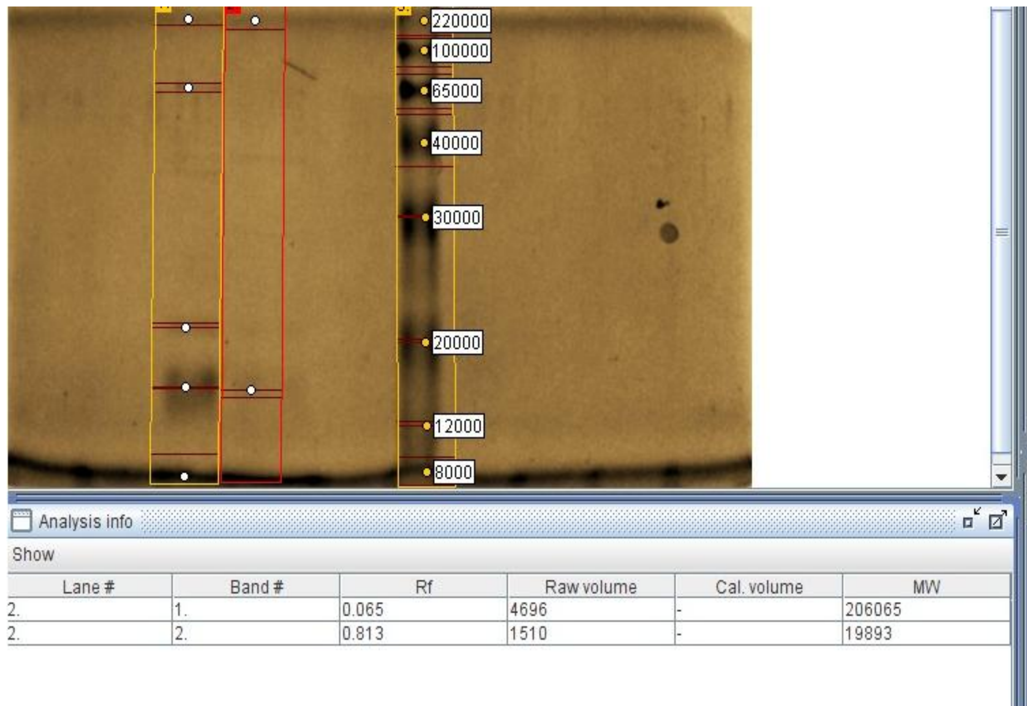
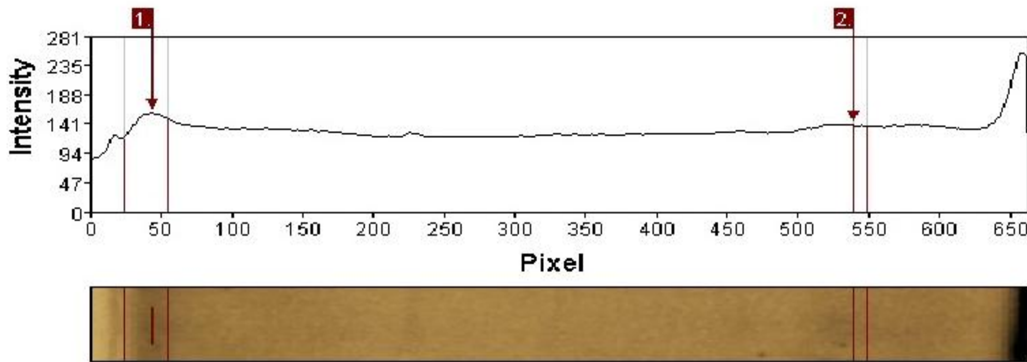
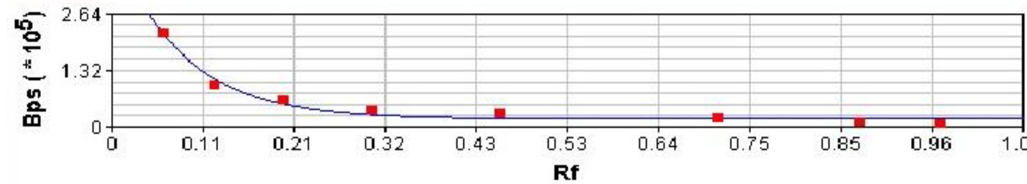


Figure 4.18 *Gel Analyzer Results for Purified Protease Lanes*



Lane #	Band #	Rf	Raw volume	Cal. volume	MW
2.	1.	0.065	4696	-	206065
2.	2.	0.813	1510	-	19893





$y = 415042.53 * e^{(-12.332483 * x)} + 19874.97$

$R^2 = 0.979$

Figure 4.19 *Gel Analyzer Results for Crude Protease Lanes*

4.5.10. Substrate specificity of partially purified protease

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 4.4), respectively. Meanwhile, collagen and gelatin were unhydrolyzed by the enzyme.

Table 4.4. *Substrate specificity calculation results*

[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	

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

The rRNA-(r-DNA)-based phylogenetic analysis, automated ribotyping (RiboPrinter® System Microbial Characterization System, DuPont™) revealed that 3 isolate including the main isolate TANN 4 resembled *Halobacillus trueperi* that exhibited 71 % similarity. Meanwhile, isolate TR 1 resembled *Virgibacillus pantothenticus* that exhibited 64 % similarity. The data compilation of automated ribotyping results are shown in Table 4.6.

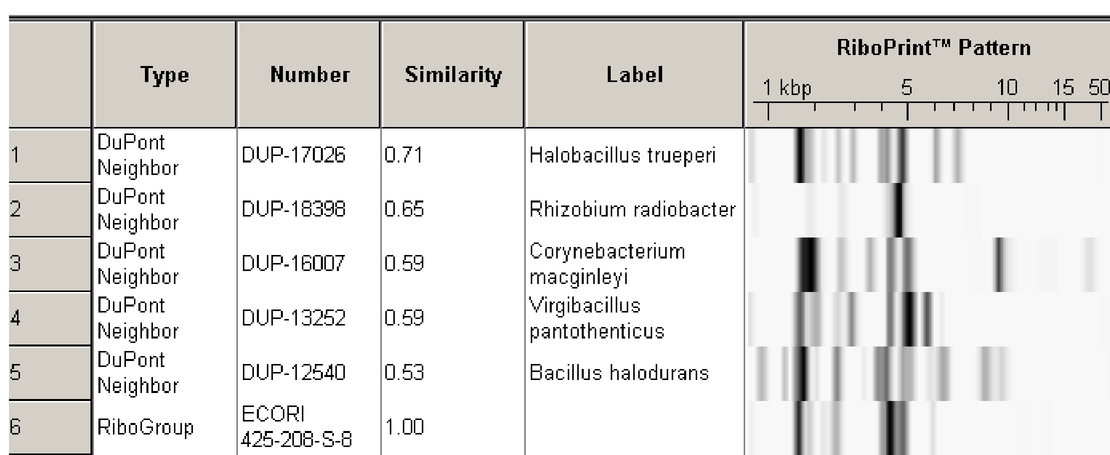
Table 4.5. *Automated ribotyping results*

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**

TAN 1A	18%MGM	Negative	Tauco	*Corynebacterium camporealensis*	0.62
TAN 1A*	18%MGM	Negative	Tauco	Chromohalobacter salexigens	0.64
TAN 1A2	18%MGM	Negative	Tauco	Chromohalobacter salexigens	0.66
TAN	MRS	Positive	Tauco	Flavobacterium species	0.71

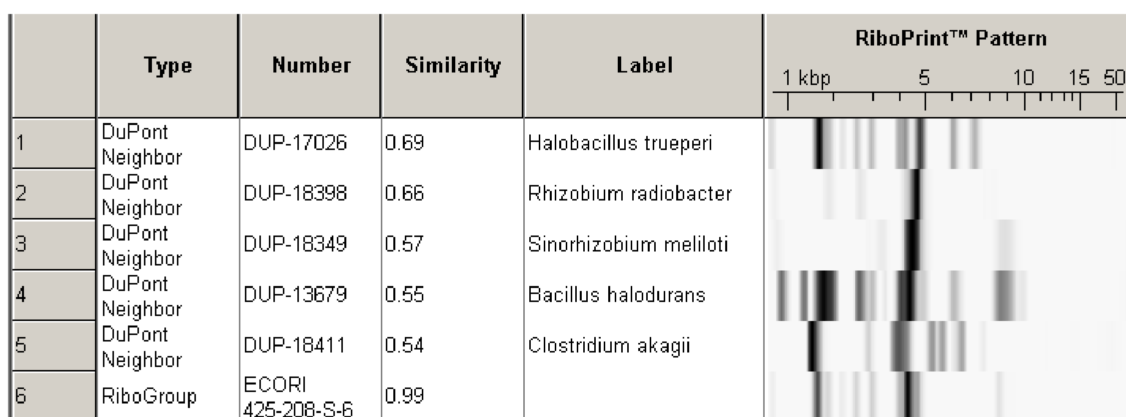
***Bold: Protease-producing Halophilic Strains**

The riboprinter profiles of the protease producing and non-producing isolates are shown in figures 4.20 to 4.26.



	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		

Figure 4.20. *Automated ribotyping result for isolate TANN 4*



	Type	Number	Similarity	Label	RiboPrint™ Pattern 1 kbp 5 10 15 50
1	DuPont Neighbor	DUP-17026	0.69	Halobacillus trueperi	
2	DuPont Neighbor	DUP-18398	0.66	Rhizobium radiobacter	
3	DuPont Neighbor	DUP-18349	0.57	Sinorhizobium meliloti	
4	DuPont Neighbor	DUP-13679	0.55	Bacillus halodurans	
5	DuPont Neighbor	DUP-18411	0.54	Clostridium akagii	
6	RiboGroup	ECORI 425-208-S-6	0.99		

Figure 4.21. *Automated ribotyping result for isolate TR 4*

	Type	Number	Similarity	Label	RiboPrint™ Pattern 1 kbp 5 10 15 50
1	DuPont Neighbor	DUP-17026	0.68	Halobacillus trueperi	
2	DuPont Neighbor	DUP-18398	0.66	Rhizobium radiobacter	
3	DuPont Neighbor	DUP-13679	0.60	Bacillus halodurans	
4	DuPont Neighbor	DUP-18349	0.59	Sinorhizobium meliloti	
5	DuPont Neighbor	DUP-18411	0.55	Clostridium akagii	
6	RiboGroup	ECORI 425-208-S-6	0.99		

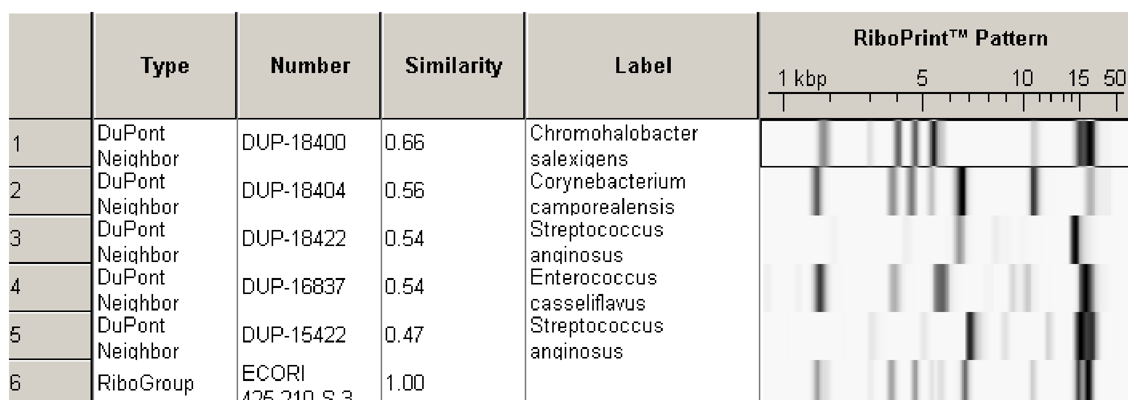
Figure 4.22. *Automated ribotyping result for isolate TR 2*

	Type	Number	Similarity	Label	RiboPrint™ Pattern 1 kbp 5 10 15 50
1	DuPont Neighbor	DUP-13253	0.64	Virgibacillus pantothenticus	
2	DuPont Neighbor	DUP-9500	0.64	Bacillus subtilis	
3	DuPont Neighbor	DUP-12568	0.62	Bacillus thermoleovorans	
4	DuPont Neighbor	DUP-13235	0.59	Bacillus licheniformis	
5	DuPont Neighbor	DUP-6103	0.59	Bacillus alcalophilus	
6	RiboGroup	ECORI 425-208-S-5	1.00		

Figure 4.23. *Automated ribotyping result for isolate TR 1*

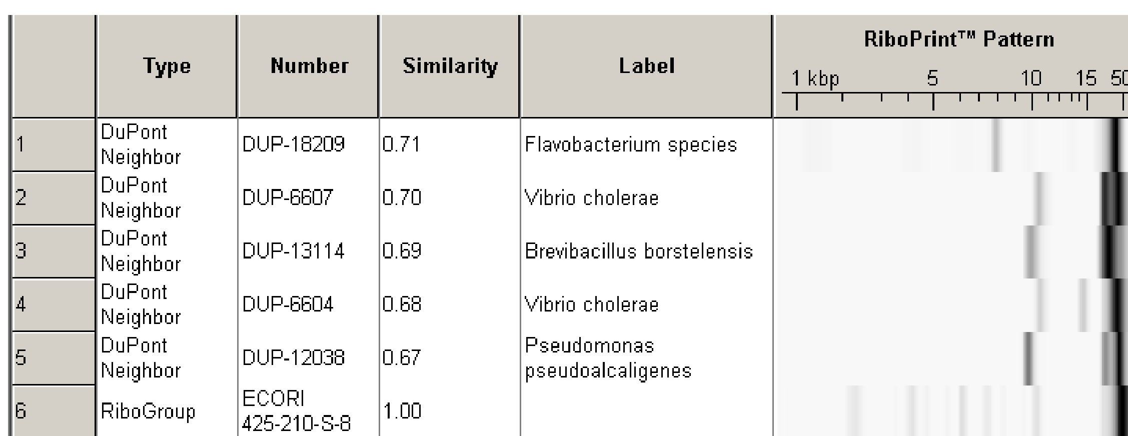
	Type	Number	Similarity	Label	RiboPrint™ Pattern 1 kbp 5 10 15 50
1	DuPont Neighbor	DUP-18400	0.64	Chromohalobacter salexigens	
2	DuPont Neighbor	DUP-18404	0.60	Corynebacterium camporealensis	
3	DuPont Neighbor	DUP-18422	0.55	Streptococcus anginosus	
4	DuPont Neighbor	DUP-16837	0.53	Enterococcus casseliflavus	
5	DuPont Neighbor	DUP-18286	0.50	Legionella pneumophila ss. pneumophila	
6	RiboGroup	ECORI 425-210-S-3	1.00		

Figure 4.24. *Automated ribotyping result for isolate TAN 1A**



	Type	Number	Similarity	Label	RiboPrint™ Pattern (1 kbp, 5, 10, 15, 50)
1	DuPont Neighbor	DUP-18400	0.66	Chromohalobacter salexigens	
2	DuPont Neighbor	DUP-18404	0.56	Corynebacterium camporealensis	
3	DuPont Neighbor	DUP-18422	0.54	Streptococcus anginosus	
4	DuPont Neighbor	DUP-16837	0.54	Enterococcus casseliflavus	
5	DuPont Neighbor	DUP-15422	0.47	Streptococcus anginosus	
6	RiboGroup	ECORI 425-210-S-3	1.00		

Figure 4.25. *Automated ribotyping result for isolate TAN 1A2*



	Type	Number	Similarity	Label	RiboPrint™ Pattern (1 kbp, 5, 10, 15, 50)
1	DuPont Neighbor	DUP-18209	0.71	Flavobacterium species	
2	DuPont Neighbor	DUP-6607	0.70	Vibrio cholerae	
3	DuPont Neighbor	DUP-13114	0.69	Brevibacillus borstelensis	
4	DuPont Neighbor	DUP-6604	0.68	Vibrio cholerae	
5	DuPont Neighbor	DUP-12038	0.67	Pseudomonas pseudoalcaligenes	
6	RiboGroup	ECORI 425-210-S-8	1.00		

Figure 4.26. *Automated ribotyping result for isolate TAN*

Table 4.6. *Automated ribotyping results summary*

Number	Label	RiboPrint™ Pattern DuPont™ RiboPrinter® System	Total Isolate
DUP-17026	*Halobacillus trueperi*		3
DUP-13253	*Virgibacillus pantothenticus*		1
DUP-18404	*Corynebacterium camporealensis*		1
DUP-18400	*Chromohalobacter salexigens*		2
DUP-18209	*Flavobacterium* species		1

V. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Discussion

The broad applications of halophilic microbial proteases in biotechnology and industries has led to an increased interest in the study of these enzymes. In this research, the protease-producing halophilic strains were screened and isolated from *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal. Their proteases were partially purified and characterized. *Tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal were chosen as the sources for isolates since research on halophiles and their enzymes of these samples among researchers are limited and out of date. For example, Surono and Hosono's study (Surono and Hosono, 1994, p. 1167) found that *terasi* starter was composed of *Bacillus brevis, Bacillus pumilus, Bacillus megaterium, Bacillus coagulans, Bacillus subtilis,* and *Micrococcus kristinae* and according to Kobayshi et al's study, species of *Tetragenococcus* are also detected in *terasi* sample (Kobayashi et al., 2003, p. 281).

Among *tauco* and *terasi* (Indonesian traditional fermented salty sauce food), Indonesian traditional salted fish and sediment soil from Bengkulu coastal, only the *tauco* and *terasi* samples yielded protease producing bacterial isolates, which were screened using 18 % MGM agar supplemented with 1% skim milk . *Terasi* describe as traditional salty fermented seafood or shrimp paste has 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, p. 1167). Surono and Hosono's study also reported that the dominant bacteria that were isolated from *terasi* were halophilic *Bacillus* sp and enzyme activities including esterase and protease were detected from these isolates by using Apizym kit (Surono and Hosono, 1994, p. 1167). 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. These hydrolysis are responsible for cheesy odor in *terasi* (Christanti A.D, 2006, p. 9). Meanwhile, *tauco* 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, p. 559). 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. On the other hand, in this study salted fish and sediment soil from Bengkulu coastal samples could not yield protease producing bacterial isolates, although several studies revealed that proteolytic activity was detected from microorganisms that inhabiting salted fish (Abd-Dalla, Omar, and El-Nagdy, 1994, p. 303; Skara et al., 2015, p. 20). This probably happened due to some reasons. First, the protease positive isolates might be do not exist in salted fish samples and sediment soil that were used in this study. Second, the protease positive isolates might be uncultured on 18 % MGM broth and agar medium. The "unculturable" indicates that the growth medium and culturing techniques that were used in this study are unable to grow a given bacterium, it also means that the culture method was failed to replicate essential aspects of bacteria environment including nutrients, pH, osmotic conditions, temperature and many more (Stewart, E.J, 2012, p. 4151).

Among the 4 halophilic protease producers isolated from *tauco* and *terasi*, the halophilic bacterial isolate TANN 4 which was isolated from *tauco* was recorded as the best protease producer. Isolate TANN 4 grew optimally at 18 % MGM broth (Modified Growth Medium) pH 7.5 at the temperature of 37 °C with agitation speed level of 150 rpm and showed maximum proteolytic activity on the 3rd day of fermentation. On 18% MGM agar plates supplemented with 1% skim milk, the color of colonies of this isolate are seen as yellow. Gram-staining analysis revealed that isolate TANN 4 is a

gram-positive, coccobacillus bacteria, a shape intermediate between cocci and bacilli. The rRNA(r-DNA)-based phylogenetic analysis, automated ribotyping (RiboPrinter® System Microbial Characterization System, DuPont™) revealed that isolate TANN 4 resembled *Halobacillus trueperi* that exhibited 71 % similarity. In addition, protease positive isolate TR 2 and TR 4 also resembled *Halobacillus trueperi* that exhibited 68 and 69 % similarity. *Halobacillus trueperi* was introduced and identified by Spring et al., at the year of 1996 describe as a rod-shaped, halophilic, gram-positive, heterotrophic bacteria. In addition, their rod cells are 0.7 to 1.4 by 2.0 to 4.5 μm and occur singly or in pairs and motile by flagella. Optimum condition for growth is in temperature range from 10 to 44 °C, with optimum temperature of 35 °C. Moreover, its optimum pH for growth is 7.5 and optimum salt concentration is around 10%. Its type strain is DSM 10404^T, which has been placed in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (Spring et al., 1996, p. 495). 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, p. 48). These automated ribotyping results actually did not reach the 85 % similarity threshold with reference riboprint patterns, so further identification for this isolates are needed, for example the phylogenetic analysis based on 16S rRNA gene sequences. Earlier studies reported that some protease were screened and isolated from *Halobacillus* sp. and *Virgibacillus* sp. For example, a serine protease were isolated from *Halobacillus* sp. SR5-3 which based on 16S rDNA sequence resembled *Halobacillus trueperi* with 97.1% similarity (Namwong et al., 2006, p. 1397). *Halobacillus trueperi* SS1 and SS3 which were isolated from soil sediment in India also reported exhibits salt dependent extracellular amylase and protease activities (Gupta et al., 2015, p 1). Additionally, Taprig, et al study also revealed that a total of 7 isolates which based on 16S rRNA gene sequences analysis

were identified as *Halobacillus* sp. and *Virgibacillus* sp exhibited protease activities (Taprig et al., 2013, p. 25).

Of the industrial enzymes, 75% are hydrolytic enzymes, one of them is protease which catalyze the total hydrolysis of proteins (Rao et al., 1998, p. 598). Microbial proteases which are accounting for approximately 60% of the total enzyme are produced by microorganisms especially bacteria (Chu, 2007, p. 241). Most of bacterial protease are extracellular enzyme, which is secreted by bacterial cell and functions outside of the cell. Extracellular enzymes can be produced in large amounts and active at a wide pH range (Ghafoori, Askari and Sarikhan, 2016, p. 120). Many research has been done on the properties of the enzymes from halophilic and halotolerant bacteria and their industrial applications, for example the extracellular lipase from a moderately halophilic *Salinivibrio* sp. and nuclease H of *Micrococcus varians* subsp. *halophilus* that have been widely applied in industries (Oren, 2010c, p. 830). In this investigation, a novel extracellular protease was partially purified from the culture supernatant of halophilic bacteria isolate TANN 4.

The novel halophilic extracellular protease produced by halophilic bacteria isolate TANN 4 was partially purified in two steps involving ammonium sulphate precipitation and dialysis. The enzyme was purified up to 25.41 fold-enrichment with 72.82 % recovery from total crude enzyme with specific activity of 301.14 U mg^{-1}. 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. 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. This metal ions serves as a nucleophile in catalysis that activated a water molecule. In current of the MEROPS database

(http://merops.sanger.ac.uk) which is a database that grouped peptidases into families based on sequence homology, metalloprotease are classified into 63 families, nine of which include but are not limited to BEMP (Bacterial extracellular metalloproteases) members. The BEMPs are distributed among M4, M5, M9, M10, M12, M13, M23, M30, and M34 families. The activity of BEMPs are inhibited by metal chelators due to the deprivation of catalytic Zn^{2+}, this inhibitor including EDTA, 1,10-Phenanthroline and EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) (Wu and Chen, 2011, p. 253).

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.* A lot of BEMPs are known as virulence factors, such as fragilysin from *Bacteroides fragilis*, pseudolysin from *Pseudomonas aeruginosa*, l-toxin from *Clostridium perfringens* and flavastacin from *Chrysebacterium meningosepticum. Myroides profundi D25,* a marine bacteria aslo reported exhibits extracellular metalloproteases activities. 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-ste 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 also used used to hydrolyze food proteins to produce flavor-enhancing peptides, like Neutrase which has been use in sausages fermentation processes. In the pharmaceutical industries, *Clostridium* collagenases have been investigated to treat Dupuytren disease and collagenases from *Vibrio* strains have potential in tissue cell dispersion, the removal of necrotic tissue from burns and ulcers. Furthermore, BEMPs have also been used in bread manufacturing, brewing industry and leather processing industry (Wu and Chen, 2011, p. 256).

Most bacterial extracellular metalloproteases only contain one catalytic domain and their molecular weight ranging from 20 to 35 kDa (Wu and Chen, 2011, p. 256). The partially purified extracellular metalloprotease showed protein band on SDS-PAGE analysis with estimated molecular weight of 19.8 kDa. The molecular mass of the partially purified halophilic protease of isolate TANN 4 is significantly lower than other halophilic metalloprotease, indicated that the partially purified extracellular metalloprotease from isolate TANN 4 is a novel metalloprotease. For example, some metalloproteases from *Salinivibrio* sp. strain AF-2004 (31 kDa) (Karbalaei-Heidari et al., 2007a, p. 237), *Halobacillus karajensis* strain MA-2 (36 kDa) (Karbalaei-Heidari et al., 2009b, p. 21), *Bacillus cereus* WQ9-2 (37 kDa) (Xu et al., 2010, p. 7991) and *Salinivibrio* sp. Strain MS-7 (21 kDa) (Shahbazi M and Karbalaei-Heidari, 2012, p. 45).

Biochemical properties of the crude and the partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 such as temperature and pH profile revealed a moderate thermoactive and alkalophilic character (optimal activity at 55 °C and pH 8.0). The crude and partially purified fraction was active at a temperature range of 25–60 °C and the maximum activity of the protease was detected at 40 °C for crude fraction and 50 °C for partially purified fraction. The differences of optimum temperature between crude and purified fraction are due to the protein interest was recovered with high yield (72.87 %) and high purity after purification, it makes the crude fraction (before purification) and purified fraction had a different particular biological activity (enzyme activity) (Berg J.M, Tymoczko J.L and Stryer, 2002). The crude and partially purified metalloprotease showed activity over a broad range of sodium chloride concentrations and pH values. The results showed that the enzyme (both crude and partially purified fraction) was active in a wide pH range from 6.0 to 10.0. The highest crude and partially purified protease activity was found at pH 8.0 and both fraction was active at salt concentrations ranging from 1 to 15 % (w/v) that showed maximal activity with 1 % (w/v) / 0.1 M NaCl. Similar results are reported from some proteases, for example metalloproteases from *Bacillus cereus* WQ9-2 (Xu et al., 2010,

p. 7991) and *Salinivibrio* sp. Strain MS-7 (Shahbazi M and Karbalaei-Heidari, 2012, p. 45) and a serine protease from *Bacillus* sp. APCMST-RS7 (Maruthiah T, Immanuel G, Palavesam A, 2015, p. 1) (optimum pH and temperature were 8.0 and 50 °C). Some studies have 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, p. 21). 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, p. 237). In comparison with another kind of protease that have quite similar characteristics, for example a purified serine protease from *Halobacterium* sp. strain HP25 showed maximum activity at pH 8.0, 60 °C, and was active at salt concentrations ranging from 5 to 25 % (w/v), with optimum activity at 17 % NaCl (w/v) (Elbanna, Ibrahim, and Revol-Junelles, 2015, p. 763).

The effect of several metal ions (2 mM) on the activity of the partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 was investigated at optimal activity conditions. Ca^{2+}, K^{+} and Mg^{2+} showed no inhibiton 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. In addition, Ca^{2+} and Mg^{2+} ions are known as stabilizers and inducers of many proteases and protect them from conformational changes (Ghafoori H, Askari M, and · Sarikhan, 2016, p. 121). Metalloproteases contain Zn^{2+}, while a few contain Mg^{2+}, Ni^{2+}, or Cu^{2+} in their active centers (Wu and Chen, 2011, p. 253). 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, which Ca^{2+}, K^{+}, and Mg^{2+} caused enhancement of the enzyme activity and Co^{2+} caused significant inhibition of the enzyme activity

(Ibrahim et al., 2015, p. 967). 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, p. 240). Elbanna's et al., study also found that ion Ca^{2+} and K^{+} had no inhibitory effect on the proteolytic activity of *Halobacterium* sp. strain HP25 (Elbanna, Ibrahim, and Revol-Junelles, 2015, p. 769). However, these findings are in contrast to those reported earlier for serine metalloprotease from *Salinivibrio* sp. strain AF-2004 in which Zn^{2+} caused enhancement of the enzyme activity (Karbalaei-Heidari et al., 2007a, p. 240).

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 V_{max} values which calculated using a Lineweaver–Burk plot were 0.0649 mM and 216.45 U mg^{-1}, respectively. This result is comparable to the results of Karbalaei-Heidari et al's study who reported for a purified metalloprotease from *Salinivibrio* sp. strain AF-2004 with K_M and V_{max} values of 1.4 mg/ml and 264 U/mg (Karbalaei-Heidari et al., 2007a, p. 237). Higher K_m and V_{max} values in the literature also reported from purified halophilic serine protease produced by *Halobacterium* sp. strain HP25 (K_M and V_{max} values were 523 μg mL^{-1} and 2500 μg min^{-1} mL^{-1}) (Elbanna, Ibrahim, and Revol-Junelles, 2015, p. 763), *Bacillus iranensis* strain X5B (K_m and V_{max} values were 0.126 mM and 0.523 mM/min) (Ghafoori H, Askari M, and · Sarikhan, 2016, p. 115) and *Bacillus* sp. strain NPST-AK15 (K_m and V_{max} values were 2.5 mg/ml and 7692.3 Umg^{-1}) (Ibrahim et al., 2015, p. 967). This is indicated that partially purified extracellular metalloprotease from isolate TANN 4 has lower substrate affinity and catalytic efficiency compared to literature reports mentioned above. This is due to the metalloprotease from isolate TANN 4 was a partially purified by ammonium sulphate precipitation with lower specific activity, meanwhile the other proteases mentioned above were purified until advance of purification step using gel filtration (*Halobacterium* sp. strain HP25), carboxymethyl (CM) column (*Bacillus iranensis* strain X5B) and gel filtration on Sephadex G.50 (*Bacillus* sp. strain NPST-AK15) with

higher specific activities. But, lower K_M indicates that the enzyme requires only a small amount of substrate to become saturated (Elbanna, Ibrahim, and Revol-Junelles, 2015, p. 765; Ghafoori H, Askari M, and Sarikhan, 2016, p. 116 ; Ibrahim et al., 2015, p. 963).

The partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 was stable and active with the addition of 1 % detergents (Ariel and OMO), resulted in residual activity of 53.61 and 42.78 % of original activity, respectively. The enzyme also highly stable in the presence of nonionic surfactant 1 and 5 % Triton X-100, retaining 84.20 and 63.85 % of its original activity. In addition, for 0.5 % and 1.0 % SDS, the enzyme retaining its activity of 44.12 and 53.46 %, respectively. The decreasing in enzyme activity is due to the conformational change of active site during the addition of SDS and detergents (Wang et al., 1995, p. 109). 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, p. 969), which retained 45.9 and of 36.2 % of its activity in the presence of Ariel and OMO, 77.1 % of its activity in the presence of 1 % Triton X-100, and 41.2 % of its activity in the presence of 0.5 % SDS. SDS and Triton X-100 are nonionic surfactant that mainly used as ingredient in detergents or cleaning products. This properties make partially purified metalloprotease from isolate TANN 4 has a possibility to use as an additive in detergent formulations.

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. This results indicate that the protease from isolate TANN 4 are not suitable for some biotechnological applications that used organic solvents in their processes, such as synthesis of peptide and ester under nonaqueous conditions (Maruthiah T, Immanuel G, Palavesam A, 2015, p. 6). On the other hand, the organic solvent tolerance metalloprotease are reported from *Bacillus cereus* WQ9-2 (Xu et al., 2010, p. 7993) and *Salinivibrio* sp. strain AF-2004

(Karbalaei-Heidari et al., 2007a, p. 241), which were highly stable in several organic solvents like hexane, methanol, ethanol, toluene, propanol, and benzene.

Substrate specifity of partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 were investigated by incubated the enzyme with several substrate such as casein, hemoglobin, collagen, gelatin and BSA. The partially purified extracellular metalloprotease from halophilic bacteria isolate TANN 4 was capable to hydrolyze casein, hemoglobin, and BSA. Meanwhile, collagen and gelatin were unhydrolyzed. The enzyme was perfectly hydrolyzed casein and hemoglobin, meanwhile BSA was not fully hydrolyzed. Earlier study reported that a serine metalloprotease from *Bacillus brevis* MWB-01 was also capable to hydrolyze BSA and egg albumin with relative activity towards casein of 74.5 and 63.2 %, while gelatin and collagen were poorly hydrolyzed (Olajuyigbe F.M and Falade A.M, 2014, p. 9). The capability of a protease to hydrolyze various substrates is one of important criteria for enzyme to be used in laundry detergent formulations. This result indicated that the protease enzyme from isolate TANN 4 can be used as additive in detergent formulations.

5.2. Conclusion

Among 4 halophilic protease positive isolates, halophilic bacteria Isolate TANN 4 which was isolated from tauco (fermented yellow soybeans) and resembled *Halobacillus trueperi* has been found to exhibit the highest extracellular metalloprotease activity. The protease then was partially purified with final yield of 72.87 % and also with 25.41 fold purity. The partially purified enzyme showed a moderate thermoactive and alkalophilic character and active at salt concentrations ranging from 1 to 15 % (w/v). The partially purified enzyme also fairly stable in several commercial detergents, surfactants and organic solvents and has been found to be able to hydrolyze casein, hemoglobin and BSA. These characteristics make this halophilic bacterial extracellular metalloprotease seems to be potentially useful for biotechnological and industrial applications.

5.3. Recommendations

Recommendations for improving this Study

The following recommendations are offered as possible ways to improve this study.

1. To improve the yield and purity of the protease enzyme, further purification processes are needed. Next purification step can be depending on the ionic properties of enzyme, for example ion exchange chromatography. Other techniques are adsorption chromatography and affinity chromatography (depending on the adsorbing properties) and gel permeation chromatography (depending on the size of enzymes).
2. To get an accurate and definitive identities of the isolates, further bacterial identification are needed, since automated ribotyping results did not reach the 85 % similarity threshold with reference riboprint patterns. For example, the phylogenetic analysis based on 16S rRNA gene sequences.
3. For commercial purposes enzyme should be manufactured in bioreactor to enhance the production. In order to be produced in bioreactor, media optimization are needed for enhanced the production of protease. It can be started with the optimization of media composition by alter one ingredient at a time until its optimum concentration is identified while the remaining ingredients are held constant. It also can be done with statistical experimental design, genetic algorithms (GAs) and particle swam optimization (PSO).
4. Several additional characterization are also needed after the enzyme are fully purified to get the accurate profile of the enzyme. For example, casein zymography and pH-temperature stability profile.

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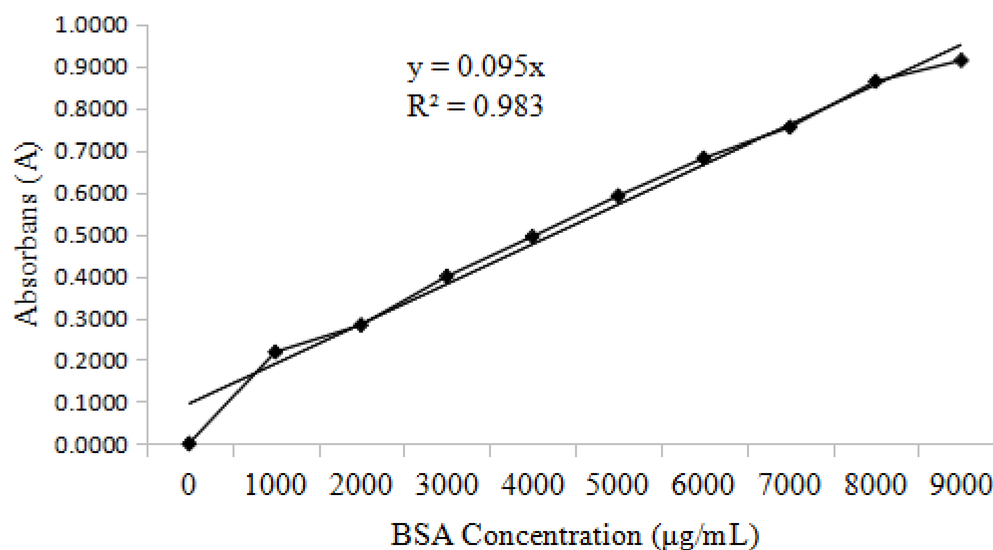
APPENDICES

Appendix 1. Protein Lowry Assay Standard Curve

Absorbance of Lowry Method

BSA (μg/mL)	A_1	A_2	A_3	$A_{average}$	$A_{corrected}$
0	0.033	0.031	0.048	0.0373	0.0000
1000	0.190	0.202	0.375	0.2557	0.2183
2000	0.320	0.322	0.318	0.3200	0.2827
3000	0.426	0.438	0.444	0.4360	0.3987
4000	0.529	0.536	0.528	0.5310	0.4937
5000	0.636	0.588	0.661	0.6283	0.5910
6000	0.670	0.733	0.752	0.7183	0.6810
7000	0.771	0.806	0.796	0.7910	0.7537
8000	0.884	0.918	0.899	0.9003	0.8630
9000	0.960	0.978	0.913	0.9503	0.9130

Standard Curve of Lowry Method



Protein Concentration using Lowry's Method

Fraction	Abs 1	Abs 2	Average	Blanko	Corrected Abs	[Protein] (mg/ml)
Crude (Isolate TANN 4)	0.369	0.378	0.3735	0.197	0.1765	1.86
Crude (Isolate TR 1)	0.389	0.385	0.3870	0.197	0.1900	2.00

Crude (Isolate TR 2)	0.450	0.451	0.4505	0.197	0.2535	2.67
Crude (Isolate TR 4)	0.416	0.413	0.4145	0.197	0.2175	2.29
Crude 1st Day TANN 4	0.352	0.367	0.3595	0.197	0.1625	1.17
Crude 2nd Day TANN 4	0.462	0.452	0.4570	0.143	0.3140	3.31
Crude 3rd Day TANN 4	0.369	0.378	0.3735	0.197	0.1765	1.86
Crude 4th Day TANN 4	0.447	0.444	0.4455	0.143	0.3025	3.15
Dialysate 85%	0.212	0.196	0.201	0.143	0.0610	0.64

Example of Calculation:

From the standard curve, the linear regression $y = 0.095\ x$, which y is corrected absorbance and x is [protein] (mg/ml).

For Dialysate 85% corrected absorbance (y) $= 0.0610$

$y = 0.095\ x$

$0.0610 = 0.095\ x$

$$x = \frac{0.061}{0.095} = 0.6421 mg / ml$$

Appendix 2. Specific Activity of Halophilic Protease from Different Isolates

Isolate	A_{Sample}	Ac_{ontrol}	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Average of specific activity (U/mg)
TANN 4	0.613	0.250	22.35	1.86	0.125	2.79	0.23	12.02	11.85
	0.621	0.268	21.73			2.72		11.68	
TR 1	0.336	0.165	10.53	2.00	0.125	1.32	0.25	5.27	5.28
	0.337	0.164	10.65			1.33		5.33	
TR 2	0.357	0.198	9.78	2.67	0.125	1.22	0.33	3.66	4.59
	0.412	0.172	14.77			1.83		5.53	
TR 4	0.443	0.418	1.54	2.29	0.125	0.19	0.28	0.67	0.55
	0.442	0.426	0.99			0.12		0.43	
TANN 6	0.280	0.124	9.60	1.71	0.125	1.20	0.21	5.61	5.90
	0.307	0.135	10.59			1.32		6.19	

Example of Calculation for Amano's Method

$$Activity\,(U/ml) = \frac{A1 - A2}{A3 - A4} x3x \frac{1.375}{0.3} x \frac{1}{10} x \frac{Dm}{Volume.Enzyme}$$

A1 : Absorbance of Sample
3 :Tyrosine quantity per 0.3 mL of Tyrosine standard solution Larutan (µg)
A2 : Absorbance of Control
A3 : Absorbance of Standard
A4 : Absorbance of Blanko
10 : Reaction time
Dm : Dilution multiple of Enzyme solution
1.375 : The final volume of the reaction mixture
0.3 : Volume of final filtrate
Vol.En : Volume enzyme (ml)

Example for isolate TANN 4 data, which is A1 = 0.613, A2 = 0.250, A3= 0.1786, A4 = 0.000

- $$Activity = \frac{0.613 - 0.250}{0.1786 - 0.000} x3x \frac{1.375}{0.3} x \frac{1}{10} x \frac{1}{0.125} = 22.35U/ml$$
- Total Activity = *Activity (U/ml) x Volume enzyme (ml)* = 22.35 U/ml *x* 0.125 ml = 2.79 U
- Total Protein = *[Protein](mg/ml) x Volume enzyme (ml)* = 1.86 mg/ml *x* 0.125 ml = 0.23 mg
- Specific Activity = $\frac{Activity\,(U/ml)}{[Protein](mg/ml)} = \frac{22.35U/ml}{1.86mg/ml} = 12.02U/mg$

Appendix 3. Purification table of the halophilic protease from isolate TANN 4

Fraction	A_{Sample}	$A_{Control}$	Dilution Factor	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Average of specific activity (U/mg)
	0.613	0.250	1	22.35			1 072.80		12.02	
Crude					1.86	48.00		89.28		11.85
Enzyme	0.621	0.268	1	21.73			1 043.04		11.68	
Dialysis	0.347	0.076	3	190.27			761.08		297.29	
am. sulphate					0.64	4.00		2.56		301.14
fraction 85%	0.355	0.077	3	195.19			780.76		304.98	

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

- Example of calculation
- *Yield (%)* =

$$\frac{Total.Activity.New(U)}{Total.Activity.Initial(U)} x100\% = \frac{770.92U}{1057.92U} x100\% = 72.87\%$$

- *Purification (fold)* =

$$\frac{Specific.Activity.New(U/mg)}{Specific.Activity.Initial(U/mg)} = \frac{301.14U/mg}{11.85U/mg} = 25.41$$

Appendix 4. Effect of fermentation period

Day -	A_{Sample}	Ac_{ontrol}	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Average of specific activity (U/mg)
1	0.199	0.167	1.97	3.54	0.125	0.26	0.44	0.56	0.68
	0.222	0.175	2.89			0.36		0.81	
2	0.452	0.229	13.73	3.31	0.125	1.71	0.41	4.14	4.18
	0.441	0.214	13.97			1.75		4.22	
3	0.541	0.216	20.01	1.86	0.125	2.50	0.23	10.75	10.41
	0.517	0.213	18.72			2.34		10.06	
4	0.447	0.285	9.97	3.15	0.125	1.24	0.39	3.16	3.18
	0.456	0.292	10.09			1.26		3.20	

Appendix 5. Effect of Temperature on Protease Activity (Crude Enzyme)

Temperature	A_{Sample}	$A_{Control}$	DF	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Average of Specific Activity (U/mg)	Relative Activity (%)
25° C	0.220	0.145	1	4.62	1.86	0.125	0.57	0.23	2.48	1.86	11.72
	0.211	0.173	1	2.34			0.29		1.26		
30° C	0.241	0.167	1	4.55	1.86	0.125	0.57	0.23	2.45	2.45	15.43
	0.266	0.192	1	4.55			0.57		2.45		
35° C	0.403	0.171	1	14.28	1.86	0.125	1.78	0.23	7.67	7.95	50.06
	0.425	0.176	1	15.33			1.91		8.24		
40° C	0.656	0.177	1	29.49	1.86	0.125	3.68	0.23	15.85	15.88	100.00
	0.637	0.156	1	29.61			3.70		15.92		
45° C	0.310	0.173	1	8.43	1.86	0.125	1.05	0.23	4.53	4.71	29.66
	0.301	0.153	1	9.11			1.14		4.89		
50° C	0.265	0.166	1	6.09	1.86	0.125	0.76	0.23	3.27	2.31	14.55
	0.201	0.160	1	2.52			0.32		1.35		

Appendix 6. Effect of Temperature on Protease Activity (Dialysate 85%)

Temperature	A_{Sample}	$A_{Control}$	DF	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Average of Specific Activity (U/mg)	Relative Activity (%)
25 °C	0.174	0.137	1	8.66	0.64	0.125	1.08	0.08	13.48	12.75	22.25
	0.169	0.136	1	7.72			0.96		12.02		
30 °C	0.181	0.142	1	9.13	0.64	0.125	1.14	0.08	14.21	14.39	25.47
	0.179	0.139	1	9.36			1.17		14.57		
35 °C	0.211	0.160	1	11.94	0.64	0.125	1.49	0.08	18.59	17.67	31.27
	0.203	0.157	1	10.76			1.34		16.75		
40 °C	0.261	0.159	1	22.47	0.64	0.125	2.81	0.08	34.99	36.08	63.87
	0.255	0.153	1	23.87			2.98		37.17		
45 °C	0.268	0.165	1	24.11	0.64	0.125	3.01	0.08	37.54	37.72	66.77
	0.272	0.168	1	24.34			3.04		37.91		
50 °C	0.321	0.167	1	36.04	0.64	0.125	4.50	0.08	56.12	56.49	100.00
	0.320	0.164	1	36.51			4.56		56.86		
55 °C	0.280	0.207	1	17.08	0.64	0.125	2.13	0.08	26.60	27.87	49.33
	0.284	0.204	1	18.72		0.125	2.34		29.15		
	0.233	0.196	1	8.66		0.125	1.08		13.48		

Appendix 7. Effect of pH on Protease Activity (Crude Enzyme)

pH	A_{Sample}	$A_{Control}$	DF	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Average of Specific Activity (U/mg)	Relative Activity (%)
6	0.373	0.289	1	5.17	1.86	0.125	0.64	0.23	2.78	2.05	13.05
	0.324	0.284	1	2.46			0.31		1.32		
7	0.574	0.265	1	19.02	1.86	0.125	2.37	0.23	10.22	9.82	62.54
	0.540	0.255	1	17.54			2.19		9.43		
8	0.694	0.219	1	29.24	1.86	0.125	3.65	0.23	15.72	15.70	100.00
	0.691	0.217	1	29.18			3.65		15.68		
9	0.494	0.275	1	13.48	1.86	0.125	1.68	0.23	7.24	7.34	46.75
	0.490	0.265	1	13.85			1.73		7.44		
10	0.407	0.253	1	9.48	1.86	0.125	1.18	0.23	5.09	5.19	33.06
	0.414	0.254	1	9.85			1.23		5.29		

Appendix 8. Effect of pH on Protease Activity (Dialysate 85%)

pH	A_{Sample}	$A_{Control}$	DF	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Average of Specific Activity (U/mg)	Relative Activity (%)
6	0.384	0.174	1	49.14	0.64	0.125	6.14	0.08	76.53	75.07	58.35
	0.377	0.175	1	47.27			5.91		73.62		
7	0.426	0.170	1	59.91	0.64	0.125	7.48	0.08	93.30	93.30	72.51
	0.428	0.172	1	59.91			7.48		93.30		
8	0.562	0.196	1	85.66	0.64	0.125	10.71	0.08	133.41	128.66	100.00
	0.534	0.194	1	79.57			9.94		123.92		
9	0.548	0.225	1	75.59	0.64	0.125	9.44	0.08	117.72	115.89	90.07
	0.529	0.216	1	73.25			9.16		114.08		
10	0.242	0.196	1	10.76	0.64	0.125	1.34	0.08	16.75	17.31	13.45
	0.252	0.203	1	11.47			1.43		17.86		

Appendix 9. Effect of NaCl on Protease Activity (Crude Enzyme)

[NaCl] (%)	A_{Sample}	$A_{Control}$	DF	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Average of Specific Activity (U/mg)	Relative Activity (%)
	0.695	0.286	1	25.18			3.14		13.53		
0					1.86	0.125		0.23		13.68	95.73
	0.685	0.267	1	25.73			3.21		13.83		
	0.670	0.249	1	25.91			3.23		13.93		
1					1.86	0.125		0.23		14.29	100.00
	0.672	0.229	1	27.27			3.41		14.66		
	0.391	0.279	1	6.89			0.86		3.70		
5					1.86	0.125		0.23		4.34	30.37
	0.411	0.260	1	9.29			1.16		4.99		
	0.298	0.211	1	5.35			0.67		2.87		
10					1.86	0.125		0.23		4.01	28.06
	0.361	0.205	1	9.60			1.2		5.16		
	0.330	0.235	1	5.48			0.68		2.94		
15					1.86	0.125		0.23		2.91	20.36
	0.306	0.219	1	5.35			0.66		2.87		

Appendix 10. Effect of NaCl on Protease Activity (Dialysate 85%)

[NaCl] (%)	A_{Sample}	$A_{Control}$	DF	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Average of Specific Activity (U/mg)	Relative Activity (%)
	0.436	0.193	1	56.87			7.10		88.56		
0					0.64	0.125		0.08		88.19	75.85
	0.430	0.189	1	56.40			7.05		87.83		
	0.492	0.173	1	74.65			9.33		116.27		
1					0.64	0.125		0.08		116.27	100.00
	0.488	0.169	1	74.65			9.33		116.27		
	0.464	0.195	1	62.95			7.86		98.03		
5					0.64	0.125		0.08		97.12	83.53
	0.470	0.206	1	61.78			7.72		96.21		
	0.363	0.165	1	46.34			5.79		72.16		
10					0.64	0.125		0.08		68.51	58.92
	0.343	0.165	1	41.65			5.20		64.86		
	0.270	0.168	1	23.87			2.94		37.17		
15					0.64	0.125		0.08		34.61	29.76
	0.248	0.160	1	20.59			2.57		32.06		

Appendix 10 .Effect of metal ions on protease activity

[Metal Ions]	A $_{Sampel}$	A $_{Kontrol}$	DF	Activity (U/mL)	Average of activity (U/mL)	Residual Activity (%)
	0.546	0.067	3	336.19		
Control 1*					335.20	100.00
	0.540	0.064	3	334.21		
	0.598	0.067	3	372.83		
2 mM $CaCl_2$*					381.61	113.85
	0.629	0.073	3	390.38		
	0.559	0.073	3	341.23		
2 mM $MgCl_2$*					342.64	102.21
	0.566	0.076	3	344.04		
	0.313	0.191	1	28.55		
2 mM $CoCl_2$*					30.18	12.42
	0.330	0.194	1	31.83		
	0.475	0172	1	70.91		
2 mM $ZnCl_2$*					82.61	34.00
	0.584	0.181	1	94.32		
	0.628	0.063	3	396.70		
2 mM KCl*					394.24	117.61
	0.619	0.061	3	391.79		
	0.419	0.061	3	251.36		
Control 2**					267.16	100
	0.472	0.069	3	282.96		
	0.502	0.105	2	185.83		
2 mM $FeCl_3$**					177.17	66.32
	0.460	0.100	2	168.51		

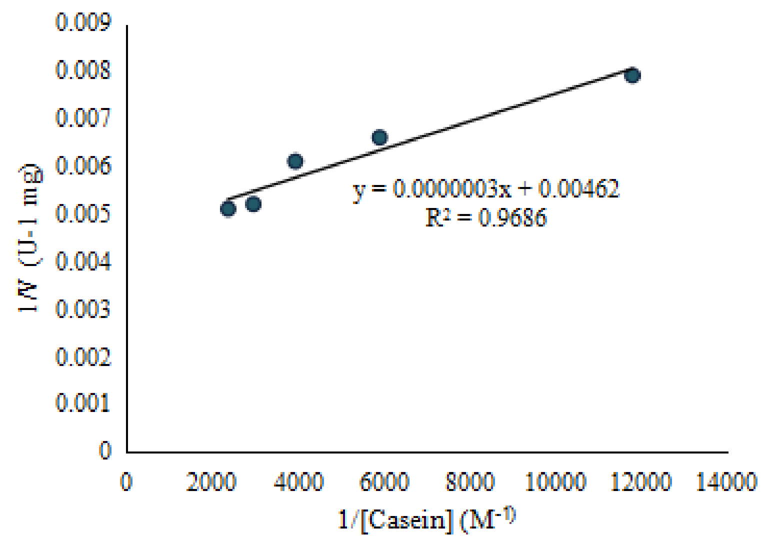
Appendix 11. Effect of commercial detergents on protease activity

[Detergent]	A_{Sampel}	$A_{Kontrol}$	DF	Activity (U/mL)	Average of activity (U/mL)	Residual Activity (%)
Control	0.293	0.178	1	26.91	27.49	100.00
	0.301	0.181	1	28.08		
1% Ariel	0.194	0.135	1	13.80	14.74	53.61
	0.205	0.138	1	15.68		
1% OMO	0.234	0.178	1	13.10	11.76	42.78
	0.230	0.185	1	10.53		

Appendix 12. Determination of Kinetics Parameter

[Kasein] (M)	A_{Sample}	$A_{Control}$	DF	Activity (U/mL)	[Protein] (mg/mL)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Average of specific activity (U/mg)
8.47×10^{-5} (0.2%)	0.565	0.199	1	85.66	0.64	0.125	10.70	0.08	133.40	125.01
	0.515	0.195	1	74.89			9.36		116.63	
1.69×10^{-4} (0.4%)	0.653	0.245	1	95.49	0.64	0.125	11.49	0.08	148.71	150.34
	0.676	0.259	1	97.59			12.19		151.98	
2.54×10^{-4} (0.6%)	0.506	0.067	1	102.74	0.64	0.125	12.84	0.08	160.00	161.83
	0.514	0.065	1	105.08			13.13		163.65	
3.39×10^{-4} (0.8%)	0.724	0.185	1	126.14	0.64	0.125	15.76	0.08	196.45	192.99
	0.725	0.205	1	121.70			15.21		189.53	
4.24×10^{-4} (1.0%)	0.719	0.192	1	123.34	0.64	0.125	15.41	0.08	192.08	193.35
	0.719	0.185	1	124.97			15.62		194.63	

[Casein] (M)	1/[Casein] (M^{-1})	V($U\ mg^{-1}$)	1/V (U^{-1} mg)
8.47×10^{-5}	11,800.80	125.01	0.0079
1.69×10^{-4}	5,900.05	150.34	0.0066
2.54×10^{-4}	3,933.33	161.83	0.0061
3.39×10^{-4}	2,950.02	192.99	0.0052
4.24×10^{-4}	2,360.05	193.35	0.0051



y = 0.00462 + 0.0000003 x

$$\frac{1}{Vo} = \frac{Km}{V\max}\frac{1}{[Casein]} + \frac{1}{V\max}$$

$$\frac{1}{V\max} = 0.00462$$

$$V\max = 216.45 U/mg$$

$$\frac{Km}{V\max} = 0.0000003$$

$$Km = 0.0000649 M = 0.0649 mM$$

Appendix 13. Effect of inhibitors and surfactant on protease activity

[Inhibitors or Surfactant]	A_{Sampel}	$A_{Kontrol}$	DF	Activity (U/mL)	Average of activity (U/mL)	Residual Activity (%)
Control 1*	0.425	0.104	2	150.25	143.69	100
	0.394	0.101	2	137.15		
1 mM PMSF*	0.443	0.104	2	158.68	164.29	114.34
	0.469	0.106	2	169.91		
5 mM PMSF*	0.438	0.108	2	154.47	146.51	101.96
	0.401	0.105	2	138.55		
1 % Triton X-100*	0.443	0.185	2	120.76	120.99	84.20
	0.440	0.181	2	121.23		
5 % Triton X-100*	0.385	0.191	2	90.81	91.75	63.85
	0.400	0.202	2	92.68		
Control 2**	0.419	0.061	3	251.36	267.16	100
	0.472	0.069	3	282.96		
1 mM EDTA**	0.236	0.210	1	6.09	5.74	2.14
	0.226	0.203	1	5.38		
5 mM EDTA**	Completely inhibited by 5 mM EDTA					
SDS 0.5 %***	0.524	0.211	1	73.25	72.90	44.12
	0.526	0.216	1	72.55		
SDS 1.0 %***	0.602	0.220	1	89.40	88.34	53.46
	0.597	0.224	1	87.29		
Control 3***	0.465	0.101	2	170.38	165.23	100
	0.433	0.091	2	160.08		

Appendix 14. Effect of organic solvents on protease activity

[Organic Solvents]	A_{Sampel}	$A_{Kontrol}$	DF	Activity (U/mL)	Average of activity (U/mL)	Residual Activity (%)
Control 1	0.465	0.101	2	170.38	165.23	100
	0.433	0.091	2	160.08		
25 % Acetone	0.311	0.231	1	18.72	19.07	11.54
	0.345	0.262	1	19.42		
25 % Ethanol	0.427	0.234	1	45.17	47.16	28.54
	0.427	0.217	1	49.14		
25 % Methanol	0.507	0.208	1	69.97	66.58	40.29
	0.516	0.246	1	63.19		
25 % Propanol	0.224	0.187	1	8.66	6.55	3.96
	0.205	0.186	1	4.44		

Appendix 15. Substrate specificity of partially purified protease

[Substrate]	$A_{corrected}$ 280 nm	$A_{corrected}$ 260 nm	DF	[Protein concentration mg/ml]	Relative Activity (%)
Casein (Control)	0.088	0.018	1	0.1227	100
BSA	0.022	0.021	1	0.0181	14.75
Hemoglobin	0.144	0.092	1	0.1533	124.94
Collagen	Unhydrolyzed				
Gelatin					

[Protein concentration (mg/ml)] casein $= (A_{280} \times 1.55) - (A_{260} \times 0.76)$

$= (0.088 \times 1.55) - (0.018 \times 0.76)$

$= 0.1227$ mg/ml