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Original Article

Test of protease activity on pigeon pea beans (*Cajanus cajan* (L.) Millsp.) fermented by *Aspergillus niger*

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Abstract

The objective of this study is to establish the effect of inoculum concentration and incubation time of protease activity on pigeon pea beans fermented by *Aspergillus niger*. The study comprises two independent variables: the inoculum concentration of *Aspergillus niger*, which consists of four levels (0 mL/g; 0.2 mL/g; 0.4 mL/g, and 0.6 mL/g), and six levels of incubation time (0 h, 24 h, 48 h, 72 h, 96 h, and 120 h). The obtained data were tested using two-way analysis of variance (ANOVA) followed by an LSD test with a significance level of 5%. The results show that the inoculum concentration of *Aspergillus niger* and incubation time do affect the activity of the protease enzyme. The highest enzyme activity of 0.298 U/ml was obtained at an inoculum concentration of *Aspergillus niger* of 0.4 mL/g, with an incubation time of 96 h (P_2K_4).

Keywords: Protease activity, enzyme, *Aspergillus niger*, *Cajanus cajan* (L) Mill.sp.

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Introduction

The advance of fermentation technology, genetic engineering and enzyme technological applications means that the use of enzymes in industry is increasingly widespread, due to their nature as a biocatalyst which is efficient, selective, economical and non-toxic, which catalyzes reactions without side products, and is environmentally friendly (Putri, 2012). One type of enzyme that has very extensive application is the protease enzyme, because of its high economic value. The countries that use these enzymes on a large scale include the U.S., Canada, Germany, the UK, France, Italy, Sweden, Austria, Poland, Russia, China, India, Japan, Indonesia, Thailand, Malaysia, Australia, Brazil, Argentina, Mexico, South Africa, the UAE, Saudi Arabia, Kuwait and Egypt. They are applied, for example, in the leather, textile, food, dairy and pharmaceutical industries, in the processing of industrial waste, and in industrial detergents, known as bio detergents (Nascimento and Martins, 2006). Protease enzymes serve as a booster technology, designed to break down the protein, starch and fat that are often found in food stains, sweat and other common stains. Proteases account for nearly 60% of the industrial market in the world (Adinarayana et al., 2003; Kranthi et al., 2012).

Protease is an enzyme that works to hydrolyse peptide bonds in proteins into oligopeptides and amino acids (Yandri et al., 2010), and can be produced by plants, animals or microorganisms (Putri, 2012). Suri et al. (2013) state that one of the microbes that has been developed commercially as a protease producer is *Aspergillus niger*, Lopes et al. (2011) explain that this is a fungus which has quite good proteolytic ability.

It secretes proteases to degrade the substrate that contains protein. *Aspergillus niger* is a filamentous ascomycota, has hyphae, branches and sects, and is abundant in nature. Fungi can be isolated from soil, plant residue, liquid and solid waste (Pujiati, 2014).

Nuts are full of protein. One type of bean with a high level of protein that can be used as a substrate for *Aspergillus niger* to produce protease is the pigeon pea (*Cajanus cajan* (L.) Millsp.). The utilisation of pigeon peas in society has not been as popular as that of peanuts or soybeans (Primiani, 2017[a]), although the protein in 100 g of pigeon peas is as high as 20.7 g (Direktorat Buakabi, 2013). Components of complex compounds in parts of plants/animals serve as raw materials for pharmaceutical industry development (Primiani, 2017 [b]). An attempt to increase the value of pigeon peas can be made through a process of fermentation that uses the *Aspergillus niger* proteolytic fungus to produce enzymes. Pigeon peas are a potential raw material in fermentation to produce the protease enzyme because their level of protein is quite high. Pigeon pea fermentation produces protease enzyme which is secreted by *Aspergillus niger* to degrade the protein of the substrate. Research conducted by Niu et al. (2011) indicated that the solid fermentation of soybeans by *Aspergillus niger* produces protease, with the highest enzyme activity at 96 h of incubation time.

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Microorganisms will have a growth period of that varies; every microorganism has a growth curve that consists of several phases (Ardhi, 2017).

Method

Sample Collection

Pigeon peas (*Cajanus cajan*) were bought from local markets in Madiun, Ponorogo and Magetan, and then milled in a hammer mill. The strain *Aspergillus niger* was obtained from the culture collection of the Biology Laboratory of Universitas PGRI Madiun, East Java, Indonesia.

Inoculum and Substrate Preparation

A culture was prepared by inoculating 1 loopful of stock culture of *Aspergillus niger* in a medium comprising sterile water 500 mL, glucose 10 g, KH_2PO_4 1.5 g and $MgSO_4$ 0.75 g, followed by shaking by an orbital rotator at 180 rpm for 20 mins. The inoculum incubated for 24 h and then used in the fermentation.

Solid State Fermentation

50 g of well-ground dry substrate (*Pigeon pea*) was placed into 500 mL conical flask and 75 mL of tap water was added; the mixture was then shaken and autoclaved at 121˚C for 20 mins. After cooling, the sterile medium could be used as a substrate. It was inoculated with different inoculum concentrations of 0 mL/g (P_0), 0.2 mL/g (P_1), 0.4 mL/g (P_2) and 0.6 mL/g (P_3) and then shaken in an orbital rotator at 180 rpm for 30 mins. The fermented medium was incubated for different incubation times, namely 0 h (K_0), 24 h (K_1), 48 h (K_2), 72 h (K_3), 96 h (K_4) and 120 h (K_5) (Niu et al., 2011).

Analytical Reducing Sugar Content Method

To analyse the sugar levels in the substrate using the Luff Schoorl method (Kowalski et al., 2013), 5 g of fermented substrate was taken from the flask every 24 h, then 50 mL of distilled water was added and mixed. The suspension was centrifuged at 4000 rpm for 20 mins, and the supernatant used for assaying the reducing sugar content. 10 mL of supernatant was put into a boiling flask, and 10 mL of the Luff Schoorl reagent was then added. The sample was boiled reflux for 10 mins, continued by the careful addition of 6 mL of KI 20% and 10 mL of H_2SO_4. The sample was titrated with $Na_2S_2O_3$ 0.1 N until it became yellow. Amylum 1% was then added and the titration was continued until the blue colour disappeared. Blank titrationwas made by using aquades as substitute of the sample. The reducing sugar content was then calculated based on Kowalski et al. (2013), as follows:

$$\text{Reducing Sugar (\%)} : \frac{AT \times Fp}{\text{weight of sample} \times 1000} \times 100\%$$

where:

AT= Luff Shoorls table values

Fp= dilution factor

The research was conducted to establish the best inoculum concentration, incubation time and their interactions in the production of protease enzyme.

Crude Protease Extraction

The crude enzyme extraction was made by taking 5 g of substrate fermentation of infusion bottle, adding 50 mL of distilled water, and then homogenizing it using an orbital rotator at 200 rpm for 30 mins. The solution was then centrifuged at 4000 rpm for 20 mins. Supernatant was taken to test the activity of the protease enzyme (Niu et al., 2011).

Measuring the Protease Activity

The procedure for measuring the activity of the protease enzyme was conducted following Walter (1984). A total of 0.1 mL of the enzyme solution was inserted into a test tube containing 0.5 mL of 1% casein and 0.5 mL of phosphate buffer (pH 7). The blank and standard treatment, were replaced with distilled water and tyrosine 0.1 M. The solution needed to be incubated at 37 ˚C for 10 mins. The hydrolysis reaction was stopped by adding 1 mL of TCA (trichloroacetic acid) 0.1 M at 40 ˚C then incubated for 10 mins, followed by centrifugation at 10000 rpm for 10 mins (The blank and the standart treatment added 0,1 mL of distilled water). A total of 0.75 mL of the supernatant was added to the test tube containing 2.5 mL of 0.4 M Na_2CO_3; 1.5 mL of Folin reagent Ciocalteau (1:2) was then added and incubated at 40 ˚C for 20 mins. The results of the incubation were measured by a spectrophotometer at 660 nm λ. The protease activity data were calculated based on Walter (1984), as follows:

$$UA = \frac{Asp - Abl}{Ast - Abl} \times P \times \frac{1}{T}$$

where:
UA : unit activity value
Asp : absorbance of sample
Ast : sample absorbance value
Abl : blank absorbance value
P : dilution factor
T : incubation time

Data Analysis

The data were analysed using two-way ANOVA. This analysis was used to determine the effect of inoculum concentration and incubation time on protease activity during the fermentation of pigeon peas (*Cajanus cajan*) by *Aspergillus niger*.

Results

Protease activity is the ability of protease to break down proteins. Protease enzyme in this study was produced by proteolytic mould, that is, *Aspergillus niger* with *pigeon peas* as a production substrate. The study used inoculum concentrations (P) of 0 mL/g, 0.2 mL/g, 0.4 mL/g and 0.6 mL/g, and incubation times (K) of 0 h, 24 h, 48 h, 72 h, 96 h, 120 h. The results can be seen in Tab 1.

Table 1. Average reducing sugar content and protease enzyme activity of fermenting pigeon peas

Treatment	Reducing sugar content (%)	Protease activity (U/ml)
$P_0 K_0$	9.25 ± 0.00	0.011 ± 0.001
$P_1 K_0$	9.25 ± 0.00	0.013 ± 0.002
$P_2 K_0$	9.25 ± 0.00	0.103 ± 0.001
$P_3 K_0$	9.25 ± 0.00	0.121 ± 0.001
$P_0 K_1$	9.25 ± 0.00	0.072 ± 0.003
$P_1 K_1$	9.25 ± 0.00	0.067 ± 0.001
$P_2 K_1$	10.23 ± 0.46	0.117 ± 0.001
$P_3 K_1$	10.23 ± 0.46	0.179 ± 0.001
$P_0 K_2$	10.55 ± 0.00	0.019 ± 0.001
$P_1 K_2$	10.55 ± 0.00	0.081 ± 0.001
$P_2 K_2$	11.85 ± 0.00	0.127 ± 0.001
$P_3 K_2$	11.85 ± 0.00	0.158 ± 0.001
$P_0 K_3$	12.15 ± 0.00	0.023 ± 0.001
$P_1 K_3$	12.50 ± 0.92	0.197 ± 0.003
$P_2 K_3$	12.18 ± 0.46	0.233 ± 0.001
$P_3 K_3$	11.85 ± 0.00	0.254 ± 0.001
$P_0 K_4$	7.98 ± 0.00	0.061 ± 0.002
$P_1 K_4$	7.98 ± 0.00	0.146 ± 0.001
$P_2 K_4$	7.98 ± 0.00	0.298 ± 0.000
$P_3 K_4$	7.98 ± 0.00	0.138 ± 0.001
$P_0 K_5$	7.98 ± 0.00	0.043 ± 0.001
$P_1 K_5$	7.98 ± 0.00	0.09 ± 0.001
$P_2 K_5$	7.98 ± 0.00	0.125 ± 0.001
$P_3 K_5$	7.98 ± 0.00	0.120 ± 0.003

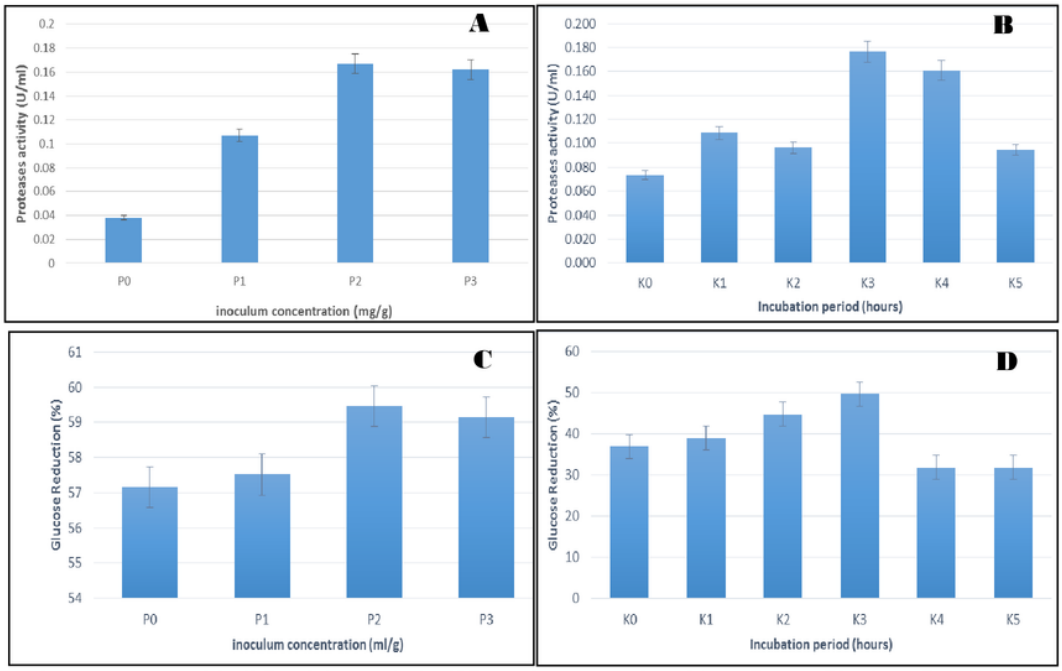

Figure 1. Average protease activity (A,B) and glucose reduction (C,D) at various concentrations of *Aspergillus niger* (P0 = *Aspergillus niger* inoculum concentration of 0 mL/g; P1 = *Aspergillus niger* inoculum concentration of 0.2 mL/g; P2 = *Aspergillus niger* inoculum concentration of 0.4 mL/g; P3 = *Aspergillus niger* inoculum concentration of 0.6 mL/g) at various incubation times (K_0 = incubation period of 0 h; K_1 = incubation period of 24 h; K_2 = incubation period of 48 h; K_3 = incubation period of 72 h; K_4 = incubation period of 96 h; K_5 = incubation period of 120 h

Fig 1A and 1B show that the lowest enzyme activity was at an inoculum concentration of 0 ml/g, tat a level of 0.038 ± 0.001 U/ml. Pigeon peas fermented at an inoculum concentration of 0 ml/g in the incubation periods of 0 h and 42 h had low average enzyme activity, but there was high activity in the incubation periods of 24 h, 96 h and 120 h.

Fig 1B shows that the lowest enzyme activity was in the 0 h incubation period, at 0.073 ± 0.000 U/ml. The incubation period of 0-24 h showed the lowest enzyme activity because this was the adaptation phase (*lag phase*), in which the microbes were still adjusting to the substrate and environmental conditions around them. The incubation time of 24 h had average enzyme activity of 0.109 ± 0.001 U/ml. The incubation period of 24-48 h was the initial growth phase. In this phase, microbe growth was still low due to the recently completed adjustment phase. The average enzyme activity in the incubation time of 48 h was 0.096 ± 0.000 U/ml, while in the incubation time of 72 h it was 0.177 ± 0.001 U/ml. The average enzyme activity in this period was at its highest. This is because the incubation period of 48-72 h was the exponential phase. The measurement of protease activity is achieved from the optical density (OD) of the enzyme activity by hydrolysing casein and also measuring the reducing sugar contents, because glucose was used as energy by *Aspergillus niger* for metabolism and growth (Roosheroe et al., 2006). Reducing sugar content is an indicator of *Aspergillus niger* metabolism and growth. The statistical analysis used two-way ANOVA, a two-way variance analysis, as shown in the variance table in Tab 2.

Table 2. Analysis of varying protease activity during the fermentation of Pigeon peas by *Aspergillus*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.254[a]	23	.011	4.9523	.000
Intercept	.670	1	.670	3.0065	.000
Inoculum concentration (P)	.130	3	.043	1.9394	.000
Incubation time (K)	.067	5	.013	6.0493	.000
Inoculum concentration * Incubation time	.057	15	.004	1.6993	.000
Error	5.3505	24	2.2296		
Total	.924	48			
Corrected Total	.254	47			

Table 3. LSD Test of inoculum concentration

Treatments	P_0	P_1	P_2	P_3
P_0		-.06808*	-.12875*	-.12342*
P_1	.06808*		-.06067*	-.05533*
P_2	.12875*	.06067*		.00533*
P_3	.12342*	.05533*	-.00533*	

P_0, P_1, P_2 and P_3 are the treatments of the inoculum concentration 0 mL/g, 0.2 mL/g, 0.4 mL/g and 0.6 mL/g (* indicates a significant difference from the treatment)

Table 4. LSD Test of incubation time

K	K_0	K_1	K_2	K_3	K_4	K_5
K_0		-.03563*	-.02300*	-.10375*	-.08775*	-.02150*
K_1	.03563*		.01263*	-.06813*	-.05212*	.01412*
K_2	.02300*	-.01263*		-.08075*	-.06475*	.00150
K_3	.10375*	.06813*	.08075*		.01600*	.08225*
K_4	.08775*	.05212*	.06475*	-.01600*		.06625*
K_5	.02150*	-.01412*	-.00150	-.08225*	-.06625*	

K_0, K_1, K_2, K_3, K_4 K_5 are the treatments of the incubation times of 0 hours, 24 hours, 48 hours, 72 hours, 96 hours, 120 hours.(* significant difference)

It was observed that the first variable of the inoculum concentration of *Aspergillus niger* (P) with a 0.05 significance level had a probability value (sig) of 0.000. This shows that sig P (1.9394) signifies that the *Aspergillus niger* inoculum concentration has an effect on protease activity during the fermentation of *Cajanus cajan* by *Aspergillus niger*. The second variable is the incubation time (K), with a significance level of 0.05 and a probability value (sig) of 0.000. This shows that sig K (6.0493) < 0.05 signifies that incubation time has an effect on protease activity during the fermentation of *Cajanus cajan* by *Aspergillus niger*. The interaction between the inoculum concentration of *Aspergillus niger* (P) and incubation time (K) (P * K), with a significance level of 0.05 and a probability value (sig) 0.000. This means that (P * K); 1.6993 <0.05) interaction between the inoculum concentration of *Aspergillus niger* (P) and incubation time has an effect on protease activity during fermentation. The results of the LSD (Least Significant Difference) test to describe the differences between each treatment are shown in Tab 3 and 4.

The LSD test showed that P_0 differed significantly from P_1, P_2, and P_3. The high value difference in the treatment of *Aspergillus niger* inoculum concentration was 0.12875 on treatment, it was P_0 compare with P_2 (Tab 3). Based on the LSD test shown in Table 4, K_0 differs significantly from K_1, K_2, K_3, K_4, and K_5, while K_2 differs significantly from K_0, K_1, K_3, and K_4 but not from K5. Based on Table 4, the biggest incubation time difference is 0.10375 in the K0 treatment with K3.

Discussions

Pigeon peas are rich in carbohydrates and proteins (Saxena et al., 2010), which are nutritious for microbes, which might cause metabolic processes in the fermentation media and produce enzyme protease to degrade proteins in the substrate. Protease enzymes produced by *Aspergillus niger* will hydrolyse soy proteins. The proteins are converted into dissolved proteins, peptides, peptons and amino acids, while the carbohydrates are converted by the activity of the amylases into reducing sugars; subsequently sugar is needed for the moulds to survive (Figs. 1C, 1D).

Inoculum concentration is an important biological factor in fermentation. Fig. 1A shows that protease enzyme activity increased along with increasing inoculum concentration. High concentrations of inoculum contained more spores, so the protein in the substrate was degraded faster and was also related to glucose content. These conditions resulted in rapid growth of the fungus, which then died quickly because the nutrients in the substrate had been completely degraded. The results of the research by Ahmed et al. (2011) showed that the higher the *Aspergillus niger* inoculum concentration, the higher the protease production, but that a further increase in the concentration resulted in a decrease of protease production due to the density of the spores.

The work of Mohanasrinivasan et al. (2012) showed that the protease enzyme which was produced by *Aspergillus niger* increased during under the 96 h incubation period. The average protease enzyme activity in the incubation period of 96 h was 0.161 ± 0.001 U/ml. In this incubation period, the average enzyme activity decreased compared to the 72 h incubation period, but it had the highest activity compared to the whole treatment. Furthemore, the highest protease activity is P_2K_4 (0.298 ± 0.000). The incubation time of 96 h was the *Aspergillus niger* stationary growth phase (*stationary phase*) (Khan et al., 2012). The sugar content also increased until the end of 72 h incubation time and then decreased. The protease activity changed because a lower inoculum level may give insufficient biomass, causing reduced product formation, whereas a higher inoculum level may produce too much biomass, leading to poor product formation (Imandi et al., 2010).

Aspergillus niger is a filamentous fungus that can produce thermostable enzymes of high scientific and commercial value, such as amylases, cellulases, proteases and lipases, by solid state fermentation (Martins et al., 2011). Longer fermentation causes reducing sugar formation, especially at 48 h or 72 h of incubation. The reducing sugar content increased because filamentous fungi were obtained during a log phase, whereas at 24 h of incubation it did not increase because the microorganisms were adapted. Moreover, a longer hydrolysis period will cause the quantity of substrate to decrease because too much of it is hydrolysed. The resulting glucose decrease or remain constant (Kodri et al,. 2013).

The average enzyme activity in the incubation period of 120 h decreased due to the death phase in the growth of the fungus. Mohanasrinivasan et al. (2012) state that after 96 h of incubation, the protease enzyme production by *Aspergillus niger* will decline significantly due to several factors, namely the reduction of nutrients in the growth medium, the aging of the fungus, and the accumulation of toxic products.

Treatment interaction with the highest enzyme activity was P2K4, according to the research conducted by Lopes et al. (2011), which showed that the maximum production of protease enzymes in keratin substrate fermentation by *Aspergillus niger* occurred in the incubation period of 96 h. The results of research by Niu et al. (2011) showed that the highest protease activity of solid fermentation of soybeans by *Aspergillus niger* occurred at an incubation period of 96 h., while Ahmed et al. (2011) found that maximum protease activity was obtained from the solid fermentation of wheat substrate by *Aspergillus niger* with 4 ml inoculum concentrations in 10 g substrate. This concentration was equivalent to the research concentration, which was 20 mL in 50 g of pigeon pea bean substrate. The finding on the incubation time of 96 h with 0.4 mL/g of inoculum concentration was that it was the optimum treatment that can be used to produce the protease enzyme.

These findings show that the P1K3 and P3K3 treatments resulted in the highest activity in the incubation period of 72 h, and subsequently decreased. Sankeerthana et al. (2013) state that the decrease in enzyme activity may be caused by the inactivation of the protease enzyme by other constituents.

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