The production of alkaline protease from Aspergillus flavus DUCC K225 on rice bran containing medium

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Abstract. Alkaline protease is an industrially important enzyme that can produce by Aspergillus flavus DUCC K225 an indigenous mold from lime soil Madura island. The production of alkaline protease by Aspergillus flavus DUCC K225 produced by submerged fermentation on modified Czapeks Dox medium containing rice bran as N source. The enzyme production was observed after 7th-day incubation, by measuring the protease activity at pH 8.5 and the temperature stability as well. The results showed that the enzyme activity is higher on the rice bran medium compared to the standard medium, with a value of 237.84 U/ml and 94.85 U/ml respectively. This alkaline protease enzyme produced also thermostable, with 89.3% stability value for 60 min at 40°C.

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 agents in the production process of various industrial products [2]. The alkaline protease is a protease that has the optimum activity at high pH. According to the published data of the market research in 2014 the needs of commercial proteases relatively high, reaching 60% of global sales of enzymes [3] and the forecast annual growth was expected to 7.8% during 2015-2020 [4]. The increasing needs of protease due to the rapid growth of the enzyme demand in the pharmaceutical industry, bioethanol processing, food processing, fine chemical industry, waste treatment and ' heavy metal ' recovery [2, 5]. Protease has currently 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 [6,7]. The protease production is greatly affected by the nutritional and environmental conditions. Studies on alkaline protease producing fungi have been conducted during the last decades, the results showed that some species of genus Aspergillus fungus can produce extracellular alkaline protease enzyme. The Aspergillus tamarii which produced extracellular alkaline proteases active at pH 9 and used for hair removal on the leather tanning process had been found [8]. On the optimization of alkaline protease production from a mutant of Aspergillus, flavus AS2 found that the highest protease activity obtained from the fermentation media containing organic nitrogen sources soya bean meal and casein media comparing to inorganic nitrogen source [9]. The nitrogen source for fungal growth can be provided in inorganic or organic form. This component needed for microorganism metabolisms especially in protein synthesis, such as enzyme productions. The cheap nutritional sources for the fermentation industry is important to establish an
economic fermentation industry. Rice bran can be one of some cheap natural nitrogen sources with continuous availability. The rice bran is a by-product of paddy or rice (*Oryza sativa* L.) milling process nearly 8% d/w is obtained from dried milled grain, it is rich in essential nutrients. Rice bran contains some valuable micronutrients like oryzanols, tocopherols, tocotrienols, phytosterols which comprise vitamin E and exhibit significant antioxidant activity.

It also contains 20% oil, 15% protein, 50% carbohydrates (mainly starch), dietary fibers like pectin, beta-glucan and gum [10]. Based on its nutrient content the rice bran supposed to be used as a nitrogen source for microorganism growth. The majority of Indonesian people consumed rice every day as a staple food. The rice production in Indonesia in 2015 is 75.36 million tons of dried milled grain (GKG) and continues to increase by approximately 4% per year, the rice bran obtained is about 8-10% w/w [11] hence in Indonesia the production of rice bran is abundance throughout the year and mainly used as animal feed. The purpose of this research was to examine the effect of rice bran in alkaline protease production from indigenous *Aspergillus flavus* DUCC K225, by determining the alkaline protease activity, the thermostability and the compatibility to local commercial detergents, thus the potential of this indigenous alkaline protease-producing fungus can be developed further and implemented to support the human welfare.

2. Materials and methods

2.1. Fungal strain and culture conditions.

The fungus *Aspergillus flavus* DUCC K225 was obtained from Diponegoro University Culture Collection, the fungus maintained on Potato Dextrose Agar slant.

2.2. Preparation of inoculum [12]

The spore inoculum prepared by culturing *Aspergillus flavus* DUCC K225 on 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) for 5 days. Ten ml of 0.1% Tween-80 in sterile distilled water was added to the culture and spores were liberated using an inoculation needle under aseptic conditions. The spore density adjusted to 108/ml spores.

2.3. Enzyme production [12,13]

The production of alkali protease on standard medium was done as follow: hundred ml of CzapekDox broth modified medium in 250 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) for 5 days. Ten ml of 0.1% Tween-80 in sterile distilled water was added to the culture and spores were liberated using an inoculation needle under aseptic conditions. The spore density adjusted to 108/ml spores.

2.4. Enzyme assay [14,15]

To examine protease activity 1 ml of culture supernatant added with 1 ml 2% casein solution, allowed to stand at 37°C for 10 min. The reaction was terminated by adding 6 ml of cold 5% trichloroacetic acid, incubated 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. Measured the absorbance of the color developed with a spectrophotometer at 660 nm. One unit of protease activity 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).
2.5. *Protease assay* [13,14]

The assay for protease activity was measured by a modified method of Keay et al. using casein as a substrate. One ml of culture supernatant was mixed thoroughly with 1 ml of 2% of casein solution, incubated at 37 °C for 10 min and the reaction was stopped by adding 2 ml of 0.4 M trichloroacetic acid and incubated for 20 min at 37 °C. The solution filtered using Whatman No. 1 filter paper. One ml of filtrate then mixed thoroughly with 5 ml of 0.4 M Na₂CO₃ and 1 ml of 0.5 N Folin phenol reagent, incubated at 37 °C for 20 min. The absorbance of the final solution was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine in 20 min at 37 °C.

2.6. *Thermostability assay* [16,17]

The enzyme thermostability examined by measuring the enzyme activity at various temperatures with an interval of 15 minutes at pH 9.8. Thermal stability determined by incubating the mix of 2 ml of culture supernatant and 2% casein solution for 60 minutes at 29, 40, 45, 50, 55, and 60°C. The non-heated enzyme was used as a control.

3. Result and discussion

3.1. *Enzyme production on rice bran medium*

The production of alkaline protease by submerged fermentation at 7 days incubation is higher on the rice bran containing medium than on standard medium, with a value of 237.84 U/ml and 94.85 U/ml respectively. A similar finding was reported by Chutmanop et al. that the protease production from *Aspergillus oryzae* obtained from rice bran in solid-state fermentation is higher than wheat bran about 1400 U g⁻¹ dry solids compared with about 1000 U g⁻¹ dry solids [18]. The replacement of natrium nitrate as nitrogen source in the standard medium with rice bran seems to provide better nutrition content not only for nitrogen source but carbon source as well because the rice contains 12.32% d/w protein and 17.92% d/w digestible carbohydrate [19], this two macronutrients provide better nutrition for the *A. flavus* DUCC K225 growth and also enzyme production. This probably explains the better enzyme production on rice bran. Similar findings were reported by Ikasari and Mitchell for enzyme production during fermentation of various solid substrates using the mold *Rhizopus oligosporus* [18].

3.2. *Effect of temperature on protease activity*

Temperature is a critical factor for maximum enzyme activity and industrial the activity and stability of the enzyme at higher temperatures is a prerequisite [20]. The protease production of *A. flavus* DUCC K225 can be observed by examined the enzyme activity. To obtain the optimum temperature of the *A. flavus* DUCC-K225 alkaline protease activity, the measurement was conducted at various temperatures (Figure 1.). The highest protease activity obtained at 40°C which is statistically at par with those at 45°C with a value of 297.64 U/mL and 294.49 U/mL respectively, while at 29, 55 and 60°C were significantly different. This result showed that the protease produce by *A. flavus* DUCC K225 is a thermostolerant enzyme, which maintains its structural integrity at above 40°C [21]. The thermostable alkaline protease produced by an *Aspergillus niger* strain has been reported stable at 40°C [14], while alkaline protease from *A. flavus* AS2 stable at 55°C [13].

![Figure 1. The alkaline protease activity of *A. flavus* DUCC-K225 at different temperatures](image-url)
Remarks: the same superscripts show no significant difference (p<0.05)

3. Thermostability of alkaline protease
The thermostability of A. flavus DUCC-K225 protease has been examined at 40°C as optimum temperature obtained from the previous examination of temperature effect on protease activity. The enzyme activity decreased along with the increase of incubation time. The result showed that the enzyme remains active for 60 minutes incubation with the retained activity value of 89.43% (Fig. 2.).

Figure 2. The stability of alkaline protease produced by A. flavus DUCC-K225 at 40°C

This result also supported the previous indication that the alkaline protease produced by A. flavus DUCC-K225 was thermostable. The previous study on the alkaline proteinases produced by Aspergillus fumigatus Fresenius TKU003 and Aspergillus terreus found similar results that are thermostable at 50°C and 60°C respectively [22]. The thermostable properties of alkaline protease produced by A. flavus DUCC-K225 make the possibility to utilize it for industrial purposes.

4. Conclusion
Rice bran can be used as a nitrogen source in the production of alkaline protease from A. flavus DUCC-K225. The enzyme was thermostolerant, the activity kept up to 60 minutes at the optimum temperature of 40°C.

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