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ISOLATION AND SELECTION OF ALKALINE PROTEOLYTIC BACTERIA FROM LEATHER PROCESSING WASTE AND ENZYME CHARACTERIZATION

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ABSTRACT

The aims of this experiment were to isolate alkaline protease producing bacteria from leather processing waste, and to study the biochemical properties of the enzyme produced by the selected bacteria.

Nine bacterial isolates incubated at 37°C, revealed proteolytic activity on skim milk containing media. Four isolates were grown at pH 9 and another four isolates at pH 10 and only one isolate at pH 11. However, in further subculture, there were only three isolates that showed proteolytic activity, namely, D2, D7, and D11. Among the three isolates, isolate D2 was the highest protease producer. The highest protease production (36.5U/L) was reached after a 36-hr fermentation at pH 9.

The optimum activity of D2 protease was observed at pH 8 and 60°C. The enzyme was stable at pH range of 7-10, and at temperature of 52-62°C. In the presence of 5mM EDTA or PMSF, the crude enzyme activity decreased to 7.04% and 25.29% respectively, which indicated that the enzyme might be a metal dependent serine protease. Zymogram analysis revealed the molecular weight of the enzyme was about 42.8kD.

Keywords: *leather / waste / protease / alkaline*

INTRODUCTION

Alkaline protease, along with other alkaline enzymes such as amylase and cellulose, has been used on an industrial scale. Microbial akaline protease is the enzyme that dominate commercial application, mainly for laundry detergents. Alkaline protease with elastolytic and keratolytic activity is used in the tannery industry for dehairing and the bating of skin and hides (Taylor *et al.* 1987). Alkaline protease from *Bacillus* spp. was reported to be used to decompose gelatin on X-ray film for silver recovery (Horikoshi 1996).

Other uses of alkaline protease are for medical purposes, food and chemical industries, as well as in waste treatment plants.

Alkaline protease has been used in industrial scales and has been an attractive option for researchers for the last three decades since the first publication concerning the enzyme by Horikoshi (1971). Studies on exploration of alkaline protease producing microorganisms, which produce novel enzymes have been extensively carried out by researchers all over the world (Banerjee *et al.* 1999; Singh *et al.* 2001; Johnvesly & Naik, 2001; Kanekar *et al.* 2002, Joo *et al.* 2002; Gessesse *et al.* 2002; Moreira *et al.* 2003).

Most of the alkaline protease producing microorganisms were isolated from natural, as well as manmade alkaline environments. Such microorganisms were also isolated from non-alkaline habitats, such as neutral, and acidic soil (Kumar *et al.* 1999).

This experiment aims to isolate alkaline protease producing microorganisms from alkaline waste of leather processing and to study the biochemical properties of the enzyme of the selected isolate.

MATERIALS AND METHODS

Isolation, screening and identification of microbial strains

The samples for screening were taken from the waste of different leather processing steps including soaking (pH 10), liming (pH 13), and deliming (pH 8.5-9.0) from PT. Muhara Dwi Tunggal Laju, and PT. Gunung Putri, in Bogor. The microorganisms were enriched on agar plates containing (gram per liter) 2 gr yeast extract, 0.4 gr $(NH_4)_2SO_4$, 1 gr $MgSO_4.7H_2O$, 0.5 gr $CaCL_2.2\ H_2O$, 0.8 gr KH_2PO_4, 40 gr agar, and 8 gr skimmed milk. The pH medium was adjusted to 8-11 using NaOH. The microorganisms were incubated at 24-40°C for 3 days. The colonies which revealed proteolytic activity were then isolated and screened further, using the same composition of medium. The isolates were screened based on proteolytic index which defined as a ratio of clear zone and colony diameter. The isolates that had the highest proteolytic index were screened further for protease production in Luria broth containing 1% peptone, 0.5 gr yeast extract and 0.5% NaCl at pH 9, 10 and 11. In the same medium composition, they were then incubated in a shaking incubator for 19 hours at 37°C. The fermented broth was then centrifuged at 4000 rpm for 20 minutes. The protease activity was finally assayed and the isolates were selected based on the protease production.

Protease assay

Protease activity was measured using a method suggested by Walter (1984). The method was based on amino acid hydrolyzed by the enzyme and was calculated as tyrosine. One unit of enzyme activity was defined as the amount of enzyme which liberate one micromol tyrosine per minute at certain condition.

Preparation of protease

The selected isolate was cultured in 5 L fermentor containing 2.5 L enzyme media production with the same composition as mentioned above with addition of 0.2% skim milk as an inducer. After a 36-hr incubation at 37°C, the fermented broth was filtered using microfilter, and then concentrated further using 10 kD membrane ultrafilter module (Minitan, Milipore).

Effect of pH and temperature studies

The effect of pH on protease activity produced by the selected isolates was determined at 37°C in 50 mM Tris-HCl buffer at pH 7-8 and in Glycine-NaOH at pH 9 -11.

To determine the effect of temperature on protease activity, the assay was performed in 50 mM Tris-HCl buffer (pH 8) at temperature range of 30-70°C.

Effect of protease inhibitors and metal ions

The concentrated enzyme solution was incubated in 2 and 5mM EDTA or PMSF in 50 mM Tris-HCl at pH 8 for 30 minutes at 5°C. The same concentration of 2 and 5 mM $MgCl_2$, $CaCl_2$, $ZnCl_2$, $MnCl_2$, $FeCl_3$ and $FeSO_4$ were also incubated with the enzyme at 5°C for 30 minutes. Residual activity was measured under optimum pH and temperature. Protease activity assayed in the absence of inhibitors and metal ions was considered as 100%.

Polyacrylamide gel electrophoresis and zymogram analysis

Zymogram method was employed to determine the molecular weight of the protease. The concentrated enzyme solution was loaded into PAGE containing skimmed milk (0.2%) which co-polymerized with 7.5% acrylamide. Electrophoresis was run at 110 V, 90 mA for 1.5 hours. After completing electrophoresis, the gel was soaked in 50 mM Tris-HCl containing 5 mM $CaCl_2$ then incubated at optimum pH and temperature of the enzyme. Visualization of the enzyme staining activity was carried out by staining the gel in Commassie Brilliant Blue solution. Clear band on the skim milk containing electrophoresis gel indicated the protein band which had shown proteolytic activity. The standard molecular weight marker used was HMW protein standard marker (Amersham).

RESULTS AND DISCUSSION

Isolation of protease-producing bacteria

There were 9 isolates which showed proteolytic activity based on clear zone formation on skim milk containing media. Four isolates grew well on media at pH 9m, another four isolates at pH 10, and only one isolate at pH 11. Table 1 shows the physical properties, proteolytic index, and protease activity of the isolates. Proteolytic activity of isolate T4 decreased in the following experiment, and therefore the isolate was excluded. Isolate D2, D7 and D11 were selected based on their proteolytic index and ability to produce protease in submerged culture.

Biochemical characteristics of the selected isolates are displayed in Table 2. Based on their physical, and biochemical characteristics, it can be concluded that the isolates (D2, D7 and D11) most likely belong to *Bacillus* spp.

Table 1. Properties of protease producing isolates

Isolate	Color (Colony)	Gram	Shape (Colony)	Shape (Cell)	Proteolytic index	Activity (U/I)
			pH 9			
M7	orange	+	round	rod	5.33	2.6
M8	white	+	round	rod	3.87	-
M9	yellow	+	round	rod	2.95	2.3
M12	yellow	-	round	rod	3.83	3.3
			pH 10			
D2	orange	+	round	rod	3.67	3.7
D7	white	+	round	rod	12	2.8
D10	yellow	-	round	coccus	3.67	1.5
D11	yellow	+	round	rod	11.08	2.7
			pH 11			
T4	yellow	-	round	coccus	3.69	0.0035

Table 2. Biochemical properties of protease producing isolates

Isolate	Biochemical test							
	Catalase	Nitrit	Urea	MR	VP	Starch	Indol	Carbohydrate fermentation
				pH 9				
M7	+	+	-	-	-	+	-	Acid producer
M8	nd	-	-	-	nd	nd	-	Not detected
M9	nd	+	-	nd	nd	nd	-	Acid producer
M12	+	+	+	-	-	-	-	-
				pH 10				
D2	+	-	+	-	-	-	-	-
D7	+	+	-	+	+	-	-	Acid producer
D10	+	+	+	-	-	-	-	Alkaline
D11	+	-	-	-	+	+	-	Acid producer
				pH 11				
T4	+	+	+		-	-	-	-

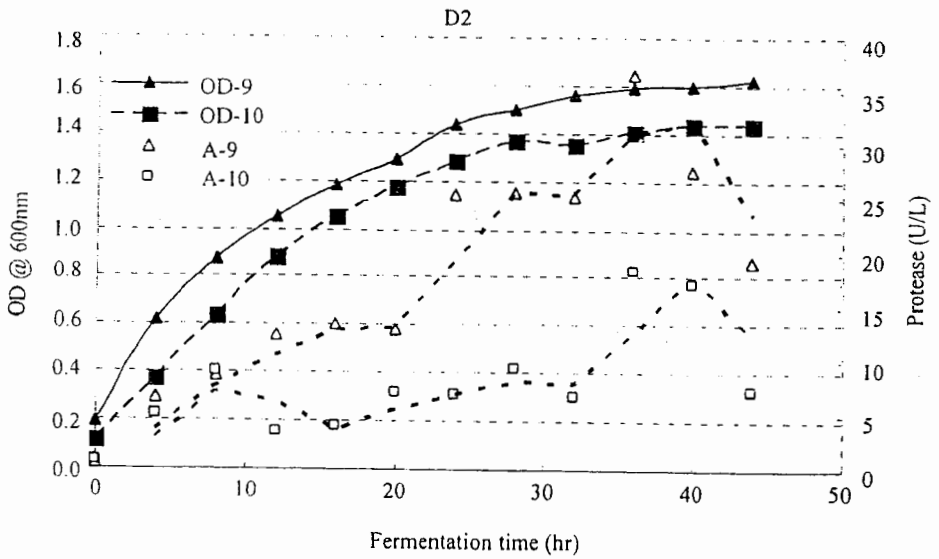
Selection of bacterial strain.

Further selection of the best proteolytic bacteria was based on the productivity of the isolate at the highest pH. All the isolates screened were grown at three different levels of pH (9; 10 and 11). However, all isolates did not grow well at pH

11. Figures 1, 2 and 3 reveal growth and protease production rates of D2, D7, and D11, respectively. The growth rate of isolate D2 was only slightly better at pH 9 as compared to pH 10. However, its protease production rate at pH 9 was higher than that at pH 10. The highest protease production of isolate D2 was at 36-hour fermentation with a protease concentration of 3.65 U/l.

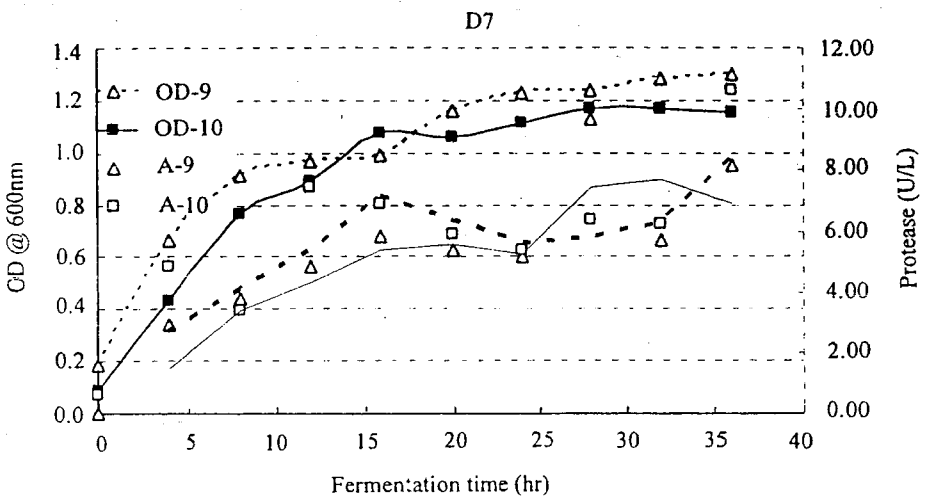
The growth rate of isolate D7 at pH 9 was slightly better than at pH 10. However, the protease production rate was higher at pH 10 after 36 hours incubation with the activity of 10.6 U/l.

Figure 3 shows that the growth rate and protease production of isolate D11 was better at pH 9 and with the highest protease production at 24 hour incubation time with enzyme activity of 5.8 U/l. The protease produced by all isolates in skim milk containing medium was much higher as compared to enzyme produced in Luria broth used in early selection of isolates. The data indicated that the enzyme produced by all isolates observed were inducible ones. Among the three isolates observed, isolate D2 produced the highest protease, which showed that the isolate was the most tolerable to alkaline condition. It was assumed that the enzyme belonged to alkaline protease family. Therefore, the enzyme of the isolate was selected for further study.



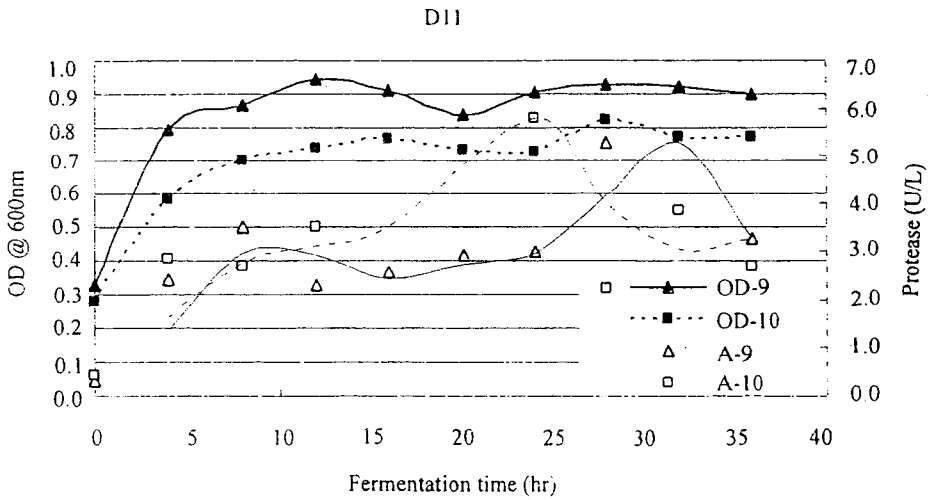
▲ : growth rate at pH 9, ■ : growth rate at pH 10, △: protease production rate at pH 9. □ : protease production rate at pH 10

Figure 1. Growth rate and protease production rate of D2 isolate.



▲ : growth rate at pH 9, ■ : growth rate at pH 10, △: protease production rate at pH 9, □ : protease production rate at pH 10

Figure 2. Growth rate and protease production rate of D7 isolate.



▲ : growth rate at pH 9, ■ : growth rate at pH 10, △: protease production rate at pH 9, □ : protease production rate at pH 10

Figure 3. Growth rate and protease production rate of D11 isolate.

Effect of pH and temperature study on protease activity

Optimum activity of D2 isolate protease was observed at pH 8 and 60°C (Figure 4). The enzyme relative activity was 70% at pH 7 and 9.5 and at 52 and 62°C. These data show that the enzyme is an alkalo and thermotolerant one.

Studies on isolation and characterization of alkaline protease have been on-going for the last three decades (Horikoshi 1996 and Kumar and Takagi, 1998). These are the most recent studies on the subject. Singh *et al.* (2001) reported that optimum activity of *Bacillus* sp. SSR1 was reached at the range of pH 8-11, 40°C, the optimum activity was shifted up to 45°C due to the presence of calcium ions.

Thermostable alkaline protease from *Bacillus* sp. JB-99 reported by Johnvesly and Naik (2001) had an optimum activity at pH 11 and 70°C. This bacterium was isolated from sugarcane molasses at pH 5.8. But with a chemically defined medium, the bacterium was able to produce alkaline thermostable protease.

Gessesse *et al.* (2002) succeeded in isolating two bacterial strains from Lake Abjata (an alkaline soda lake in Ethiopia), using chicken feather meal as the sole source of nitrogen and carbon. The bacteria were identified as *Nesternkonia* sp. AL-20 and *B. pseudofirmus* AL-89. The optimum enzyme activities of AL-20 and AL-89 were observed at pH 10, 70°C and at pH 11, 50°C, respectively. The AL-20 protease was calcium independent whereas the AL-89 was calcium dependent. Both enzymes were indicated as serine protease.

Bacillus. horikoshii protease had optimum activity at pH 9, 45°C, and the enzyme was stable at the pH range of 5.5-12 and temperature up to 50°C (Joo et al, 2002).

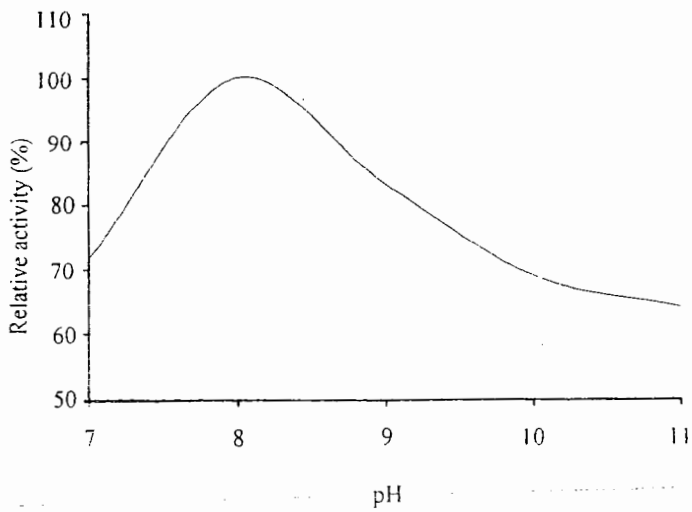


Figure 4. Effect of pH on protease activity of isolate D2

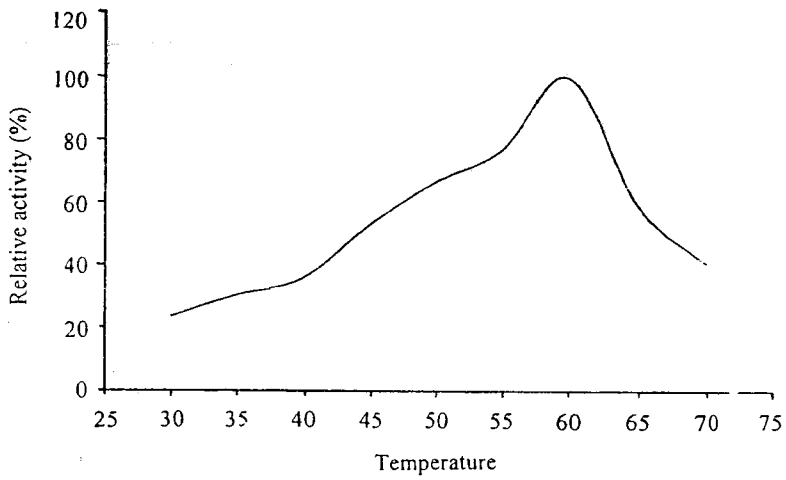


Figure 5. Effect of temperature on protease activity of isolate D2

Effect of protease inhibitor and divalent ions

The presence of 5 mM phenyl methyl sulfonyl fluoride (PMSF) reduced the protease activity down to 23.3% which indicated the enzyme belongs to serine protease. Five mM EDTA almost totally inhibiting the enzyme with a remaining activity of 7% only, which showed that the enzyme activity depends on the presence of metal ion.

The study on the effect of several metal salts revealed that calcium, zinc and manganese ion increased the activity of the enzyme up to 117.2%, 111.3%, and 114.4%, respectively. Whereas, magnesium, ferry and ferrous ions at 5 mM decreased the enzyme activity down to 91.9%, 64.8%, and 71.3%, respectively.

Previous studies on alkaline proteases reported that for maximum activity, some serine alkaline proteases were dependent on divalent ion such as calcium, magnesium and manganese (Kumar & Takagi, 1998). However, Gessesse *et al* (2002) reported that serine alkaline protease activity produced by *Nesternkonia* sp was not dependent on the presence of calcium ion. The enzyme was not affected by PMSF, but, it was inhibited by another serine protease inhibitor 3,4-dichloro-coumarine at concentration of 100 µM,.

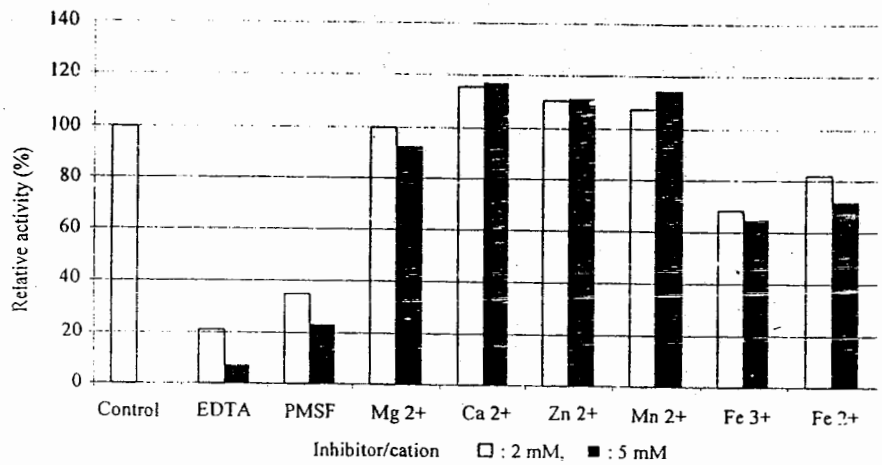


Figure 6. Effect of protease inhibitor and cation on enzyme activity

Molecular weight of the enzyme

Zymogram analysis revealed that the molecular weight of the enzyme was approximately 42.8kD (Figure 7). That molecular weight of most *Bacillus* alkaline proteases reported ranges from 15 to 45 kD (Kumar & Takagi, 1998). A more recent report by Singh *et al* (2001) also stated that the molecular weight of serine alkaline protease produced by *Bacillus sp* SSR1 was 29kD which was also lower than the previous reports.

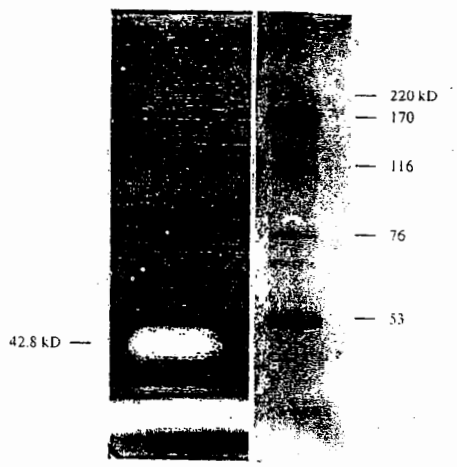


Figure 7. Zymogram of isolate D2 protease

CONCLUSION

In this exploration of protease-producing bacteria from leather processing waste, only few bacteria that produce alkaloprotease were discovered. Among the three isolates screened. Isolate D2 produced the highest protease in submerged culture medium. Based on physical and biochemical properties of the isolate, D2 most likely belongs to *Bacillus* sp. The optimum activity of the enzyme produced was observed at pH 8, 60°C. The enzyme retained 70% of its activity at the range pH of 7-10. At pH 8, the enzyme also retained more than 70% of its activity at the range temperature of 52-62°C. The enzyme was identified as serine protease and its activity was affected by the presence of calcium, zink and manganese ion. The molecular weight of the enzyme as determined using zymogram analysis was approximately 42.8 kD.

Further investigation is needed to identify the isolate using more reliable means such as 16rRNA sequencing

The enzyme can be used in a condition that is slightly alkaline at around pH 8 and at 50-60°C. However, to find suitable application of the enzyme, other considerations have to be taken into account such as type of substrate of the respected process. Hence, it is necessary to investigate different kinds of protein substrates such as hemoglobin, collagen, gelatin, keratin etc. Selecting the best medium to produce the enzyme is also necessary, as well as more thorough characterization of the enzyme such as the stability of the enzyme toward pH, temperature, and some additives that might be used in enzyme application.

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