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Thermostable Alkaline Protease Activity from *Aspergillus flavus* DUCC- K225 and Its Compatibility to Local Detergents

ALMALINA NABILA SULISTYO RINI, ISWORO RUKMI*, AND SRI PUJIYANTO

Department of Biology, Faculty of Science and Mathematics, Universitas Diponegoro
Jl. Prof Soedharto, SH, Tembalang, Semarang 50275, Indonesia.

Protease important enzyme in many industries, including detergent. *Aspergillus flavus* DUCC-K225 is a thermotolerant indigenous molds isolated from Madura island which is potential in producing thermostable alkaline protease enzymes. The enzyme produced by submerged culture on modified Czapeks Dox liquid medium containing glucose as carbon source and 1% of casein. The aims of this study were to determine the activity and stability of thermostable alkaline protease produced by *A. flavus* DUCC-K225 at various temperatures, also the compatibility to 5 local detergents. Research were conducted using Completely Randomized Design in triplicate, with temperature variation for protease activity as treatment. The results showed that the highest activity of thermostable alkaline proteases was 214.503 U/mL, with retained activities up to 78% in 60 minutes at 55°C. This enzyme compatible with 5 local detergents tested, with the retained activity varied 59.48%-99.48% at 29°C and 62.83%-98.05% at 55°C. The compatibility to all detergent tested were confirmed by the increament of blood solubility.

Key words: alkaline protease, *A. flavus* DUCC-K225, compatibility, detergent, thermostable

Protease merupakan enzim yang penting untuk industri, termasuk industri deterjen. *Aspergillus flavus* DUCC-K225 merupakan kapang termotoleran indigenous dari Pulau Madura, yang berpotensi menghasilkan enzim protease alkalis termostabil. Produksi enzim dilakukan dalam kultur terendam pada medium Czapexs Dox broth yang mengandung glukosa sebagai sumber karbon, dan kasein 1%. Tujuan dari penelitian ini untuk mengetahui aktivitas dan stabilitas ensim protease alkalis yang dihasilkan oleh *A. flavus* DUCC-K225 pada berbagai suhu, serta kompatibilitasnya terhadap 5 jenis deterjen lokal. Hasil yang diperoleh menunjukkan bahwa ensim tetap stabil pada suhu 55°C yang menunjukkan bahwa ensim ini termostabil, Aktivitas tertinggi ensim ini adalah 214.503 U/mL, dengan aktivitas yang dipertahankan sebesar 59.48%-99,48% pada suhu 29°C dan 62.83%-98,05% pada suhu 55°C. Ensim ini kompatibel terhadap semua deterjen yang diujikan, karena dapat meningkatkan kelarutan noda darah pada kain.

Kata kunci: *A. flavus* DUCC-K225, deterjen, kompatibilitas, protease alkalis, termostabil

Protease (EC 3.4) is an enzyme that able to hydrolyze the peptide bonds between amino acids in proteins (Nallusami, Remya & Al-Bahri 2009). The availability of proteases is important for several industries, especially the proteases which are stable at high temperature and pH According to Gupta *et al*. (2002) and Chimbekujwoa *et al*. (2020) some industries need thermostable alkaline proteases which is active at pH 8-12 and temperatures of 50°C-70°C. The presence of alkaline proteases is one of the 3 major groups of enzymes needed in industrial processes, which have a contribution of 60% in the sale of enzymes worldwide (Sahib 2009; Lanka, Anjali, & Pydipalli 2017, Chimbekujwoa *et al*. 2020). Protease enzymes can be obtained from various sources such as plants, animals and microorganisms that are widespread in nature, including in extreme environment (Coral *et al.* 2003). Some microorganisms have the potential to produce thermostable alkaline proteases, among others are fungi that can produce many kinds of extracellular enzymes. The enzyme production from fungi has several advantages, because the enzymes are more easily obtained in submerged fermentation, easily separated from the medium, and can be grown on a cheap medium (Souza *et al.* 2015; Soares *et al. 2*010). According to Souza *et al.* (2015) the mold of the genus *Aspergillus* widely known as a largest producer of alkaline proteases, such as *Aspergillus flavus* which has been known as a species that generally has a high ability in producing extracellular protease. The production of microbial enzymes were influenced by the medium composition, while the enzyme activity affected by the incubation temperature (Packianathan *et al.* 2008). The aims of this study were to examined the production of thermostable alkaline protease from

*Corresponding author: Phone: +62-811288428; Fax: +62-;
E-mail: mgisworo rukmi@lecturer.undip.ac.id

indigenous thermotolerant fungi *Aspergillus flavus* DUCC-K225 isolated from lime soil in Madura Island, East Java using glucose as carbon source and examined the effect of temperature on the enzyme activity, as well as the enzyme compatibility to local detergents.

MATERIALS AND METHODS

Culture Maintenance and Inoculum Preparation. The mold *A. flavus* DUCC-K225 was maintained on PDA Agar slant, and sub cultured before used in the study. The spore inoculum was prepared by adding sterile distilled water containing 1% of Tween 80 into 5 days old mold culture on PDA (Coral *et al.* 2003).

Enzyme Production. The fungal alkaline protease from *A. flavus* DUCC-K225 produced according to Coral *et al.* (2003) and Packianathan *et al*. (2008) by submerged fermentation in the modified Czapeks Dox medium composed of 30 g/L glucose; 0.5 g/ L KCl; 0.01 g /L $FeSO_4$; 0.5 g/L $MgSO_4$; 1 g/L K_2HPO_4; 2 g/L $NaNO_3$, and 1 % casein. The media pH was arranged at 9. One percent fungal spores suspension of *A. flavus* DUCC-K225 (10^8/ml) inoculated into the medium, the flasks were then incubated on rotary shaker with 120 rpm for 7 days at room temperature. The fungal cultures filtered using Whatman paper. No. 1, the supernatant obtained centrifuged at 4000 rpm for 20 minutes and use as crude enzymes.

Protease Assay. The protease activity was measured by modified method of Keay *et al*. (1970 *in* Coral *et al. 2*003; Rani & Prasad 2013; Chimbekujwoa 2020) using casein as substrate. One ml of culture supernatant was mixed thoroughly with 1ml of 2% of casein solution, incubated at 37°C for 10min and the reaction was stopped by adding 2ml of 0.4M trichloroacetic acid and incubated for 20min at 37°C. The solution filtered using Whatman No. 1 paper. One ml of filtrate then mixed thoroughly with 5ml of 0.4M Na_2CO_3 and 1ml of 0.5N Folin phenol reagent, incubated at 37°C for 20min. The absorbance of the final solution was measured at 660nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine in 20min at 37°C.

Thermostability Assay. The enzyme thermostability examined according to Kamoun *et al.* (2008) by measuring the enzyme activity for 1 hour at various temperatures with interval of 15 minutes at pH 9.8. Thermal stability of enzyme was determined by incubating 2 mL of culture supernatant mixed with 2% casein solution for 60 minutes at 29°C, 40°C, 45°C, 50°C, 55°C, and 60°C. The unheated enzyme was used as control.

Compatibility to Commercial Detergents. The alkaline protease of *A. flavus* DUCC-K225 was tested for its compatibility to 5 commercial detergents i.e. B, D, S, R, and J, according to Choudhary (2012). A detergent solution of 0.7g/100mL were heated at 100°C for 1 hour, to destroy indigenous protease that might be present. The detergent solution mixed with the culture supernatant in a ratio of 1:1 (v/v) incubated at 40ºC for 20 min. The residual protease activity examined using standard assay procedure as mentioned previously. A mixture of culture supernatant and tap water (1:1) used as control 100%. The relative enzyme activity was expressed as percentage activity considering the activity of control. The enzyme activities were examined in 3 treatments, i.e. 0.3mL of culture supernatant mixed with 5.7mL of detergent solution (0.7g/100mL); 0.3mL of detergent solution (0.7g/100mL) mixed with 5.7mL of distilled water; and 0.3 mL of culture supernatant mixed with 5.7mL of distilled water. The enzyme assays conducted at room temperature (29°C) and the optimum temperature of protease activity which previously obtained (55°C).

Cleansing Power as Additive in Local Detergents. The cleansing power of the enzyme carried out based on Niyonzima & More (2015) by soaking a piece of 4x4cm square white cloth which have been stained with blood in 4 solutions, containing (1) 50mL of distilled water, 1mL of culture supernatant and 1mL of detergent solution (0.7g /100 mL) which has been heated before at 100°C for 1 hour; (2) 51mL of distilled water and 1 mL culture supernatant; (3) 51mL of distilled water and 1mL of detergent solution (0.7g /100mL) heated at 100°C; and (4) 52mL of distilled water as control. The bloodstained clothes were soaked in the solution at room temperature (29°C) and 55°C for 10 minutes. The clothes were then dried and visually observed for the stain removal. The blood solubility in the soaking solution were also observed by measuring the absorbance at λ_{200}.

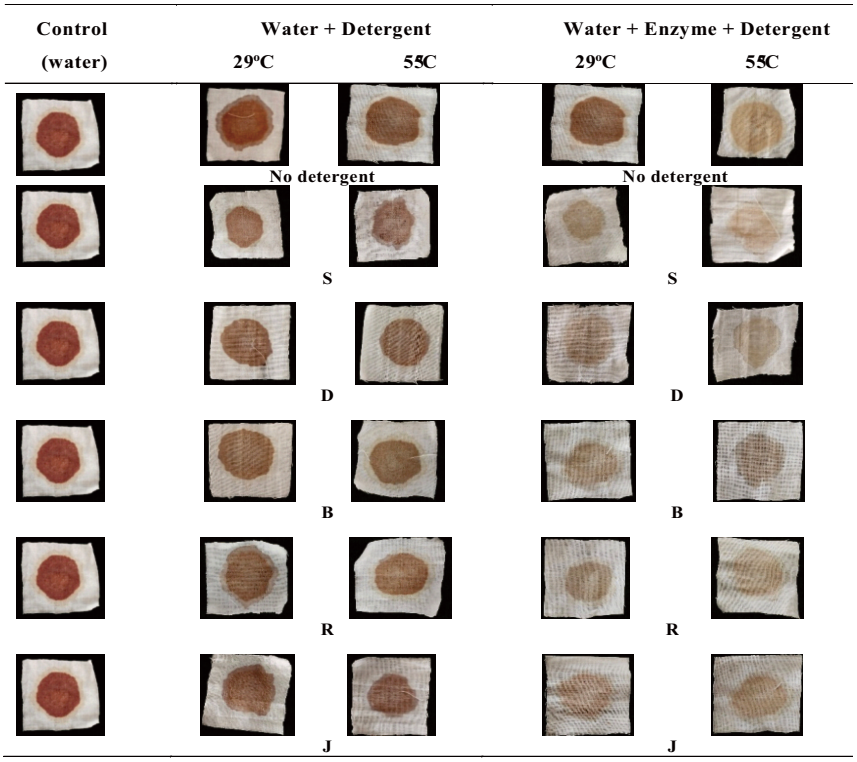
RESULTS

Effect of Temperature on Protease Activity. The protease activity examination at various temperatures carried out to obtain the optimum temperature of *A. flavus* DUCC-K225 protease. Figure 1, showed that the activity of alkaline protease increased along with the temperature increment. The highest protease activity reached at 55°C with the value of 214,50U/mL,

Table 2 The compatibility of *A. flavus* DUCC-K225 alkaline protease to various detergents

Detergents*	Protease activity (U/mL)		Retained activity (%)	
	29°C	55°C	29°C	55°C
Enzyme	66.76 ± 1.46	111.77 ± 2.70	100.00	100.00
B	61.55 ± 2.83	102.23 ± 1.33	92.19	91.47
D	57.01 ± 1.29	99.42 ± 2.19	85.39	88.96
J	48.11 ± 1.78	85.43 ± 2.83	72.07	76.44
R	39.71 ± 2.70	70.22 ± 3.10	59.48	62.83
S	66.41 ± 2.66	109.58 ± 1.66	99.48	98.05

Table 3 The compatibility of *A. flavus* DUCC-K225 alkaline protease to various detergents



although it is statistically at par with 50°C and 60°C.

Thermostability of Alkaline Protease. The enzyme thermostability have been examined at the optimum temperature of 55°C, which obtained from previous examination of temperature effect on protease activity. The result showed that the enzyme was still active for 60 minutes with retained activity value of 77.8 – 78.8% (Fig 2).

Compatibility with Detergents. The detergent compatibility test was carried out at room temperature (29°C) and 55°C to determine the ability of *A. flavus* DUCC-K225 thermostable alkaline protease as additive for commercial detergents. The enzyme compatibility to detergents determined by the retained activity value of enzyme in the detergent solutions. Table 2 showed that the retained activity of the enzyme in the detergent solution were varied. in three commercial detergents, which is in D, J and R were slightly higher at 55°C than at 29°C, while for B and S detergent were slightly higher at 29°C than at 55°C (Table 2), but not different significantly.

Cleansing Potential as Detergent Additive. The effect of alkaline protease produce by *A. flavus* DUCC-K225 on detergent's cleansing power was carried out by soaking the blood stained fabrics in the detergent solution mixed with enzyme at two different temperature. The visually examination found that the enzyme increased the detergent's removing power of the blood stain from the fabrics (Table 3). The measurement of soaking solution's absorbance at λ_{200} were carried out to ensure that the blood dissolved to the water. The obtained of absorbance values were higher at 55°C than at 29°C indicated that more dissolved blood occurred at higher temperature (Fig 3). The highest absorbance showed on the enzyme and S detergent mixed solution, this result in line with the

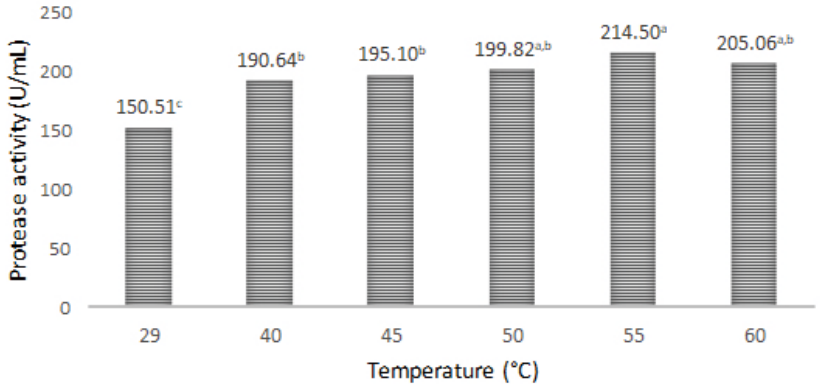


Fig 1 The effect of temperature on the alkaline protease activity of *A. flavus* DUCC-K225.
Remarks: the same superscripts show no significantly difference ($p<0.05$).

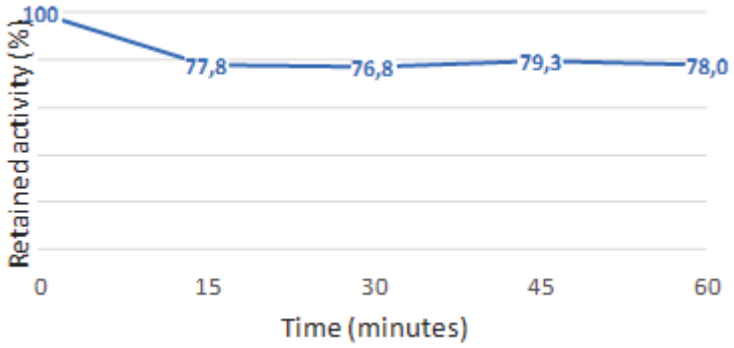


Fig 2 The stability of alkaline protease enzyme produced by *A. flavus* DUCC-K225 at 55°C.

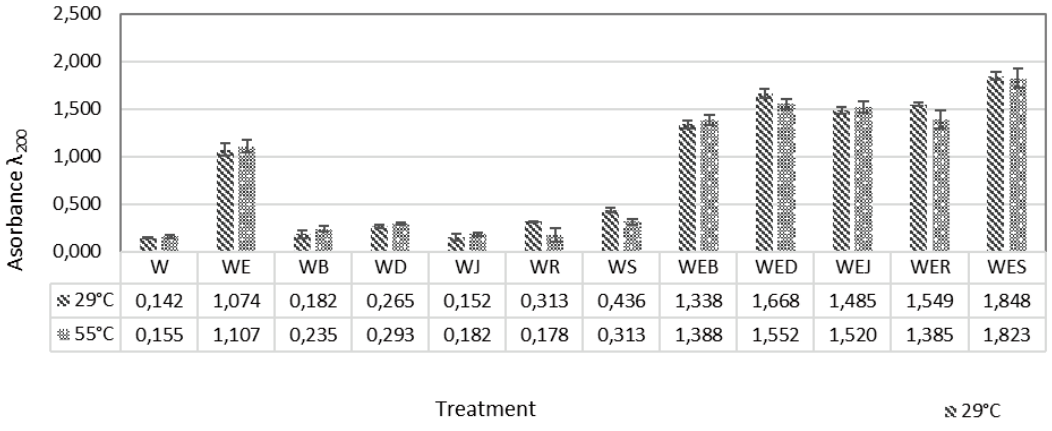


Fig 3 The blood stain solubility in the soaking water.
W- water; E – enzyme; B,D,J,R,S - detergents.

value of detergent's compatibility (Table 2).

DISCUSSION

Aspergillus flavus DUCC K225 is an indigenous mold isolated from lime soil of Madura Island. The temperature of the sampling site was 38°C with the pH of 8.3. This fungus can grow up to 45°C at the pH 8 on PDA medium and showed proteolytic activity with the index of 1.38. Based on the data obtained, study in the production of alkaline thermotolerant protease from this isolate were conducted, to examine on the thermotolerant characteristic and the compatibility to comercial detergent. The result of protease activity from *A. flavus* DUCC K225 examination showed that the enzyme is a thermostable one. The enzyme activity at 55°C-60°C higher than at lower temperatures (Fig.1.), this indicates that the enzyme structure remains stable up to 60°C. According to Yeoman *et al.,* (2010) the thermostable enzymes can maintain their

structural integrity at above 55°C. Coral *et al*. (2003) reported the thermostable alkaline protease produced by an *Aspergillus niger* strain which is stable at 40°C, while alkaline protease from *A. flavus* AS2 studied by Rani & Prasad (2013) found stable at 55°C. Based on previous data obtained in this study, the temperature of 55°C was stated as optimum temperature of this protease. The observation at 55°C showed that this enzyme still active for 60 minutes with 78% retained activity (Fig. 2.). This result supported the previous indication that the alkaline protease produced by *A. flavus* DUCC-K225 was thermostable. The similar result also found in previous study on the alkaline proteinases which produced by *Aspergillus fumigatus* Fresenius TKU003 and *Aspergillus terreus* which is thermostable at 50°C and 60°C (Nirmal *et al*. 2011). The enzyme retained activity value of the alkaline protease produced by *A. flavus* DUCC-K225 in five commercial detergents (Fig 3), indicated that this enzyme compatible with all detergents tested. Choudhary (2012), Niyonzima & More (2015) and Devi *et al* (2008) have been reported that the alkaline protease from several *Aspergillus* species were compatible to detergents. The stability of enzyme was strong related to the retained activity in detergent solutions. The differences in enzyme stability of *A. flavus* DUCC K225 in the five detergent solution used, may corelated with detergent ingredient. Surfactant is one of the main ingredient in commercial detergents, which have the ability to interact with proteases in increasing or inhibiting protease activity (Zhang & Zhang 2015). The detergent S, B, and D contained Sodium Alkyl Benzene Sulfonate as surfactant with the concentrations of 22%, 20% and 15% respectively, while J and R detergents contained 16% and 19% surfactant respectively, but the types of surfactant were not clearly defined. According to Samanta & Mitra (2004) there are 4 groups of surfactants, i.e. anionic surfactants, non-ionic surfactants, cationic surfactants, and amphoteric surfactants. An anionic surfactants such as Sodium Alkyl Benzene Sulfonate also known as Linear Alkyl Benzene Sulfonate (LAS) have the greatest effect on protease activity, compared to other anionic surfactants such as Sodium Duodesyl Sulfate (SDS) and Sodium Lauryl Sulfate (SLS) (Zhang & Zhang 2015). The protease activity will be increased or persists constantly when the concentration of anionic surfactants increases (Barberis *et al* 2013). The low concentration of LAS in water solution (<20%) caused low protease activity, whereas in the high concentration (>45%) caused enzyme denaturation. The highest compatibility of the alkaline protease from *A. flavus* DUCC-K225 shown in S detergent which is contained 20% LAS as surfactant, with the activity retained 99.48% and 98.05% at 29°C and 55 °C respectively. The ability of alkaline protease of *A. flavus* DUCC-K225 in removing blood stains on cloth indicates that this enzyme has the potential to be used in detergent formulas (Tambekar & Tambekar 2013). Shahid & Ahmed (2016) stated that proteases capable in removing proteinic stains such as blood, often used in various detergent industries. The alkaline protease of *A. flavus* DUCC-K225 showed highest compatibility to S detergent, hence the *A. flavus* DUCC-K225 alkaline protease potentially developed to be applied as detergent additives.

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