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Exploration and Characterization of Extracellular Protease Producing Bacteria from Bledug Kuwu and Its Potential in Degrading Chicken Feathers

Suharti Suharti*, Nur Faridah, Surjani Wonorahardjo and Mieke Alvionita
Chemistry Department, Faculty of Mathematics and Sciences, Universitas Negeri Malang,
Jl. Semarang No. 5, Malang 65145, Indonesia
*Corresponding author (e-mail: suharti.fmipa@um.ac.id)

High salt tolerant extracellular proteases are enzymes that play an important role in industries that require high salt conditions. High salt tolerant extracellular proteases can be produced by halophilic microbes. This research has succeeded in isolating several halophilic microbes capable of producing extracellular proteases from Bledug Kuwu volcanic mud, Grobogan, Central Java, Indonesia. Five isolates tested had bacillus cell shape and were moderate halophilic bacteria. These isolates also produced extracellular proteases on skim milk substrate media in the pH range of 6-9 and the optimum fermentation time was 3-4 days. The extracellular proteases produced by these isolates worked optimally in the pH range of 8-9, the temperature of 40-52 °C, and tolerant at 3-10% NaCl. Extracellular proteases from isolates BK1A and BK1C worked optimally in the presence of Mg^{2+}. BK1H extracellular protease activity works optimally in the presence of Ca^{2+}. All isolates produced extracellular proteases that have keratinase activity that can degrade keratin in chicken feathers with an optimum incubation time of 3-4 days, as proven by the % weight loss on day 5 of 12.2%; 20.9%; 12.3%; 16.4%; and 35.8%. Based on these characteristics, these isolates showed a potential to be utilized in producing extracellular proteases that can be used in industrial processes such as the detergent industry which requires alkaline proteases, and leather tanning which requires proteases (keratinase) that are resistant to moderate salinity conditions.

Keywords: Protease; chicken feather; halophilic; keratinase

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Halophilic microorganisms are classified according to their optimal growth conditions in various salt concentrations, namely as moderate halophiles between 3%-15% (~0.6 M) NaCl, extreme halophiles above 15% (>5 M) NaCl [1-5], and 'slight halophiles' were able to grow on media with 1%-3% NaCl [6-9]. Various microorganisms have been isolated from saline soils, seawater, hypersaline lakes, high-salt marshes, brines from underground salt springs, saltwater lakes, salt ponds, deep-sea sediments, and fermented fish sauces [10-12]. Bledug Kuwu is a mud volcano accompanied by eruptions of gas from the ground that lasts periodically between 2 and 3 minutes, located in Kuwu Village, Kradenan, Grobogan, Central Java. Analysis of geological data shows that Bledug Kuwu is included in the Randublatung zone and has an average temperature of 32 °C; pH 7.5; sulfur concentration 62.883 x102 mg/Kg; and salinity 57.15 mg/L (salt content 5.72%) [13].

Halophilic moderate and light halophilic or halotolerant microbes use the Compatible Solute mechanism by collecting small molecular weight organic solutes in the cell (called compatible solutes) and removing as much salt from the cytoplasm as possible to balance the osmotic pressure [14,15]. Compatible solutes function as stabilizers that maintain the active conformation of various macromolecules in cells to adapt to extreme environmental conditions [16]. Cellular membranes of halophilic microbes also increase the proportion of phospholipids, thereby helping maintain the cytoplasmic membrane's hydration state [17]. In addition to this unique way of adapting, halophilic bacteria can live at high salt levels because they have stable extracellular enzyme at high salt levels.

These are hydrolase enzymes, namely amylase, nuclease, phosphatase, and protease [9]. Protease, which can survive in a wide range of salt concentrations, is very useful in several industries with high salt conditions in the process, such as the detergent industry, fish canning, and leather tanning. Proteases as keratin degraders have different characteristics. These characteristics provide maximum protease activity in an optimal conditions. Several halophilic bacteria are reported to be able to produce proteases, including *Halobacillus* sp. CJ4 produced a protease with optimal activity at 0.4 M NaCl; pH 9.0; and 45 °C [18], *Chromohalobacter japonicus* BK-AB18 produced a protease with optimal activity at 7.5% NaCl; pH 9; and

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45 °C [19], *Bacillus luteus* H11 had a protease with optimal activity at 7.5% NaCl; pH 10.5; and 45 °C [20], and *Halobacterium* sp. HP25 produces proteases with optimal activity at pH 8; and 60 °C [21].

Eight isolates isolated from Bledug Kuwu mud were known to produce extracellular proteases and were purified by the streak quadrant method [18]. These isolates need to be analyzed for optimal growth conditions under various conditions of NaCl concentration and extracellular protease production. The protease produced also needs to be analyzed for its characteristics, so that it can work optimally.

A microorganism can produce more than one type of protease to process the nutrients available in its environment. One of the proteases produced is keratinase. Keratinase (EC.3.4.99) is a serine protease or metalloprotease that can hydrolyze peptide bonds and disulfide bonds in keratin [22]. Feathers contain about 91% keratin protein which has the potential to be a source of production of high value products consisting of keratin protein or keratin fibers [23]. Feathers can be used as a substrate source to analyze the ability of isolates to produce keratinase.

METHOD

Optimization Growth of Isolates

Growth of Isolates on The Variation of NaCl Concentration

The isolates used are BK1A, BK1B, BK1C, BK1D, and BK1H bacteria isolated from the Bledug Kuwu Mud [24]. The isolates were regenerated at skim milk-agar media before being used for protease production. Methods from Alshubaith and Gilmour, 2017 [24-26] with modifications. The isolates were inoculated on liquid LB media and incubated at 37°C with 100 rpm aeration for 16 hours, and the culture was used as a starter. Then 1% (v/v) starter was inoculated on LB liquid medium with various concentrations of NaCl (from 5 to 25%) and incubated at 37 °C with 100 rpm aeration for 24 hours. Bacterial growth was measured and observed with optical density (OD) values at 600 nm every 2 hours.

Growth of Isolates on The Variation of pH and Incubation Time

The preculture and production media used to refer to the research conducted by [27] with a modified substrate, skimmed milk. The culture that has been incubated is taken as much as 1% (v/v) and inoculated into each production medium with pH variations in the buffer (from 5 to 10). Then incubated at 37 °C with 100 rpm aeration for 5 days. The incubated cultures were centrifuged at 4 °C at 10,000 rpm for 10 minutes to separate the enzyme from the other mixtures. The results of centrifugation were in the form of supernatant and pellets. The supernatant is also called crude protease extract whose activity will be determined. Determination of the optimum production time was carried out by measuring the extracellular protease activity every 24 hours.

Protease Assay

Extracellular protease activity was measured by a modification of the Anson method [28]. 200 L Supernatant (protease crude extract) added 1 mL of Tris-HCl buffer solution pH 8, 500 L 1% casein solution, and vortexed. The mixture was incubated at 40 °C for 30 minutes. Then 1 mL of 10% trichloroacetic acid (TCA) solution was added and incubated for 15 minutes at room temperature. Then the mixture was centrifuged for 10 minutes at 10,000 rpm. The resulting supernatant was put into a test tube containing 2 mL of 0.5 M Na_2CO_3, added with 1 mL of Folin Ciocalteu reagent, vortexed until homogeneous, and incubated in the dark for 30 minutes. Absorbance measurements were carried out at a wavelength of 660 nm. The negative control used the same method but the addition of 10% TCA solution was carried out before the addition of 1% casein substrate.

Protease Activity on Variation of Temperature

Determination of the optimum temperature was carried out according to the procedure in the protease activity test, but there were differences in incubation temperature variations, namely 34ºC to 52ºC. The buffer solution used is following the optimum pH that has been determined in the previous step.

Protease Activity on Variation of pH

The determination of the optimum pH for the enzyme activity test is the same as the protease activity test procedure. The difference in the pH of the buffer solution used varies from 5 to 10. And then compared each enzyme activity.

Protease Activity on Variation of NaCl Concentration

Determination of enzyme resistance to NaCl concentration was carried out by measuring protease activity with the addition of 50 mM Tris-HCl buffer (pH according to the optimum protease pH) with varied NaCl levels, namely NaCl 3%, 5%, 8%, 10% and 13% [29].

Protease Activity on Variation of Divalent Metal Ion

Determination of the effect of divalent metal cations on protease activity was carried out according to the enzyme activity test procedure. The difference lies in the variation of divalent metal ions, namely Ca^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+} ions at a concentration of 5mM [11]. The addition of divalent metal ions was carried out before adding 1% casein. Determination of the effect of divalent metal cations on the activity of crude protease extract was carried out by calculating the

percent of residual activity due to the effect of the divalent metal cations.

Potential Isolate in Production of Keratinase with Chicken Feather as Substrate

The ability of isolates to produce keratinase can be seen by the hydrolysis of keratin in chicken feathers using the SSF method [30]. The microbial preparation needed was fresh isolate inoculated on liquid LB media and incubated for 16 hours at 37°C with an aeration speed of 100 rpm (starter/preculture). Next, 1 mL of culture was inoculated on SSF keratinase production medium with 500% humidity, the composition of the production medium: 1 g chicken feathers and salt solution (0.2 g/L KCl; 0.2 g/L $MgSO_4.7H_2O$; 0.1 g/L $(NH_4)_2PO_4$; 20 g/L $CaCO_3$; 0.5 g/L KH_2PO_4; 0.5 g/L K_2HPO_4 dissolved in 100 mL aquadest) pH according to the optimum pH for extracellular protease production. Then incubated at 37°C for five days (without aeration). Determination of activities enzyme carried out every 24 hours for five days. At each incubation time, the mass of chicken feathers remaining after the degradation process and the keratinase activity produced during the degradation process were analyzed.

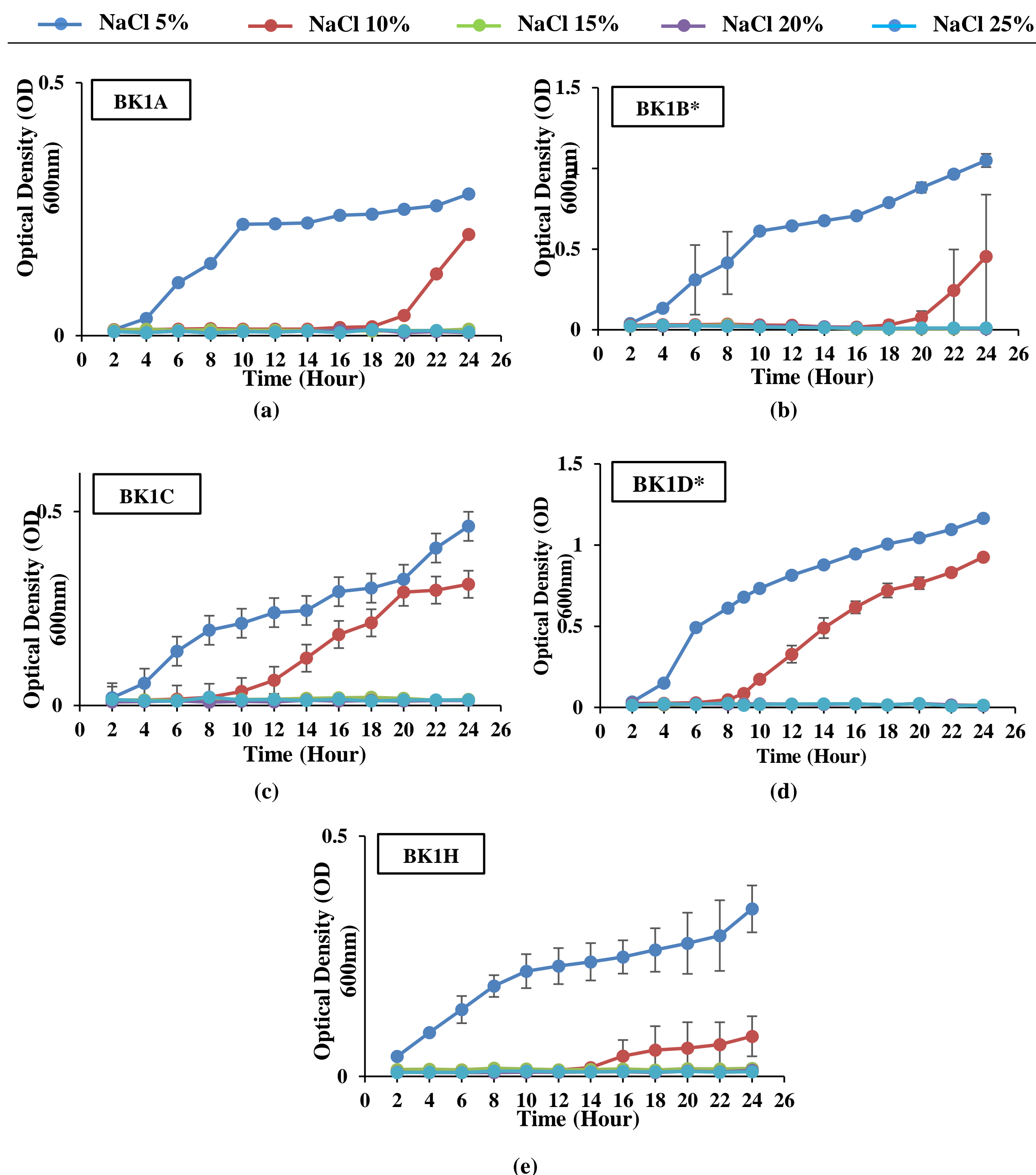

Figure 1. OD600nm curves depicting the growth of isolates at various concentrations of NaCl: (a) BK1A, (b) BK1B[24], (c) BK1C, (d) BK1D[24], and (e) BK1H.

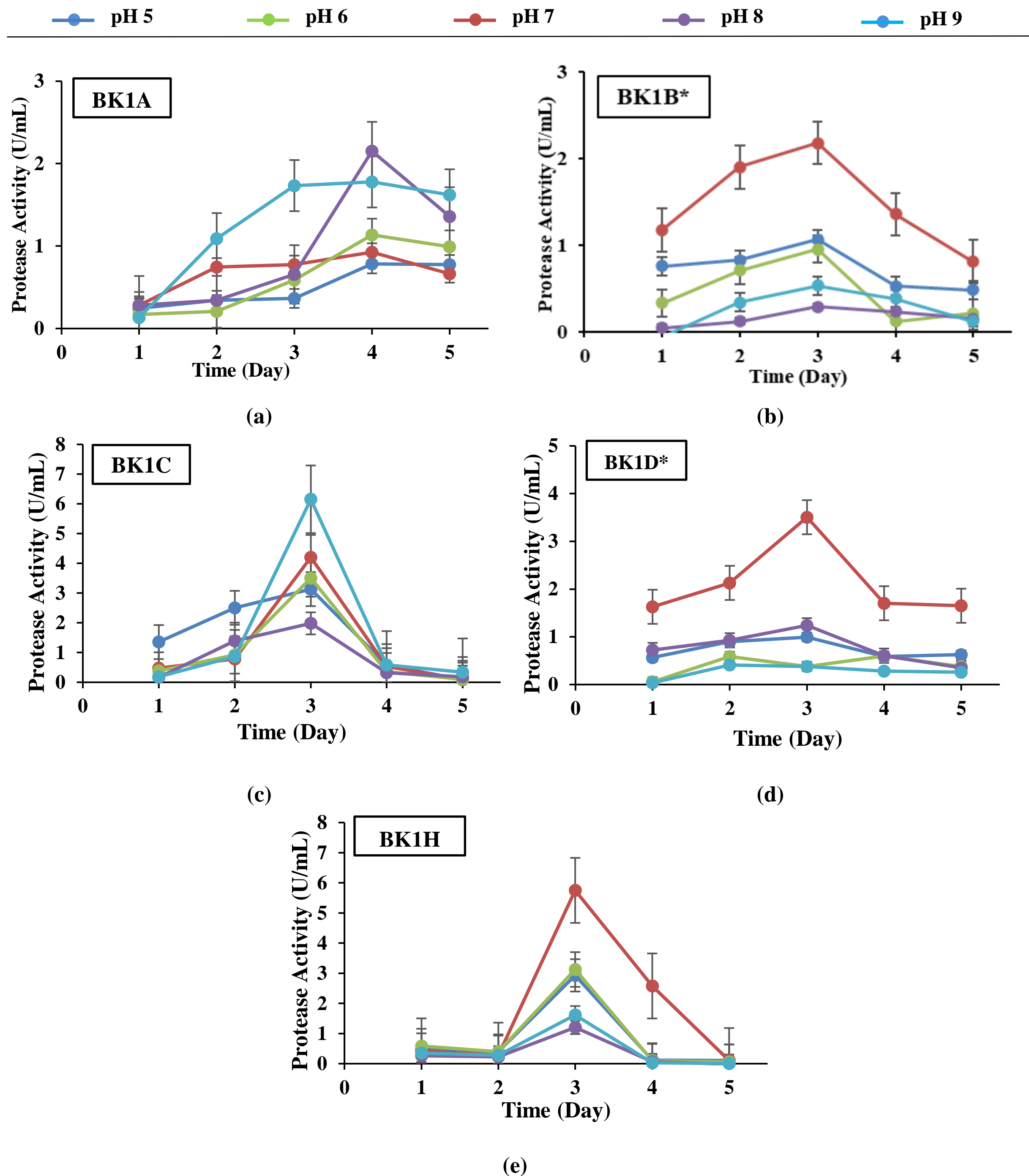

Figure 2. Extracellular protease production curves of each isolate with an incubation period of 5 days and variations in pH of the media with skim milk as substrate. (a) BK1A, (b) BK1B[24], (c) BK1C, (d) BK1D[24], (e) BK1H.

RESULT AND DISCUSSION

Optimization Growth of Isolates

Growth of Isolates on The Variation of NaCl Concentration

Halophilic moderate and light halophilic or halotolerant microbes use the Compatible Solute mechanism by collecting small molecular weight organic solutes in the cell (called compatible solutes) and removing as much salt from the cytoplasm as possible to balance the osmotic pressure [14]. Compatible solutes function as stabilizers that maintain the active conformation of various macromolecules in cells to adapt to extreme environmental conditions [16]. Cellular membranes of halophilic microbes also increase the proportion of phospholipids, thereby helping to maintain the hydration state of the cytoplasmic membrane [17].

Based on the results of observations, isolates BK1A, BK1B, BK1C, BK1D, and BK1H can live and grow optimally at a concentration of 5% NaCl, marked by a significant increase in the OD value of 600 nm and requires a slower lag phase (adaptation) on medium 10% NaCl. In 5% NaCl media all isolates still experienced a stationary phase after incubation for 24 hours. To facilitate the reading of the data, it can be seen the difference in the required phase time of each isolate in Figure 1. The use of a starter can shorten the lag phase, as indicated by the exponential phase that has been started from each BK isolate in the first 2 hours of OD measurement. In 15%, 20%, and 25% media, the OD value is very small and tends to be unstable, it is possible that bacteria are not able to adapt and undergo cell lysis, so they cannot grow and develop on these media.

Based on the data in Table 4.6, it is known that each isolate has a different time in passing through each phase. This also shows the ability of isolates to process the nutrients provided in the media to meet their needs in dividing cells. From the OD measurements made from BK1A isolates, if the measurements were continued at 2, 4, 6, 8, or 10 hours later, BK1A was likely to continue to divide and the OD value in 10% NaCl media could be higher than in 5% NaCl media. Thus, BK1A isolates may have the most optimal salt resistance, but the number of cells is low in LB media. The use of media with suitable substrates may accelerate cell division, thereby increasing the number of cells of BK1A.

Growth of Isolates on The Variation of pH and Incubation Time

Bacteria have different growth capacities in completing each phase of their life, so the optimum time for the production of extracellular proteases from each bacterium is also different. The growth curve in Figure 2, shows that the extracellular protease activity of each isolate (in the optimum pH medium) on the first day increased, which means that the isolates had been able to adapt to the media conditions. The value of extra-cellular protease activity continued to increase significantly until the optimum incubation time of each isolate was reached (day 3 or 4). Then the protease activity decreased drastically on the 4th day (BK1B, BK1C, BK1D, and BK1H isolates) or 5th day (BK1A isolates). This is presumably due to the fact that the nutrient supply in the medium has been exhausted, the presence of toxic compounds resulting from the metabolism of isolates that can interfere with or inhibit cell growth, and some cells may still metabolize and divide, but more viable cells are lost than those obtained. thereby losing viable cells [31]. This situation is called the death phase, so the optimum production time is the best time to harvest extracellular proteases.

In addition to incubation time, the varying pH of the medium can also affect the production of extracellular proteases. The optimum pH of the media of each isolate can be seen in Table 1. Based on the curve in Figure 2, it is known that the highest protease activity was obtained in the medium with the optimum pH. The pH of the culture media and the composition of the media can affect the type and amount of proteases produced [32]. According to Sharma et al. [33], the pH of the medium can affect all enzymatic processes and the transport of various components across the cell membrane. Extracellular proteases have many side groups that can be protonated and deprotonated, so changes in pH can also change enzyme conformation, substrate binding, and the catalytic activity of groups on the active site of the enzyme [32, 34]. According to Mokashe et al. [11], during the protease production process among halophilic microbial cells, pH controls metabolic responses, genetic regulatory mechanisms, product/by-product formation, and nutrient transport across cell membranes and contributes to maintaining active conformation and enzyme stability. So that the enzymes produced by halophilic bacteria may be able to survive in extreme conditions that are not in accordance with their optimum conditions.

Protease Assay

Protease Activity on Variation of pH

The catalytic activity of enzymes is highly dependent on the pH of their environment (medium). Variations in pH result in changes in the ionic form of the active site which affect the reaction rate [35, 36]. The pH value at optimal activity corresponds to the pH when the enzyme's active center presents an adequate (stable) ionic conformation to carry out catalysis with high efficiency [37]. Based on Figure 3 isolates BK1A, BK1C, and BK1D showed optimal activity at pH 9. At pH 8 the extracellular protease activity decreased by 12.010%; 9,590%; 6,440% and pH 10 of 20,460%; 1,520%; 79.210%. Isolates BK1B and BK1H showed optimal activity at pH 8. At pH 7 the extracellular protease activity of isolates BK1B and BK1H decreased by 81.270%; 24,630%; and pH 9 of 84.240%; 2,210%. The high percentage of decreased protease activity of BK1D isolates indicated that the protease had a high sensitivity to changes in pH. Several halophilic/ halotolerant bacterial strains produce alkaline proteases with optimal pH between 8.0 and 11.0 [38]. The extracellular proteases produced by isolates BK1A, BK1B, BK1C, BK1D, and BK1H are basic proteases.

Protease Activity on Variation of Temperature

The next factor that greatly affects the activity of extracellular protease enzymes is temperature. As the temperature increases, the rate of movement of molecules increases and hence the rate of reaction

increases, but at the same time progressive inactivation occurs due to denaturation of the protein enzymes [39]. Because each enzyme is different in structure and the bond between amino acids and peptides, so the optimum temperature and denaturation temperature are specific for each enzyme. Proteases from halophilic/halotolerant microorganisms are active in a wide range (30 to 80°C) [14]. FIG 4. shows the curve of the effect of pH on extracellular protease activity and Table 4.9 shows the optimum pH data for each isolate.

Table 1. Summary of Protease Activity at Optimum pH Media and Optimum Fermentation Time.

Isolate	pH Optimum Media	Optimum Fermentation Time (Day)	Protease Extracellular Activity (U/mL)
BK1A	8	4	2,152 ± 0,021
BK1B*	6	3	2,175 ± 0,301
BK1C	9	3	6,153 ± 0,391
BK1D*	6	3	3,506 ± 0,243
BK1H	6	3	5,750 ± 0,026

*Faridah & Suharti, 2021[24]

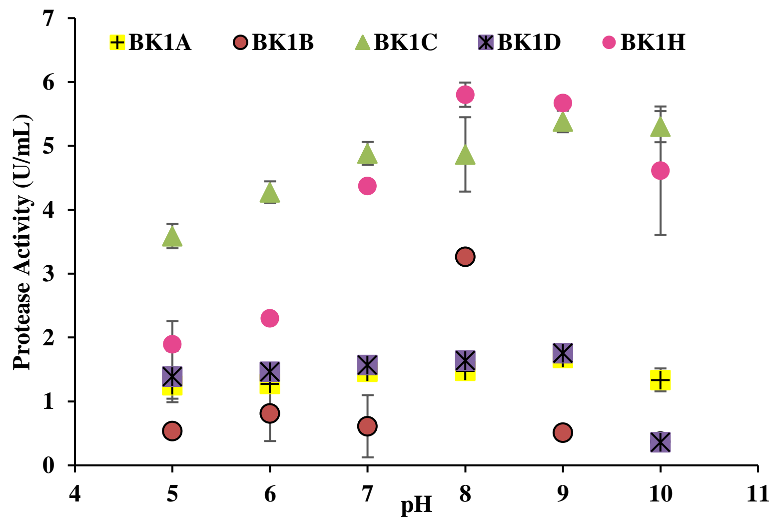

Figure 3. The curve of the effect of pH on the extracellular protease activity produced by each isolate.

Table 2. Data summary effect of pH variation on extracellular protease activity.

Isolate	pH Optimum Protease Extracellular	Protease Extracellular Activity (U/mL)	Decreased Activity at pH (One Level) Below Optimum pH (%)	Decreased Activity at pH (One level) Above Optimum pH (%)
BK1A	9	1,681 ± 0,032	12,010	20,460
BK1B*	8	3,267 ± 0,053	81,270	84,240
BK1C	9	5,383 ± 0,169	9,590	1,520
BK1D*	9	1,756 ± 0,032	6,440	79,270
BK1H	8	5,802 ± 0,190	24,630	2,210

*Faridah & Suharti, 2021[24]

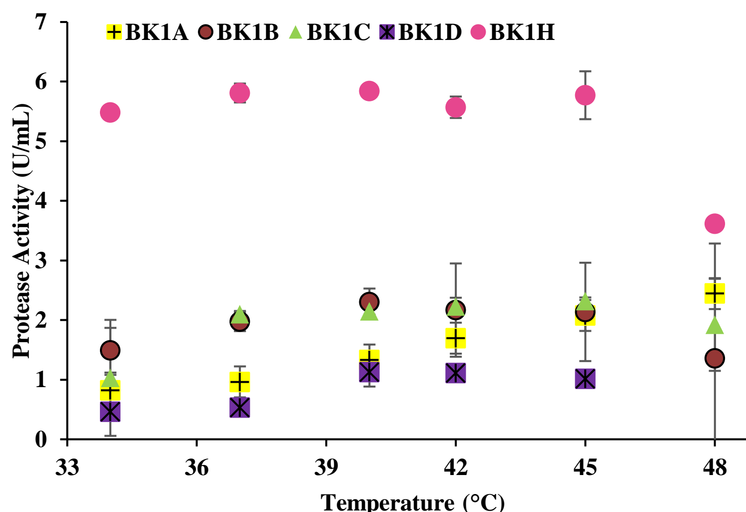

Figure 4. The curve of the effect of temperature on the protease activity produced by each isolate.

Table 3. Optimum temperature and value of extracellular protease activity produced by each isolate.

Isolate	Temperature Optimum Protease Extracellular (°C)	Protease Extracellular Activity (U/mL)	Decreased Activity at pH (One Level) Below Optimum Temperature (%)	Decreased Activity at pH (One level) Above Optimum Temperature (%)
BK1A	52	2,713 ± 0,011	10,03	-
BK1B*	40	2,302 ± 0,227	14,29	5,86
BK1C	45	2,317 ± 0,063	3,88	17,09
BK1D*	40	1,128 ± 0,243	53,06	1,24
BK1H	45	5,772 ± 0,402	3,50	37,31

*Faridah & Suharti, 2021[24]

Based on Figure 4, the extracellular protease activity decreased at a pH above the optimum pH. At 48 °C the extracellular protease activity of BK1A decreased by 10.03%; 2.68%, the measurement was stopped at 52 in this condition the extracellular protease activity of the two isolates was still increasing, so that the optimum temperature of the enzyme was not known. At 37°C the extracellular protease activity of isolates BK1B and BK1D decreased by 14.29%; 53.06 % and at a temperature of 42 5.86%; 1.24%. At 42°C the extracellular protease activity of isolates BK1C and BK1H decreased by 3.88%; 3.50% and at a temperature of 48 17.09; 37.31. TG-4 isolate decreased protease activity at 45 and 52 by 10.19% and 2.08%, respectively.

Protease Activity on Variation of NaCl Concentration

Halophilic and halotolerant moderate groups of bacteria utilize the Compatible Solute survival mechanism in the presence of compatible solutes [14]. Compatible solutes function as stabilizers that maintain the active conformation of various macromolecules in cells to adapt to extreme environmental conditions, so that the extracellular proteases produced by halophilic bacteria have resistance to variations in NaCl concentration [16]. Figure 5 shows the relationship between extracellular protease activity and NaCl concentration.

Based on Figure 5, the protease activity of each isolate had a different effect on the NaCl concentration. BK1B isolate produced unstable protease activity at any concentration of NaCl, at 3% NaCl protease activity reached 4.740 U/mL, but protease activity decreased at 5-10% NaCl, and increased again at 13% NaCl. The extracellular protease activity of isolates BK1D and BK1H was optimum at 10 and 8% NaCl, but their activity did not show a significant increase or decrease in value. The isolates BK1A and BK1C produced

extracellular proteases with the highest activity at 3% NaCl and continued to decrease at 5-13% NaCl. The addition of salt can precipitate enzymes. The precipitation is due to decreased enzyme solubility. The addition of salt concentration contributes to increasing the surface tension of water and induces competition between protein molecules and salt ions for hydration, so that water molecules bound to the hydrophilic surface of proteins will bind to salt [38], called salting out. This situation can cause a decrease in protease activity. Even higher salt concentrations can even cause protein denaturation. However, the enzymes produced by halophilic bacteria require salt to increase their catalytic activity [38]. Salt molecules can stabilize protein molecules by reducing electrostatic energy between protein molecules which increases protein solubility, so that proteins can still work optimally, called salting in. The condition at the optimum NaCl concentration indicates the presence of salting in.

Protease Activity on Variation of Divalent Metal Ion

Metal ions have varying effects on enzyme activity. Some enzymes require metal ions either as catalytic centers (e.g. Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, or Co^{2+}) that are tightly bound as enzyme cofactors or as structural stability to form active conformations (e.g. Na^{+}, K^{+}, or Ca^{2+}) [14,40]. The majority of halophilic/ halotolerant bacterial enzymes require the presence of Na^{+}, K^{+}, or Ca^{2+} to compensate for the excess negative charge oriented due to the characteristic amino acid sequence. FIG 6. shows a diagram of the relationship between divalent metal cations and the extracellular protease activity produced by each isolate.

Based on Figure 6, some isolates produce extracellular proteases with the highest activity on the tested divalent metal cations (Ca^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+}). Still, there are also isolates that produce extracellular proteases that are not affected by the presence of these divalent metal cations. The extracellular protease produced by isolate BK1A only increased in the presence of Mg^{2+} reaching 111.33% and decreased activity in the presence of metal cations Co^{2+}, Mg^{2+}, and Zn^{2+} which indicated an inhibition of enzyme activity. Extracellular proteases from isolate BK1B were not affected by the divalent metal cation but decreased activity on Zn^{2+}. Extracellular proteases from BK1C isolates had the highest activity in the presence of Mg^{2+}, reaching 289.47%. The presence of Ca^{2+}, Co^{2+}, and Zn^{2+} cations also increased the extra-cellular protease activity of BK1C to 249.76 %; 265.90 %; and 248.17%. Extra-cellular protease from BK1D isolate was not affected by the presence of divalent metal cations, the addition of the four divalent metal cations inhibited enzyme activity as indicated by decreased extracellular protease activity. Extracellular proteases from BK1H isolates had the highest activity in the presence of Ca^{2+} reaching 260.00%. The presence of Mg^{2+} increased activity by 126.19%, while in Co^{2+} and Zn^{2+} there was no visible effect.

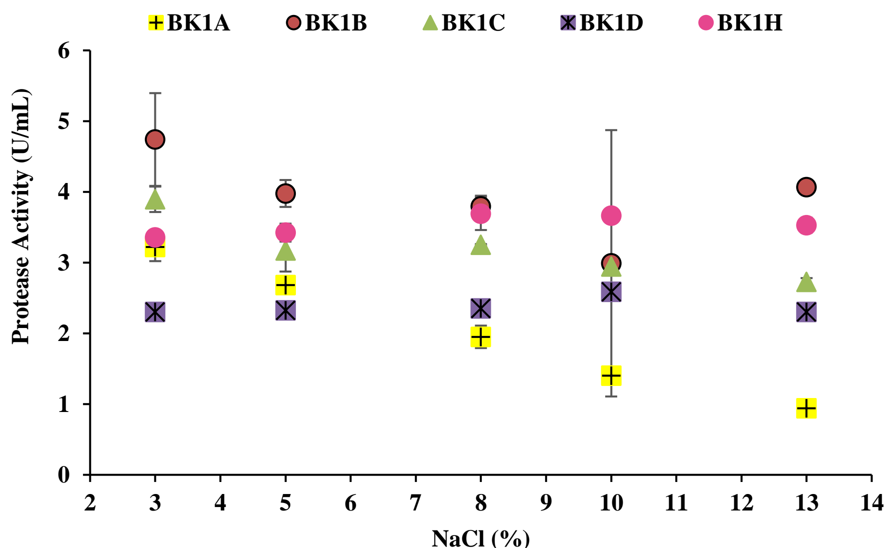

Figure 5. The curve of the effect of NaCl concentration on the protease activity produced by each isolate.

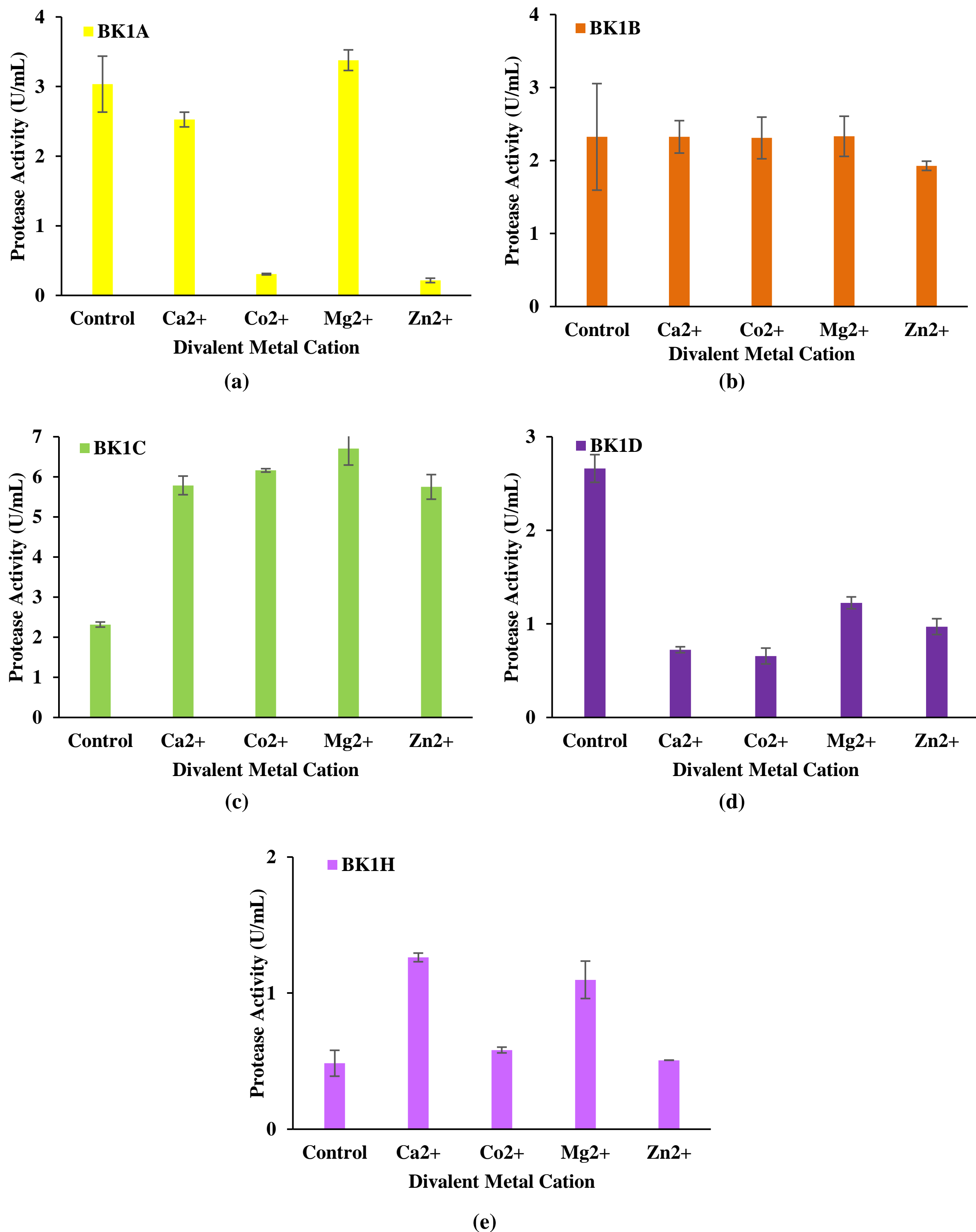

Figure 6. Graph of the effect of divalent metal cations on protease activities produced by each isolate: (a) BK1A, (b) BK1B, (c) BK1C, (d) BK1D, and (e) BK1H.

Potential Isolate in Production of Keratinase with Chicken Feather us Substrate

This test was carried out in the production of extracellular proteases with the pH of the media in accordance with the optimum pH of production, but with the SSF method with chicken feather substrate. Bacteria can produce more than one type of enzyme depending on the nutrients available in their environment. The enzymes produced can be the same group of enzymes, but with different types and functions. Based on the types of proteases, extracellular proteases produced by the obtained isolates may have the character of keratinases that

can degrade keratin. Figure 7 shows the relationship curve between the length of incubation time and the protease activity of each isolate. The extracellular protease profile data produced by each isolate can be seen in Table 4.

Based on Figure 7 and Table 4, each isolate produced the highest extracellular protease activity on the 3rd or 4th day. This optimum time was different from the optimum time of production with skim milk substrate from each isolate. Isolates BK1A produced proteases with the highest activity on day 3, while isolates BK1B, BK1C, BK1D, and BK1H were on day 4. This shows that changes in the substrate can affect the bacteria's survival mechanism. Bacteria will produce enzymes that can process the nutrients provided as a source of C and N to survive, so the enzymes produced must be in accordance with the available nutrients. Keratin is 80% of the protein in chicken feathers [41], so bacteria require keratinase to degrade keratin in chicken feathers. The results of the degradation of chicken feathers by keratinase from each isolate can be shown by the value of % weight loss of chicken feathers after an incubation period of every 24 hours for 5 days. The ability to degrade chicken feathers by each isolate can be seen in the % weight loss. The %weight loss value continues to increase from day 1 to day 5 measurement. This indicates that each isolate has been excreting keratinase to hydrolyze the keratin present in chicken feathers. The data for the 5th day of %weight loss can be seen in table 5.

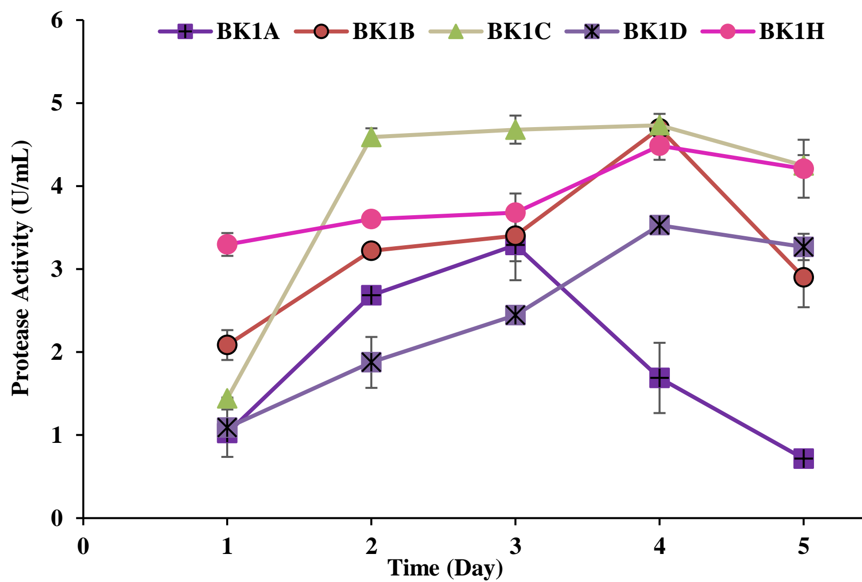

Figure 7. Keratinase production curve of each isolate with an incubation period of 5 days and chicken feather as substrate.

Table 4. The resulting keratinase profile of each isolate.

Isolate	Optimum Fermentation Time (Day)	Protease Activity (U/mL)
BK1A	3	3,289 ± 0,423
BK1B	4	4,695 ± 0,085
BK1C	4	4,732 ± 0,137
BK1D	4	3,528 ± 0,085
BK1H	4	4,486 ± 0,169

Table 5. 5th Day Chicken Feather Weight Loss Data from Each Isolate.

Isolate	*%Weight Loss* 5th Days
BK1A	12,200 %
BK1B	20,900 %
BK1C	12,300 %
BK1D	16,400 %
BK1H	35,800 %

CONCLUSION

Five isolates tested had bacillus cell shape and were moderate halophilic bacteria. These isolates also produced extracellular proteases on skim milk substrate media in the pH range of 6-9 and the optimum fermentation time was 3-4 days. The extracellular proteases produced by these isolates worked optimally in the pH range of 8-9, the temperature of 40-52 °c, and tolerant at 3-10% NaCl. Extracellular proteases from isolates BK1A and BK1C worked optimally in the presence of Mg^{2+}. BK1H extracellular protease activity works optimally in the presence of Ca^{2+}. All isolates produced extracellular proteases that have keratinase activity that can degrade keratin in chicken feathers with an optimum incubation time of 3-4 days, as proven by the % weight loss on day 5 of 12.2%; 20.9%; 12.3%; 16.4%; and 35.8%. Based on these characteristics, these isolates showed a potential to be utilized in producing extracellular proteases that can be used in industrial processes such as the detergent industry which requires alkaline proteases, and leather tanning which requires proteases (keratinase) that are resistant to moderate salinity conditions.

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