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Isolation and Characterization of Organic-Solvent Stable Protease Isolated by *Pseudomonas stutzeri* BK AB-12

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Abstract

In this study, proteases have been isolated from halophilic bacteria, *Pseudomonas stutzeri* BK AB-12. The bacteria produced proteases as exhibited by the appearance of clear zones resulted from casein hydrolysis. Based on the protease specific activity, 70–80% ammonium fraction exhibited the highest activity. Protease in this fraction has highest activity at pH 8.0 and 55 °C and its activity of protease was enhanced by the addition of several metal ions. The addition of Fe^{3+} ion also resulted in a shifting of the optimum pH from 8 to 9 and the optimum temperature from 55 to 60 °C. Protease of *P. stutzeri* BK AB-12 in the fraction of 70–80% is not likely metallo-, cysteine-, or serine-protease because it is not inhibited by EDTA, β-mercaptoethanol, and PMSF. Proteases of this fraction was sensitive to ionic strength, where the highest activity was observed at concentrations at NaCl concentrations of 2.5 M. Beside influenced by the ionic strength, protease activity was also sensitive to solvent polarity. The protease wasstable in the presence of different organic solvents, which enables its potential use for the synthesis of peptides.

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Keywords:protease; Pseudomonas stutzeri; halophiles

1. Introduction

Proteases which catalyze the hydrolysis of proteins are an industrial enzyme that has been applied in wide range of production process, such as bioactive component of detergent, biocatalyst in cheese production, bread production, medicines production, leather processing, etc. In order to fulfill wide variety of industrial needs, proteases have been isolated from various microorganisms, ranging from mesophiles to extremophiles[1].

One of extremophiles type that is currently being explored intensively is halophilic microorganism. Halophilic are microorganisms that can live in hypersaline environments. As a result of adaptation to high salt environment, most

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halophilic microorganisms produce proteases that were stable in some organic solvents. It is due to the property of salt that reduces water activity is similar to organic solvent system, hence halophilic protease may be used as a biocatalyst in organic media[2]. The common applications of proteases in organic solvent system include ester synthesis, acylation of taxol, transesterification, galactosylation, and synthesis of oligosaccharide propionates[3]. However, most of proteases are typically not stable when exposed in organic solvent because of the higher denaturation rate for the enzyme conformation in this solvent system. It is, therefore, in the present work, we isolated protease from halophilic bacteria *Pseudomonas stutzeri* BK AB-12 originated from the mud crater located at Bledug Kuwu village, Purwodadi, Central Java[4]. We showed here that protease isolated from this halophilic bacteria has extraordinary stability in both polar and nonpolar organic solvents.

2. Materials and Methods

2.1 Microorganism

The protease was isolated from halophilic bacteria, *Pseudomonas stutzeri* BK AB-12. This bacterium has been published by Parwata et al. (2014)[4].

2.2 Materials

All chemicals used were of analytical grade from Mercks.

2.3 *Protease assay*

The protease activity was spectrophotometrically determinedas described previously by Gaur et al. (2010) with some modifications[5]. The reaction was carried out in reaction mixture containing 100 µL of 1% (w/v) casein with 50 mM Phosphate buffer (pH 7) and 50 µL of the diluted enzyme for 20 min incubation at 40 °C. The reaction was stopped by adding 300 µL of TCA solution [5% (w/v)], followed by 30 min incubation at 4 °C and centrifugation (15652 ×g, 5 min). To the supernatant, 500 µL of 0.4 M Na_2CO_3 solution and 200 µL of Folin-Ciocalteau reagent were added and mixed thoroughly and the absorbance was measured at 660 nm. One unit of protease activity was defined as amount of enzyme required to liberate 1 mmol of tyrosine per min. The specific activity is expressed in the units of enzyme activity per milligram of protein.

2.4 Screening of extracellular protease produced by bacteria

Isolated bacteria were cultivated in Luria-Bertani (LB) medium containing 5% NaCl, 0,5% yeast extract, and 1% tripton with vigorous shaking (150 rpm) at 37 °C for 24 h. The culture then centrifuged at 10,017×g for 15 min. Bacteria potential in producing extracellular protease was evaluated by inoculating the free cell supernatant from the overnight culture on casein containing medium (2% casein and 2% bacto agar) at 37 °C. Proteolytic activity of enzyme solution was detected by the appearance of clear zones resulted from casein hydrolysis.

2.5 The growth and extracellular protease activity profile

1% of inoculums from an overnight grown culture in LB medium was added to Tryptic Soy Broth (TSB) medium (30 g/L TSB; 14,19 g/L Na_2HPO_4; 13,61% (g/L) $CH_3COONa{\cdot}3H_2O$; and 5% NaCl) and then incubated with vigorous shaking (150 rpm) at 37 °C. The culture was withdrawn aseptically at a certain time interval and cell density was measured at OD_{600} spectrophotometrically along with enzyme activity was monitored as described above.

2.6 Partial purification of protease enzyme

Ammonium sulfate was added to the cell free supernatant at 70-80% saturation, precipitates were centrifuged at 25714×*g* for 20 min at 4°C. The obtained precipitation was dialyze against Tris-Cl buffer at pH 7.0. The obtained protein was then assayed for enzyme activity and its concentration.

2.7 Protein concentration determination

Total protein content was measured by Bradford[6] method using bovine serum albumin (BSA) as a standard protein.

2.8 Enzyme characterization

Effect of metal ion

The effect of different metal ions on proteolytic activity of protease was investigated by incubating the enzyme in the presence of 5 mM metal ions at room temperature for 10 min. Metal ions, such as Ni^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, and Fe^{3+}ions, were used for the study. The residual enzyme activity was calculated in percentage referred to the activity of the enzyme without metal ions.

Effect of pH on activity of protease

The pH effect on the activity of protease was determined both in the absence and presence of Fe^{3+}ion.The optimum pH of protease was studied over a range of pH 7.0-12.0 at 40 °C with casein 1% (w/v) as substrate. The following buffer systems were used: potassium phosphate buffer for pH 7.0; Tris-HCl for pH 8.0 and glycine-NaOH buffer for pH 9.0-12.0.

Effect of temperature on activity of protease

The effect of temperature on the protease activity was examined at different temperature ranging from 25 to 65°C for 20 min at pH 8 (without Fe^{3+}) and pH 9 (with Fe^{3+}).

Effect of inhibitors on protease activity

The inhibitors that were used in this research are phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, and ethylenediaminetetraacetic acid (EDTA). Enzyme was preincubated with 30 mM Fe^{3+} for 10 min at room temperature and then incubated with 5 mM inhibitors for 30 min. The protease activity was assayed using casein as substrate. Residual activities in the presence of the inhibitors were compared with the controls without inhibitor.

Effect of organic solvents on protease activity

Enzyme was preincubated with 30 mM Fe^{3+} for 10 min at room temperature. Organic solvent was added (1:1) to the mixture and incubated for 20 min at room temperature. The substrate was casein (1%) in buffer pH 9. Activity was expressed as the remaining proteolytic activity relative to the organic solvent-free controls.

Effect of NaCl concentration on protease activity

Protease that incubated with 30 mM Fe^{3+} was performed at different NaCl concentrations using casein as substrate.

3. Results and Discussion

3.1 Assay for the potential of Pseudomonas stutzeri BK AB-12 in producing extracellular protease

The initial assessment to evaluate the capability of *Pseudomonas stutzeri* BK AB-12 in producing potential extracellular proteases was carried out by dropping the cell-free supernatant resulted from the overnight culture into the solid agar medium containing casein as the substrate.Protease containing supernatant was able to hydrolyze casein and formed a clear zone on casein agar (Fig. 1). The area of the clear zone can be directly correlated to the amount of proteases produced by bacteria[7].

Fig.1. Proteolytic activity of free-cell supernatant *P. stutzeri* BK AB-12 when plated in casein agar

3.2 The growth and extracellular protease activity profiles

Synthesis of enzymes in a microorganism is associated with the growth of the microorganism cells[8] (6). The growth profile of *Pseudomonas stutzeri* BK AB-12 was generated by monitoring the cell density every period of time and at the same time protease activity was also measured. The result showed that the growth profile of the bacteria hasrather long a log phase, which was from 5 up to 25 hours before finally entering the stationary phase (Fig. 2). Proteases have been produced since the beginning of bacterial growth and achieve the highest activity at the end of the log phase of bacterial growth, which was at 17th hour (Fig. 2).

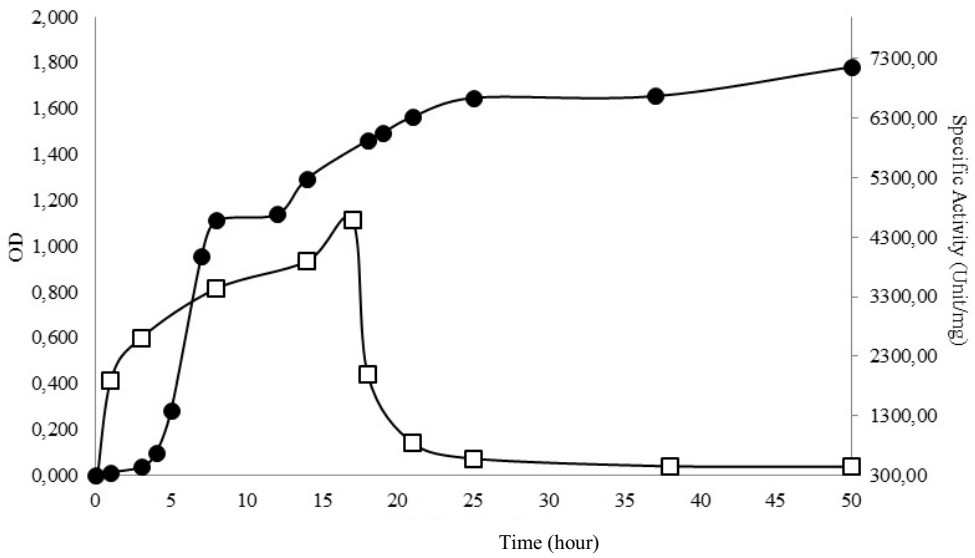

Fig 2. Growth (●) and protease activity (□) of *P. stutzeri* BK AB-12

3.3 Production and purification of protease

A summary of the protease activity at each purification procedure is given in Table 1. The crude enzyme was precipitated at three levels of ammonium sulfate saturation, and the fraction of 70-80% ammonium sulfate has the highest specific activity (2230,128 Unit/mg) and 10 fold purification (Table 1).This fraction was used in the characterization.

Table 1 Summary of protease activity at each purification steps

Ammonium sulfate Concentration range	Total Protein (mg)	Unit activity (Unit)	Specific Activity (U/mg)	Level of purity
Crude	11.878	2639.62	222.23	1
0 – 60%	0.824	478.83	581.18	3
60 – 70%	0.615	523.59	599.47	4
70 – 80%	2.141	4775.29	2230.13	10

3.4 Effect of metal ion on protease activity

The initial characterization is to determine metal ions effect on protease activity in the fraction of 70-80% ammonium sulfate. The result of the characterization showed that metal ions have different effect on protease activity (Fig.3). The addition of ions Ni^{2+}, Mg^{2+}, and Ca^{2+}ions decreased the activity of protease. Similar results were observedto protease from *Pseudomonasaeruginosa* MCM B-236[9].Zn^{2+}ion increased the activity of protease in this study up to nearly 200%. The metal ions thatgave the highest enhancement to the enzyme activity was Fe^{3+} ion, the activity was elevated up to 218%. This is the first report that protease from the genus *Pseudomonas* can be enhanced its activity by Fe^{3+} ion. Previously it was reported to be observed to protease isolated from*Bacillus subtilis* strain FP-133[10].

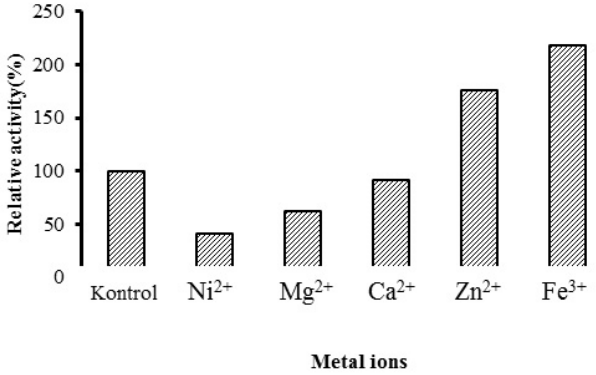

Fig. 3. Effect of metal ions on the activity of protease from *P. stutzeri* BK AB-12

3.5 Effect of pH and temperature on protease activity

Proteases that have been isolated from *Pseudomonasstutzeri BK AB-12* had a maximum activity at pH 8 and 55 °C (Fig.4a). Proteases produced by P. stutzeri BK AB-12 does not lost its activity at higher pH, even retained 67% activity at pH 12. Protease activity in this study had higher stability in alkaline environments compared to protease isolated from halophilic bacteria *Pseudoalteromonas sp.* CP76 strain[2], in which protease from *Pseudoalteromonas* has lost its activity by 70% at pH 10. The addition of Fe^{3+}ion to the enzyme solution increased its activity at each pH.In addition,the optimum pH shifted to pH 9. This was likely occurred due to the roleof Fe^{3+}ion to prevent protein denaturation in the alkaline pH.The optimum temperature of protease from *Pseudomonasstutzeri* BK AB-12 is

higher than the proteases isolated from *Bacillus cohnii* APT5[11], which has an optimum temperature of 50 °C. This protease was also active at temperatures between 25 and 65 °C, and retained 60% of its maximum activity at 65°C (Fig. 4b). The addition of Fe^{3+}ion also shifted in the optimum temperature from 55 to 60 °C. The phenomenon of a shifting in temperature optimum of protease due to the addition of the metal ions is also observed at the protease isolated from *Bacillus licheniformis* RP1[12].

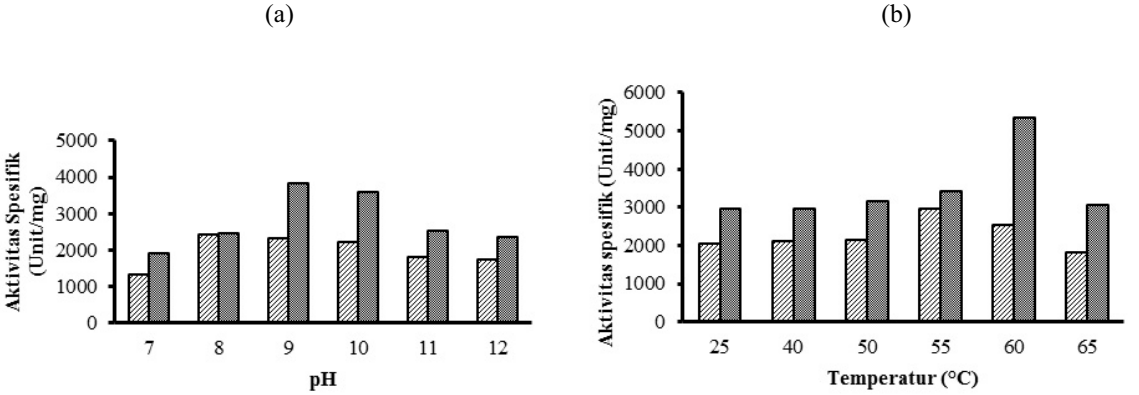

Fig. 4. Effect of pH (a) and temperature (b) on the activity of protease from *P. stutzeri* BK AB-12 in the absence (▨) and presence (▩) of Fe^{3+} ion

3.6. Effect of inhibitors on protease activity

Protease inhibition test give an insight into the nature of an enzyme, its cofactor requirements, and the enzyme active site[13]. Disruption caused by the presence of inhibitors may cause changes in the structure of the enzyme, resulting the decrease of its activity. The addition of PMSF and β-mercaptoethanol did not significantly decrease the activity of protease (Fig. 5). This suggests that the protease isolated from *P. stutzeri* BK AB-12 may not be included in the group of serine and cysteine proteases. The addition of other inhibitors, EDTA, also did not decrease the activity of protease *P. stutzeri* BK AB-12 significantly so that it can be concluded that the protease is not among the group metalo protease.

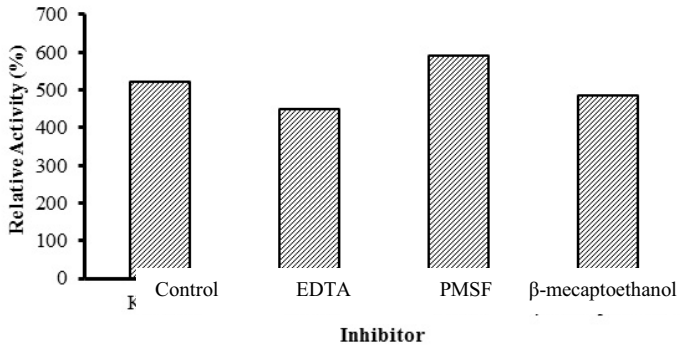

Fig. 5. Effect of inhibitors of protease from *P. stutzeri* BK AB-12

3.7 Effect of organic solvents on protease activity

Protease isolated from *Pseudomonas stutzeri* BK AB-12 is expected to have a high stability in nonaquous solvent (organic). It is based on the fact that the bacteria original habitatwas from the hypersalineenvironment. Salts are capable to bind water so it can be said that the bacteria was able to live in the environments with less water activity. Such circumstances can make the bacteria produce enzymes that able to work in the environment with less water or organic media. In order to evaluate the ability of protease produce by *Pseudomonas stutzeri*BK AB-12 to work in organic solvents, we measured its activity in 1:1 water/organic solventby varying the organic solvent component from the polar to the nonpolar ones. The enzymewas very stable in the solvent n-propanol, n-hexane, ethyl acetate and acetone but became less active in 2-propanol and chloroform (Table 2).Protease that has high stability in organic solvents can be used in the fermentation process and other reactions that use organic solvents. Besides this protease can also prevent contamination from microbes during the ongoing reaction[14].

Table 2Effect of organic solvents on the activity of protease from P. stutzeri BK AB-12

Organic Solvents	Log P	Relative activity (%)
Water	-	100
Acetone	-0.24	128
2-propanol	0.05	77
n-propanol	0.29	141
Ethyl acetate	0.73	128
Butanol	0.88	105
Chloroform	2.0	79
Benzene	2,13	111
n-hexane	3.98	131

3.9 Effect of NaCl on protease activity

Pseudomonas stutzeri BK AB-12 is halophilic bacteria, thus, we want study how salt affecting the activity of extracellular protease produced by this bacteria. The activity was measured from 0 M up to 4 M NaClconcentration. The result showed that the highest activity was attainedatNaCl 2.5 M (Fig.6) and at 3 and 4 M NaCl concentration, the remaining activity was about 26% and 17%, respectively. Such stability was better than protease isolated fromhalophilic bacterium *Bacillussp*[15], in which its maximum activity was noticed at 1 M NaCl and at 3MNaClthe proteolytic activity was almost undetectable.

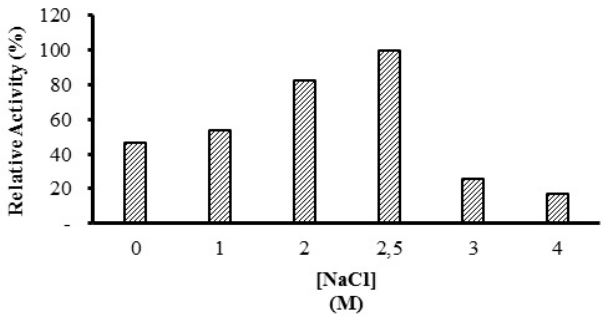

Fig.6. Effect of NaCl on the activity of protease from *P. stutzeri* BK AB-12

4. Conclussion

Pseudomonas stutzeri BK AB-12 produced high activity protease at 70-80% ammonium sulfate saturation that was highly stable at alkaline pH and showing optimum activity without and with Fe^{3+} ion at pH 8 and 9, respectively.

The addition of Fe^{3+}ion also causing the shifting of optimum temperature from 55 to 60 °C.the catalytic power was enhanced in 2.5 M NaCl and in the presence of n-propoanol or n-hexane. Considering the high activity in the presence of various organic solvents, the protease may find potential industrial application particularly those involving reaction in organic solvents.

Acknowledgements

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References

1. Gomes J, Steiner W. The biocatalytic potential of extremophiles and extremozymes. *Food TechnolBiotechnol* 2004;**42**(4):223-35.
2. Ruiz D, Castro R. Effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilicarchaeon*Natrialbamagadii, J IndianMicrobiolBiotechnol* 2007; **34**:111-15.
3. Sinha R, Khare S. Effect of organic solvents on the structure and activity of moderately halophilic*Bacillus sp.* EMB9 protease, *Extremophiles* 2004; **18**:1057-66.
4. Parwata IP, Asy'ari M, Hertadi R. Organic Solvent Stable Lipase from Moderate Halophilic Bacteria *Pseudomonas stutzeri* Isolated from the Mud Crater of BledukKuwu, Central Java, Indonesia. *J Pure App Microbiol* 2014; **8**(1): 31-40.
5. Gaur S, Agrahari S, Wadhwa, N. Purification of Protease from Pseudomonas thermaerum GW1 Isolated from Poultry Waste Site, The Open *Microbiol J* 2010, **8**: 67-74.
6. Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem* 1976. **72**:248–54.
7. Najafi MF, Deobagkar D, Deobagkar D., 2005, Potential application of protease Isolated from *Pseudomonas aeruginosa* PD100, *J Biotechnol*2005; **8**:197─203.
8. Kaur M, Dhillon S, Chaudhray K., Singh R, 1998, Production, purification and characterization of a thermostable alkaline protease from *Bacillus polymyxa*. *Indian J Microbiol* 199;**38**: 63─67.
9. Zambare V, Nilegaonkar S, Kanekar P. 2011, A Novel Extracellular protease from *Pseudomonas Aeruginosa* MCM B-327 : enzyme production and its partial characterization, *New Biotechnol*2011; **28**:173─81.
10. Endang S., Ju KY, Shinji T, Shuichiro M, Kenji A. *Purification and Characterization of a Halotolerant Intracellular Protease from Bacillus subtilis strain FP-133*. Kobe University Repository 2006.
11. Tekin N, Cihan Ç, Takaç Z S, Tüzun Y, Tunç K, Çökmus C, 2012, Alkaline protease production of *Bacillus cohnii* APT 5, *Turkey J Biol* 2002; **36**:430─40.
12. Sellami KA, Haddar A, Ali NE, Ghorbel FB, Kanoun S, Nasri M. Stability Of Thermostable Alkaline Protease From *Bacillus Licheniformis* RP1 in Commercial Solid Laundry Detergent Formulations, *Microbiological Research* 2008; **163**:299─306.
13. Ellaiah P, Srinivasulu B, Adinarayana K, 2002, A Review on Microbial Alkaline Proteases, *J Scien and Indust Res* 2002; **61**: 690─704.
14. Najafi MF, Deobagkar D, Deobagkar D. Potential application of protease isolated from *Pseudomonas aeruginosa* PD100, *Electronic J Biotechnol* 2005; **8**:197─203.
15. Kamekura M, Onishi H, 1973, Protease formation by a moderately halophilic*Bacillus* Strain, *App Microbiol* 1973; **27**: 809─10.