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Production and Preliminary Characterization of Alkaline Protease from *Aspergillus flavus* and *Aspergillus terreus*

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Abstract: Proteases are being an industrial candidate, which are widely used in food, bakery, and beverage and detergent industry. In leather industry, alkaline proteases are exhibiting a prominent role in unhairing and bating processes. An extensive use of filamentous fungi, especially *Aspergillus species* has been studied elaborately. Although, the significant application of alkaline protease produced from these strains in leather industry is being limited. *Aspergillus flavus* and *Aspergillus terreus* found as the potential strains for production of tannery protease in submerged fermentation. To improve the productivity of this enzyme in liquid broth, various media ingredients have been optimized. The crude and partially purified proteases preliminarily characterized and used for unhairing processes at lab scale in tannery. The protease obtained from these strains showed the good activity in wide alkaline condition at 50 ^{o}C suggesting the possibility of using in leather and detergent industry.

Keywords: Alkaline protease, *Aspergillus flavus, Aspergillus terreus,* Tannery applications.

Introduction

Protease is the most important industrial enzyme interest accounting for about 60% of the total enzyme market in the world. It is extensively used in a wide of industries *viz*., detergent, baking, tanning and food industry[1]. Though most of filamentous fungi are capable of producing proteolytic enzymes, a demand for strains selection and fermentation media is an essential target in biotech industry[2-3]. The production of alkaline protease from *Aspergillus species* has been reported by many workers[2-5], however, only few of them are emerged as potential candidates to commercialization[1]. Traditionally, sodium chloride and sodium sulfide are used for removing hairs from cattle skins and hides. The wastes generated from these usages are polluting the soil health to become infertile. Enzymes will probably play a key role for environmental friendly cleanup processes because of biodegradability and efficient processing in leather industry.

Thus, this present study investigated the optimized fermentation media for the production of alkaline type proteases by *Aspergillus flavus* and *Aspergillus terreus.* This work was also aimed to test alkaline proteases produced from these organisms in removing hair from hides.

Experimental

Aspergillus flavus and *Aspergillus terreus* were isolated from soil around leather industry, identified morphologically and maintained on potato/dextrose/agar slant at 4 ^{o}C with periodic regeneration.

Submerged fermentation

For producing protease, 50 mL of *Aspergillus* minimal medium[6] containing carbon (1%, w/v) and nitrogen source (0.5%, w/v) in 250 mL flask was used. Casein (1%, w/v) was used as a substrate. The conidial spore suspension was prepared by adding 5 mL sterile distilled water with drops of Tween-80 to 7 days old slant, scraped the spores and homogenized. The fermentation was initiated by adding 1 mL of inoculum containing $1x10^6$ spores/mL and then incubated at 28 ^{o}C for 120 h in an orbital shaker. The influences of various carbon and nitrogen sources were checked by replacing the respective media sources in the production medium. Culture broth was withdrew periodically, filtered, and then centrifuged at 4 ^{o}C for 15 min. This cell-free culture supernatant was used for assaying protease activity.

Protease activity assay

The enzyme produced in culture broth was partially purified by ammonium sulfate fractionation (54%), followed by dialysis with the same buffer. Protease activity was determined by incubating a reactive mixture containing 2 mL azo-casein (40 mg/mL), as a substrate, in 0.1 M tris-HCl buffer (pH 8.5) and 0.5 mL enzyme source at 37 ^{o}C for 30 min. One enzyme activity was expressed as the amount of enzyme required to release one micromole of azo-dye/mL from substrate under the assay condition.

Results and Discussion

The growth pattern of *A. flavus* and *A. terreus* on protease formation in broth culture is shown in Figure 1, it suggesting the optimum incubation time for obtaining maximum enzyme production was at 72 h. Then after, enzyme production gradually declined with fermentation time. Though a maximum enzyme yield attained at same time, protease activity of *A. flavus* showed greater than activity of *A. terreus*.

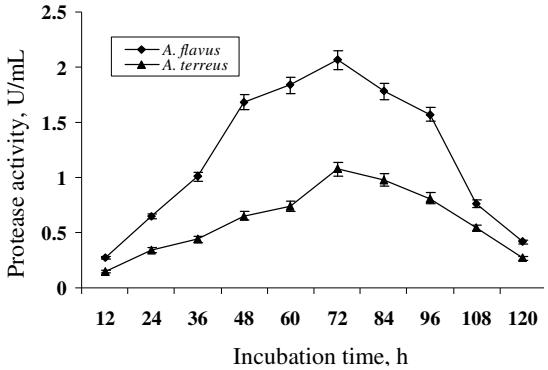

Figure 1. Effect of incubation time on protease production by *A. flavus and A. terreus.*

Table 1 are listed the effect of carbon and nitrogen source on protease production by these strains. It reported that fructose and glucose proved to the best carbon sources for improving the productivity of protease from *A. flavus* (2.048 U/mL) and *A. terreus* (1.006 U/mL) respectively. Although, beef extract was alone served as the best organic nitrogen source for both strains, other sources could not bring considerable protease activity in culture broth apart from these carbon and nitrogen sources[7-8].

Table 1. Influence of carbon and nitrogen sources on *A. flavus* and *A. terreus* for alkaline protease production in minimal medium (MM) at 72 h.

Production media	Enzyme activity, U/mL	Production media	Enzyme activity, U/mL
A. flavus		*A. terreus*	
MM + Fructose + Yeast extract	1.107	MM + Glucose + Yeast extract	0.697
MM + Fructose + Beef extract	2.048	MM + Glucose + Beef extract	1.006
MM + Ribose + Beef extract	1.302	MM + Ribose + Beef extract	0.365
MM + Ribose + Yeast extract	2.002	MM + Ribose + Yeast extract	0.735

As shown in Figure 2(a), the crude and partially purified enzyme obtained from *A. flavus* showed pH optimum in the range of 9-11; in contrast *A. terreus* protease gave maximum activity only at mild alkaline condition (pH 8.0). It revealed that the protease produced from *A. flavus* is acting well in wide alkaline pH, which is greater than protease excreted by *A. terreus*. The effect of temperature profiling on protease enzyme activity are shown in Figure 2(b). This reported that there was optimum activity for both strains at temperature of 45-60 ^{o}C beyond that enzyme lost its activity. When the protease treated with different protease inhibitors, *A. flavus* protease exhibited its residual activity with great extent in presence of TPCK. However, protease activities of both strains have completely inhibited by EDTA. This clearly indicated the presence of serine metallo-type proteases in culture broth of both strains. Generally, *A. flavus* found to be producing alkaline protease in submerged fermentation[2-4], but its applicability to leather industry is studied with less extent.

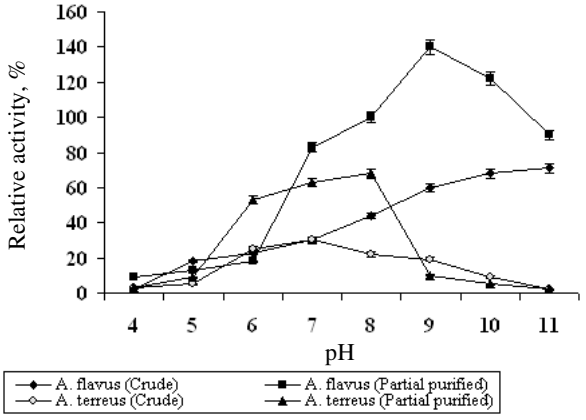

Figure 2(a). Effect pH on protease activity.

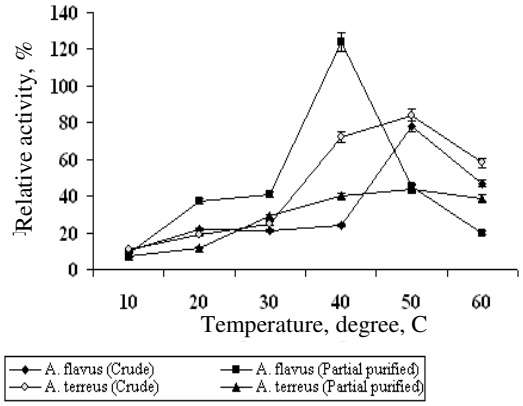

Figure 2(b). Effect of temperature on protease activity.

The culture filtrates of these strains were tested for unhairing using goat skin (10 cm^2) on a lab scale at CLRI tannery. It found that *A. flavus* protease (6699 U) showed more efficient for unhairing from goat skin than *A. terreus* protease (1023 U). *A. flavus* alkaline protease (pH optima 9) is acted as a depilation agent in tannery[4], and *A. tamarii* protease is effective in removing hairs from cattle hide[5].

Conclusions

Overall, we suggest that *A. flavus* is a potential strain capable of producing alkaline protease in broth culture, with environment friendly use on hairs removing from cattle hide in leather industry. This preliminary attempt will hopefully be helpful to replace traditional chemicals used in leather processing. Moreover, as alkaline protease taking a potential role in hydrolyzed food products (yeast extract, casein, soya bean meal, meat extract *etc*.,) production, it would also be needed for many food industry.

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