The effect of molase and urea on the production of alkaline protease from indigenous *Aspergillus flavus* DUCC K225.

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Abstract. Protease is one of industrial important enzyme which can be produced by microorganisms. including fungi. Alkaline protease had physiological and commercially important. this enzyme used in detergent industries as additives. The indigenous mold *Aspergillus flavus* DUCC K225 isolated from lime soil of Madura island demonstrate the ability in producing extracellular protease at pH 8. The aim of this study was to examined the production of alkaline protease from *A. flavus* K225 on Czapeks Dox modified liquid medium containing molase as C-source and urea as N-source. The study were done in submerged culture on rotary shaker. The production of alkaline protease were observed after 7 days incubation. by examined fungal biomass, protease activity, protein content and protease specific activity. The examination of enzyme activity conducted at pH 8.5 done on crude enzyme and ammonium phosphate fractions of 0-20%, 20-40%, 40-60%, 60-80% and 80-100%. The results showed that the fungal biomass gained was 14.27 g/L. while the highest protease specific activity found at 40-60% ammonium sulphate fractionation (71.75 U/ml) and 4.2 purification fold.

Keywords: Aspergillus flavus, alkaline protease, fractination, urea, molase.

1. Introduction
Protease is hydrolysis enzyme that catalyze the breakdown of protein to peptide or free amino acids. The application of this enzyme in industrial, agricultural and medicinal purposes has been widely known [1]. As heterothrophic organisms. microbes including bacteria and fungi has the capability in producing extracellular enzymes. including protease. The extracellular protease become the important enzyme for commercial purposes in recent years. due to the widely use in various industry, like leather processing, food industry, pharmaceutical [2]. The requirement of protease for commercial needs reach more than 50% fom all enzyme needs. Studies on alkaline protease from fungi have been done by some researcher to get potential producing alkaline protease fungal isolates. Extracellular alkaline proteases produced by fungi such as *Aspergillus niger*. *A. flavus* and *A. oryzae* [3]. Studies on the fungal alkaline protease were conducted extensively by researchers in the last few years. The fungus *Aspergillus flavus* DUCC K225 is an indigenous mold isolated from limestone proven as a potential alkaline protease producer. The aims of this study was to examined the fungal alkaline protease produced by *A. flavus* DUCC K225 in the fermentation medium containing mollasses and urea.
2. Experimental Method

2.1. Inoculum preparation

The mold A. flavus DUCC K225 maintained on Potato Dextrose Agar as stock culture. subcultured for 5 days on Czapeks Dox Agar slants for inoculum preparation. The fungal spores inoculum prepared by adding 5 ml of 0.85% NaCl containing 0.01% Tween 80 to release fungal spores [4].

2.2. Enzyme production

The protease enzyme produced by submerged culture on production medium i.e. modified Czapeks Dox broth containing (g/L) 30.0 molase; 0.5 KCl; 0.01 FeSO₄; 0.5 MgSO₄.7H₂O; 1.0 K₂HPO₄ ; 2.0 urea and 1% casein. The pH of fermentation medium was 8.0. One percent of spore suspension (10⁸/ml.) was inoculated into production medium. incubated on rotary shaker 120 rpm for 7 days at ambient temperature [4,5]. At the end of the incubation period. the mycelium were filtered through Whatman no.1 filter paper. the crude enzyme obtained were then centrifuged at 10,000 rpm for 20 minutes at 4 °C.

2.3. Enzyme purification

The crude enzyme obtained from filtration and centrifugation were then gradually purified by ammonium phosphate fractionation of 0-20%, 20-40%, 40-60%, 60-80% and 80-100%. The deposition obtained were liberated from ammonium sulphate by dialysis using cellophane tube in the same buffer. [6; 7; 8].

2.4. Protease activity

Protease activity of purified and crude enzyme were done by mixing 1 ml casein 2% in Tris-HCL 0.1M buffer pH 8.5 with 1 ml of enzyme. incubated at 40°C for 10 min. Reactions stopped by adding 3 ml cold 10% TCA incubated for an hour at room temperature. The deposition formed were filtered using Whatmann no. 1 filter paper. An amount of 1 ml filtrate added with 5 ml of 0.4M Na₂CO₃ and 0.5 ml 0.5N Folin phenol reagen. homogenized using vortex mixer and reincubated for 20 min. at room temperature. The absorbances were then measured at 660 nm. One unit activity of protease expressed in units/ml. define as the amount of enzyme used to release 1 μg tyrosine in 20 minutes at 37°C[ 7; 8].

2.5. Protein content

The protein content of crude and purified enzymes were determined by Lowry method using BSA standard [5]. The determination of the enzyme specific activity carried out by calculating the value of enzyme activity divided by the protein content.

3. Result and Discussion

The mold isolates of A. flavus DUCC K225 used for the alkaline protease production was maintained on PDA slant. for inoculum preparation the mold was subcultured on CDA medium are incubated at room temperature for 5 days. During 7 days incubation period. the mold A. flavus DUCC K-225 looks to grow well on the production medium containing molasses as a source of C and urea as a N source N (Fig 1.). with biomass reached 11.04 g/L. while in the control medium Czapekz Dox modified broth containing 1% casein the biomass obtained was 2.83 g/L.

Figure 1. The A. flavus DUCC K225 culture in molasses-urea fermentation medium at 7 days incubation time. (A) before incubation and (B) at the end of incubation time.
The significant increment of fungal biomass may caused by the better nutrition content in the production medium. Cane molasses which is used in this experiment as organic C source were also rich of nitrogenous compounds. According to Curtin [9] molasses contained 46% total sugars, 3% of crude protein and 63% of nitrogen free extract. Figure 2. below showed the results of the alkaline protease from A. flavus DUCC K225 on molasses-urea medium in 7 days incubation, showed the activity of the alkaline protease of crude enzyme and gradually ammonium sulphate fractionated enzyme in molasses-urea medium.

![Figure 2](image1.png)

**Figure 2.** The alkaline protease activity of crude and fractionated enzyme from *A. flavus* DUCC K225 on urea-molasses medium at 7 days incubation.

The crude enzyme has the highest protease activity compared to all fractionated one (Fig.3). This result in accordance with previous studies using *A. flavus* AS2 [7], and *A. clavatus* [10], which is also showed that the crude enzyme have the highest protease activity. It showed the opportunity to use the crude enzyme directly without purification. Examination of total protein content of crude and fractionated enzymes obtained that the highest levels of total protein found in crude enzyme and declined in accordance with the increasing of ammonium sulphate percentage used in gradually fractionation. i.e. 0-20%; 20-40%; 40-60%; 60-80% and 80-100%. The same results were also presented in some previous studies.

![Figure 3](image2.png)

**Figure 3.** The protein content of crude and fractionated alkaline protease from *A. flavus* DUCC K225 on urea-molasses medium at 7 days incubation.
The total protein content of the alkaline protease were varied between the crude enzyme and its fractionation results. The highest found in crude enzyme, while the lowest found in 40-60% ammonium sulphate fraction (Fig 3). The value of total protein obtained used further to determine the specific activity of the alkaline protease, which is indicated the enzyme purity level. The specific activity stated as U/mg, which is determined by dividing the value of protease activities (U/ml) with protein (mg/ml).

![Figure 4](image_url)  
**Figure 4.** The specific activity of crude and fractionated alkaline protease from *A. flavus* DUCC K225 on urea-molasses medium at 7 days incubation

The value of specific activity of alkaline protease from crude enzyme and its fractions showed at Figure 4. The 40-60% ammonium sulphate fraction showed the highest specific activity, this indicated the purest alkaline protease found in this fraction. This results are still within the range of previous studies that showed the gradually ammonium sulfate fractionation on alkaline protease of *A. flavus* present on the 70% ammonium sulfate fraction [7]; other studies found 40-75% ammonium sulphate fraction on alkaline protease of *A. clavatus* [10] and 40-80% ammonium sulphate on alkaline protease of *A. niger*. [6]

![Figure 5](image_url)  
**Figure 5.** The purification fold of alkaline protease from *A. flavus* DUCC K225 on urea-molasses medium. at 7 days incubation.

The highest purification fold present in the 40-60% ammonium sulphate fraction with the purification factor value of 4.282 (Fig. 6). The study on alkaline protease from *A. clavatus* also get the value of purification factor value was 4.04 [10]. The different results of this study compared to other
previous fungal alkaline studies happened due to the different species and different strain of fungi used.

4. Conclusion
The alkaline protease produced by *A. flavus* DUCC K225 have a prospect for industrial purposes. Molasses and urea can use as cheap component in production medium for alkaline protease from *A. flavus* DUCC K225. that can reduce the production costs.

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5. References
[1] Niyonzima F N and More M M 2015 3 *Biotech* 5 61
[2] Khan F 2013 *Innov. Res. Chem.* 1 1
[3] Preetha P 2012. *Discovery life* 118. www.discovery.org.in/dl.htm
[4] Charles P, Devanathan V, Anbu P, PonnuSwamy M N, Kalaichelvan P T and Byung-Ki H  *J. Bas. Microbiol.* 48 347
[5] Coral G, Arikan B, Unaldi M N and Guvenmez H 2003 *Ann. Microbiol.* 53 491
[6] Devi M K, Banu A R, Gnanaprabhal G R, Pradeep B V and Palaniswamy M 2008 *Ind. J. Sci. Technol.* 1 1
[7] Roja-Rani M. and Prasad NN 2013 *Res. J. Biotech.* 8 58
[8] Sankheerthana C, Pinjar S, Jambagi R T, Bhavimani S, Anupama S, Sarovar B and Inamdar S R 2013 *Int. J. Curr. Eng. Technol.* 143
[9] Curtin L V 1983 *Molasses – General consideration*. National Feed Ingredients Association (Est Des Moines Iowa) p 6
[10] Tremacoldi C, Monti R, Selistre-De-Araujo H S and Carmona E 2007 *W. J. Microbiol. Biotechnol.* 23 295