The effect of coconut water and tofu wastewater as nitrogen source on the production of alkali protease from *Aspergillus flavus* DUCC K225

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Abstract. Protease is one of important industrial enzyme produced by microorganisms, including fungi. *Aspergillus flavus* DUCC K225 is an indigenous mold isolated from lime soil Madura island, has been verified as potential producing protease at pH 8. The production of alkaline protease by *A. flavus* DUCC K225 was studied under submerged fermentation. Molasse and glucose were used to replace sucrose as carbon source. The effect of medium components was observed on the 7th day's incubation, by measuring biomass, protease activity, protein content and protease specific activity at pH 8.5. The examination were done for crude enzyme, 0-20%, 20-40%, 40-60%, 60-80% and 80-100% ammonium phosphate fractionation. The results showed that highest biomass found in glucose medium (14,27 g/L); the highest protease specific activity was found in 60-80% ammonium sulfate fractionation of molasses medium (120,17 U/ml) and 9.4 purification fold.

1. Introduction.
Protease is an enzyme that degrades protein by hydrolysis of peptide bonds to produce amino acids and other smaller peptides [1]. Extracellular proteases have commercial interests and have used in a wide range of industries, such as for protein degradation agent in the production process of various industrial products [2]. Protease can be classified into 3 namely, acidic, alkaline and neutral protease based on the optimum activity pH. Some microbial species can produce more than two kinds of protease, although these may not be generated in the same condition [3]. The needs of commercial proteases relatively high, reaching 70% of all sales of enzymes across industry sectors, such as detergent ‘laundry’, leather processing, food industry, pharmaceutical industry, waste treatment and ‘heavy metal’ recovery [2; 4]. Protease is the most widely produced to meet the needs of industrial detergents, beside of alkaline cellulase, amylase, and lipase which are also added to detergent to enhance its cleaning action [5]. Protease had become one of the components of various detergents[6]. The alkaline protease can be produced by various microorganisms including fungi from the Aspergillus genera, such as *A. niger*, *A. flavus*, and *A. oryzae*, etc. [7]. The research of alkaline protease producing fungi by many researchers has proved that fungi from genus Aspergillus, mainly has the ability to generate extracellular alkaline protease enzyme. The extracellular alkaline proteases from *A. tamarii* that can be used for hair removal on the leather tanning process, at pH 9 had been found [8]. Optimization alkaline protease production from the mutant of *A. flavus* AS2 has been done
with various sources of nitrogen, including casein, yeast extract, urea, soya bean meal. The results indicated that the highest protease activity obtained from soya bean meal and casein media [9]. Research on the exploration of indigenous alkalophilic thermotolerant fungus that produced protease from limestone of Madura island has been done by Rukmi et al. [10] and found that fungal isolate of *Aspergillus flavus* DUCC K225 has the highest protease activity, with specific activity of 54.8 U/mg protein at pH 9 [11]. The purpose of this research was to enhance the enzyme production by nitrogen source modification and examined the alkaline protease activity; thus the potential of indigenous alkaline protease-producing fungus can be developed and implemented to support the social welfare.

2. Materials & Methods

2.1. Fungal strain and culture conditions

The *Aspergillus flavus* DUCC K225 was obtained from the isolation of lime soil from Madura island and used as the enzyme source.

2.2. Preparation of inoculum [12]

Ten ml of sterile distilled water containing 0.1% Tween-80 was added to a 7-day-old fully sporulated fungal culture grown on a CzapekDox agar slant containing (g/l) sucrose, 30; KCl, 0.5; FeSO₄, 0.01; MgSO₄, 0.5; K₂HPO₄, 1.0; and NaNO₃, 2.0 (pH 8.0). The spores were liberated using an inoculation needle under aseptic conditions, and the suspension was diluted with sterile distilled water to a density of 10⁸/ml spores.

2.3. Enzyme production [12,13]

Hundred ml of CzapekDox broth modified medium in250 ml flasks containing (g/l): sucrose 30; KCl 0.5; FeSO₄ 0.01; MgSO₄ 0.5; K₂HPO₄ 1.0; NaNO₃ 2.0; and casein 1% (pH 8.0) were inoculated with a 1% fungal spore suspension. Tofu water waste and coconut water in the concentration of 1% v/v were used as the nitrogen source to replace NaNO₃. The flasks were incubated on a rotary shaker with 120 rpm for 7 days at 30°C. At the end of the incubation, fungal mycelia were removed by filtration through Whatman no. 1 filter paper. The filtrate was centrifuged at 10,000 rpm, and 4°C for 20 min and crude enzyme obtained used as the crude enzyme.

2.4. Ammonium Sulphate Precipitation [14,15,16]

The enzyme was precipitated from the crude enzymet by the gradual addition of solid ammonium sulfate with gentle stirring with the saturation of 0-20%, 20-40%, 40-60%, 60-80%, and 80-100% ammonium sulfate. The suspension was centrifuged at 10,000 rpm for 15 minutes. The precipitate from each ammonium sulfate concentration was collected and, the crude enzymet obtained were further fractionation with higher ammonium sulfate saturation, as mention before. All precipitates obtained after ammonium sulfate fractionation were dissolved in 25 mMNaCl; 10Mm Tris-HCl buffer put in cellophane semi-permeable bag and kept immersed in Tris-HCl buffer for dialysis at 4°C. After dialysis, the enzyme was taken out and centrifuged at 8000 rpm for 15 minutes. The crude enzymet is collected as the pure enzyme after dialysis.

2.5. Enzyme assay [15, 16]

Protease activity was determined by mixing 1 ml of culture crude enzymet with 1 ml 2% casein solution, and the mixture was allowed to stand at 37°C for 10 min. The enzyme reaction was terminated by adding 6 ml 5% trichloroacetic acid, allowed to sit for 10 min, and filtered through Whatman filter paper No. 1. One ml of the filtrate, 3 ml of 0.2 M Na₂CO₃, and 1 ml of 0.5 N Folin phenol reagent were mixed thoroughly and incubated at 37°C for 30 min. The absorbance of the color that developed after the incubation was measured in a spectrophotometer at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine in 20 min at 37°C, expressed as units per μ mole substrate (U/μmole).
2.6. Protein content [13]
The protein concentration of the fungal filtrate was determined by the Lowry method, using bovine serum albumin as the standard.

2.7 The specific activity of protease [15,16]
The specific activity of the fungal crude protease was calculated as a ratio of total protease activity and an estimate of total protein.

3. Results & Discussions
The fungal biomass obtained from three production media were varied at seven days incubation (Fig. 1.). The smallest amount of fungal biomass obtained from coconut water medium, this medium alleged to have had a less balanced nutrition content. The nutrition content of coconut water were 7.3% carbohydrates, and 0.3% protein, while in the standard medium itself contained 30 g/L sucrose. Coconut water will increase the C/N ratio of the medium, that can interfere the fungal growth. The growth and production of microbial enzyme were highly influenced by C/N ratio on the medium [17].

![Figure 1. Biomass of 7 days old of A. flavus DUCC K225 culture in different N-sources media](image)

The alkali protease activity of crude enzyme of A. flavus K225 from 3 kinds of production media, showed that the highest (344.58 U/ml) found in medium containing tofu wastewater as nitrogen source was, followed by medium with coconut water (313.90 U/ml), and the lowest is 7.13 U/ml found in standard medium contain natrium nitrate (Fig.2). The protease activity both in two modified N-sources was increased more than 44 times from that in standard medium (Fig.2.). Study on A. niger found that this fungus produced alkaline protease 325 U/ml in the media containing organic nitrogen sources, i.e., peptone, and casein hydrolysate [19]. The ingredient of liquid tofu waste were 90.72% water, 1.8% protein, 1.2% fat, 7.36% coarse fibre, and 0.32% ash, while the coconut water contained of 91% minerals, 0.3% protein, 0.15% lipid, 7.3% carbohydrate and 1.06% ash [18]. This is indicated that tofu wastewater will give a better nitrogen source needed for enzyme synthesis. Coconut water also improved the nutrition content of the media. From the results of this research, coconut water was suggested for carbon source replacement instead of nitrogen source replacement, due to the higher content of carbohydrate than protein.
Gradual ammonium sulfate fractionation was carried out to purify the crude enzyme from *A. flavus* DUCC K225 submerged culture. All enzyme fractions obtained from gradual ammonium sulfate fractionation of crude enzyme were then examined for their specific activity, which can be indicated the purity of enzyme fraction. Like the protease activity, the highest specific activity found in the 40-60% ammonium sulfate fractionation (Fig. 3). The results showed that the fraction of 40-80% ammonium sulfate precipitation has the highest specific activity (55.1 U/mg) among these three production media, meaning that the highest purity protease alkalies obtained from this fraction. Tofu wastewater contains nutrients that are good for the production of protease enzyme, since the nitrogen content was quite a lot, this compound crucial for protein synthesis, including the enzyme. This finding result was higher compared to the result of research of *A. flavus* AS2 strain protease purification conducted by Rani and Prasad [15] which found that protease specific activity from 70% ammonium sulfate fraction was 2.55 U/mg, whereas the specific activity of the crude enzyme was 0.81 U/mg.

**Figure 2.** Enzyme activity of 7 days *A. flavus* DUCC K225 culture in different N-sources

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Figure 3. Effect of N-source on the specific activity of protease produced by A. flavus DUCC-K225 in 7 days incubation

Figure 4. Effect of N-source on purification fold of protease produced by A. flavus DUCC-K225 in 7 days incubation.

Based on the specific activity observed from crude enzymet and all enzyme fractions obtained from gradual ammonium sulfate fractionation, the purification fold of the alkaline protease from A. flavus DUCC K225 can be detected. The highest purification fold found in 40-60% ammonium sulfate fractionation which is 5.8 times (Fig.4), indicated that this fraction was the purest one. The purer the enzyme, the better enzyme quality produced.

4. Conclusion.
Tofu wastewater and coconut water increased the production of alkaline protease from A. flavus DUCC K225. The purest alkaline protease was obtained from 40-60% ammonium sulfate fractionation, showed 5.8 purification fold and 55.1 U/mg for the specific activity.
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References
[1] Banik M and Prakash M 2006 Ind. J. Biotechnol. 5 380
[2] Khan F 2013 Innov. Res. Chem 1 1
[3] Kladwang W, Bhumirattana A and Hywel-Jones N 2003 Fungal Diversity 13 69
[4] Sangmuang S 2010 Thesis Master of Science Silpakorn University Bangkok Thailand.
[5] Fujinami S and M Fujisawa 2010 Environ.Chem. 31 845
[6] Mikhailova R V 2011 Mikrobiol. Biomek 3 579
[7] Preetha P 2012 Discovery life 1 18
[8] Anandan D, Marmer W N and Dudley R L 2007 J Ind Microbiol Biotechnol 34 339
[9] Rani R M, Prasad N N and Sambasivarao K R S 2012 Asian J. Exp. Biol. Sci. 3 565
[10] Rukmi I, Wuryanti and Trilunggani A 2013 Diponegoro University Research Report Semarang Indonesia 20
[11] Rukmi I, Wuryanti & A Trilunggani A 2014 Proc 9th Join Conf. on Chem. Semarang 408
[12] Charles P, Devanathan V, Anbu P, Ponnuswamy M N, Kalaichelvan P T and Hun B-K. 2008 J Bas. Microbiol 48 347.
[13] Coral G, Arikan B, Unaldi M N and Guvenmez H 2003 Ann Microbiol 53 491
[14] Devi M.K., Banu A R, Gnanaprabhal G R, Pradeep B V & Palaniswamy M 2008 Ind J Sci Technol 1 1.
[15] Rani R M and Prasad N N 2013 Res J Biotechnol 8 58
[16] Sankheerthana C, Pinjar S, Jambagi R T, Bhavimani S, Anupama S, Sarovar B and Inamdar S R 2013 Proc Nat Conf on ‘Women Science & Engineering. Dharwat. India. (http://inpresco.com/category/ijcet.).
[17] Sahib A 2009 Iraqi J. Sci., 4 476
[18] Trismilah 2001 Proseding Seminar Keanekaragaman Hayati dan Aplikasi Bioteknologi Pertanian. BPPT Jakarta 110