Isolation and Characterization of Extracellular Protease Producing Fungi from Tannery Effluent.

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Abstract: The isolation and characterization of extracellular protease producing fungi from tannery effluent was examined, the result reveals the isolation of 2 fungi. *Aspergillus niger* and *Mucor* sp. They were tested for their ability to produce extracellular protease *Aspergillus niger* demonstrated the ability at 72 hours with enzyme activity of 0.43 ug/ml,. The highest enzyme activity of 0.61 ug/ml was demonstrated on the 120 hours. The effect of temperature and pH reveals that a niger at 60^{o}C and pH 7 respectively, However the production of proteases by *Aspergillus niger* shows the potentiality of these organism to be used for industrial purpose, thus saving the huge foreign exchange used for its procurement.
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1. Introduction.

Protease is an enzyme that breaks the peptide bonds of proteins (Mitchell *et al., 2007).* Protease breaks down peptide bonds to produce amino acids and other smaller peptides. It can be isolated from a variety of sources such as plants, animals and microbial (fungi and bacteria) *(Oyeleke et al., 2010).* Its application is very broad and has been used in many fields for years, and is mainly used in food and detergent industries (Yandri *et al.,* 2008). Protease work best in acidic conditions except alkaline proteases which has optimal activity shown in alkaline (basic pH (Mitchell *et.al.,* 2007).

Proteases are one of the most important classes of industrial enzymes and account for about 60% of commercial enzymes in the world (Barrette and Rawlings, 2003). With respect of properties of the enzyme and its capacity for degradation of different protein source, theses protease having a long history of application. They find application in a number of biotechnological processes, viz in food processing and pharmaceuticals, leather industry, detergent industry etc (Nascimento and Martins, 2004). Two third of the industrial produced proteases are from microbial sources (Ellaiah and Adinarayama, 2002). Microbial proteases are derived from a wide variety of microorganism such as bacteria, fungi, yeasts, and actinomycetes (Madan et al., 2002; Devi et al., 2008). Molds of the genera *Aspergillus, Penicillium* and *Rhizopus species* are especially useful for production of proteases, as several species of these genera genetically regarded as safe (*Devi et al.,* 2008). *Aspergillus clavatus ESI* has recently been identified as a producer of an extra-cellular bleaching stable alkaline protease (Hajji *et al.,* 2008). Yeast proteases are mainly intra-cellular in nature and therefore, these enzymes have not gained significant commercial interest (Olajuyigbe and Ajele, 2005).

Oyeleke *et al.*,(2011) study the production of extra-cellular protease by *Aspergillus flavus* and *Aspergillus fumigates,* and revealed that both organisms were good producers of extra-cellular protease. A fungal cultlure of *Conidiobolus* species, produces high yields of extra-cellular alkaline protease (Srinivasan *et al.,* 2009). The enzyme is active at pH 10.0 and is been tried for many industrial applications. A process has been patented for the activation of pancreatic enzymes by the use of acid protease from *Aspergillus fumigates.* Srinivasan *et al.,* (2009) reported the isolation of an organism identified as *Bacillus* species from Tannery effluents and found to produce thermostable protease.

It's fact that microbial proteases from bacteria and fungi are used in the pre-tannin processes of leather manufacturing. The most important criteria for their selection are their specificity. pH activity range as well as their thermal stability. Mainly neutral and alkaline proteases are obtained from bacteria, which differ in their pH activity range. Fungal proteases are also classified according to the pH activity range (Kretovich, 2010).

Fungal acid proteases net between pH 2.5 and 6.0 and can be derived from *Aspergillus satoi* (Kamini *et al.,*2008). Fungal alkaline proteases

belong to the same group of serine proteases as alkaline bacteria proteases. However, these are more heat sensitive and quickly deactivated at 60°C. Fungal natural proteases are mainly obtained from *Aspergillus* or *Penicillium* species (Kamini *et al.,* 2008). Apart from bacterial and fungal proteases, specific proteases like Keratinases (Noval and Nickersen, 1959) are known. Keratinases which hydrolyese keratins are obtained from *Streptomyces fradie (*Kamini *et al.,*2008).

2. Material and Method.

2.1 Isolation and Identification.

One litre of tennary effluents was collected in a sterile bottle from Erena local tennary industry, in Shiroro Local Government Area of Niger State, Nigeria for preparation of initial cultures. Zero point two ml(0.2 ml) of the tannery effluent was dropped at the centre of sabouraud dextrose agar (SDA) plates and incubated at room temperature (±25°C) for 72 hours – 7 days. The growth of fungal colonies was observed after incubation. The individual colonies were then subcultured. The cultures were identified on the basis of color and nature of the hyphae a seasoned by Chaesbongh (2006) and Oyeleke and Manga(2008).

2.2 Screening for proteolytic activity of the microbial isolates.

The fungal isolates were screened for their ability to produce protease enzyme as described by Oyeleke *at al* (2010). The isolates were plated onto skim milk agar plates and were incubated at ±25°C for four (4) days. Clear zones of skim milk hydrolysis gave an indication of protease producing organisms. The zones of clearance were measured.

2.3 Optimization of media for extraction of fungal protease.

Cultivation was carried out in the Czapek Dox medium as described by Bertrand *et al.,*(2004) with the following composition (g/1)KH_2PO_4. 1; $M_gSO_4.7H_2O$, 0.5; KC1, 0.5; $FesO_4$. $7H_2O$,0.01; sucrose, 30; casein, 1% (W/V). The cultivation of the fungal isolates was carried using the Czapek Dox medium. The fungal isolates were cultured in 250ml Erlenmeyer flasks for five days at ±25°C on a rotary shaker set at 250 revolutions per min. The culture was centrifuged at 10,000 rpm for 15 min and the supernatant thus obtained was used as crude enzyme extract.

2.4 Fungal protease enzyme assay.

The fungal protease enzyme assay was done according to the method described by Bartrand *et al*2004), Oyeleke *et al.,* (2010). 3cm^3 of reaction mixture containing 0.5% casein in 2.95ml of 0.1 M Tris-HC1 buffer, pH 8.5. and 0.1 milliliters of enzyme was incubated at 40°C. After 30 min, the reaction was stopping by adding 3 cm^3 of cold 10% trichloroacetic acid. After 1 hour, the cultured filtrate was centrifuged at 8,000 rpm for 5 min to remove the precipitate. The absorbance of the supernatant was read using spectrophotometer set at 540nm. Enzyme activity was calculated by measuring the milligram of tyrosine equivalent released and compared with the standard.

2.5 Effect of pH on protease production.

The effect of pH on protease production by the fungal isolates was examined using different pH ranges like 3, 4, 5, 6, 7, 8, and 9 as described by Bertrand *et al* (2004) respective. The optimization media with the respective pH were inoculated with the test samples and the protease assay was done after 72 hours. The best pH was concluded by reading the absorbance at 540nm in a spectrophotometer.

2.6 Effect of temperature on protease production.

The effect of temperature on protease production was examined by taking various temperatures ranges like 40°C, 50°C, 60°C, 70oC, 80°.C, and 90°C. The optimization media was inoculated with the test samples at different temperatures and the protease assay was done after 72 hours, and the as smyance reading was done at 540nm in a spectrophotometer.

3. Result Analysis and Discussion.

The production and characterize ation of extracellular produce by fungi isolated from tannery effluent was examined, the result reveals the presence of too fungal isolate; *Aspergillum niger* and *Mucor* specie. The protease by *Aspergillus niger* shows enzyme activity of 0.43ug/m1 on the their day, 0.55 µg/ml on the fourth day, a high enzyme activity of 0.61µg/ml on fifth day, 0.44µg/ml, and a decline in activity of 0.36µg/ml on the sixth and seventh days respectively (fig 1). This result compared favourably with the report of Srinivasan *et al* (2009) and Oyeleke *et al* (2010) in which *A. fumingatus* has the highest activity on the fifth day.

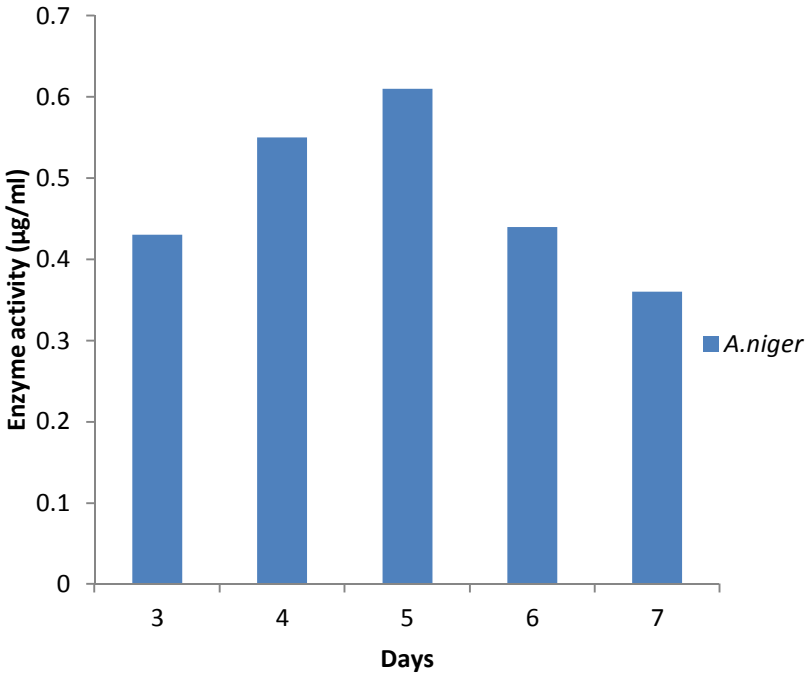

Figure 1. Protease activity produced by *Aspergillus niger*

Oyeleke *et al*., (2010) and Srinivasan *et al*., (2009) study the production of extra-cellular protease by *Aspergillus flavus* and *Aspergillus fumigatus*, and revealed that both organisms were good producers of extra-cellular protease.

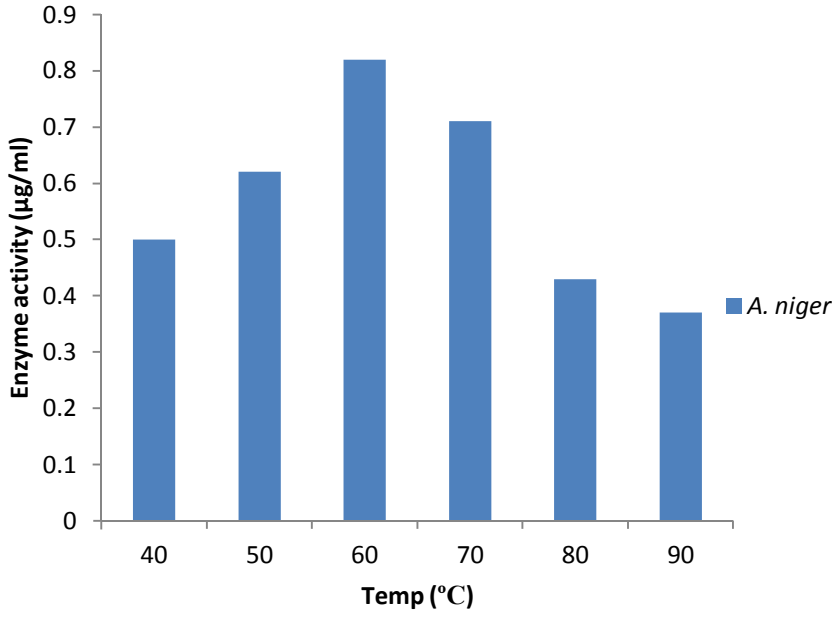

Figure 2: Effect of temperature on protease activity produced by *Aspergillus niger*.

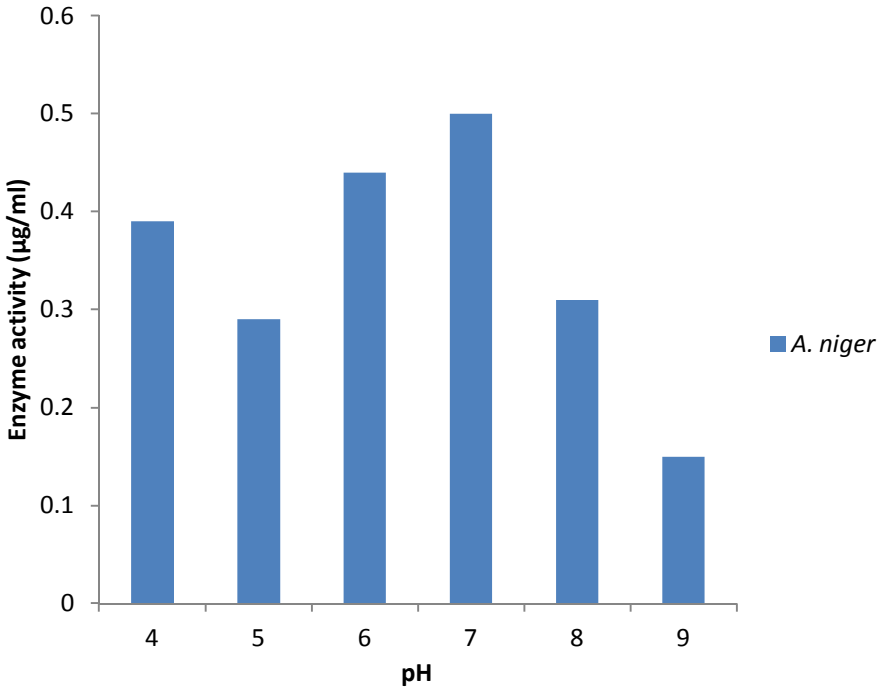

Figure 3: Effect of pH on enzymatic activity of protease produced by *Aspergillus niger*

Optimum temperature for protease produced by *Aspergillums niger* had an optimum temperature at 60°C, this was followed by a sharp decrease in enzyme yield with increase in temperature at 70°C, 80°C and 90°C (0.71 µg/ml, 0.43 µg/ml, respectively (Fig 2). Temperatures above 70°C, led to decrease in protease activity. *Aspergillums niger* had enzyme yield of 0.50 µg/ml, at pH 7 (Fig. 3). This result agrees with protease production as reported by Oyeleke *et al.,*(2010) who recorded protease production by *Aspergillums flavus* (pH 8) with protease activity of 0.74 µg/ml and by *Aspergillums fumigates* (pH 5) with protease activity of 0.70 µg/ml,. *Mucor* spp had the least enzyme activity as such there is no prospect for its use. The production of protease by *A niger,* will go along way to reduce the use of foreign exchange to procure protease for industrial usage.

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