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Isolation and Characterization of Proteolytic Enzyme Produced from Fungi

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Abstract:

Protease is an enzyme that conducts proteolysis and receives high attention as the most important enzyme in many industries. The enzymes find applications in waste treatment, bioremediation processes, detergents, and the leather industry. However, the search for microbial sources of novel proteases in nature is a promising area of interest for researchers. In the present research work, protease-producing microorganisms were isolated from soil samples collected from two different locations in UNIZIK, from the surface and at depths of 15 cm. The isolated organisms were screened for their protease-generating abilities by using the skim milk agar experiment. The isolates that gave high positive results in screening were identified as *Aspergillus sp.* and *Penicillium sp.* by analysing the colonies morphology and using the lacto-phenol cotton blue (LPCB) staining technique. Of these isolates, *Aspergillus sp*. that was isolated from the soil surface demonstrated the highest protease activity, as indicated by the clear zone around the colony that was incubated at room temperature for 96 hours. The results showed that among the isolates, two fungi had great potential to be used for the production of protease enzymes and upscaled for industrial production.

Keywords: Protease, *Aspergillus sp.*, *Penicillium sp.*, Enzyme

INTRODUCTION

Animals, plants, and microorganisms produce protease and have an important role in numerous physiological as well as pathological processes like catabolism of protein, cell growth and migration, blood clotting, estimates of tissue morphogenesis in development, tumour growth and metastasis, inflammation, hormone production, activation of zymogens, and the pharmacologically active peptides from the precursor protein and that protein's transport across the membrane (Sawant and Nagendran, 2014; Souza *et al., 2015).* Extracellular proteases help catalyse the proteins hydrolysis in the form of minor peptides as well as amino acids for final cell immersion, forming a highly significant step in the nitrogen metabolic processes (Sabotiè and Kos, 2012; Souza *et al., 2015*). Microorganisms have the ability to interact, and utilize

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substances leading to structural changes in materials (Agu and Odibo, 2021; Agu *et al.,* 2022; Orji *et al.,* 2022).

According to a recent study, the vast majority of industrial enzymes currently used are hydrolytic in action and are used in the degradation of natural materials. Protease enzymes remained the dominant type because they are used by the vast majority of industries. Microorganisms are the most common source of commercial enzymes due to the nature of their physiology and biochemistry, facile cultural conditions, and ease of manipulation of the cells. Moreover, the fungus is generally considered a good strain for the production of protease enzymes. They are simpler to regenerate from mould fermentation broth of the genus *Aspergillus, Penicillium*, and *Rhizopus (Vishwanatha et al.,* 2009; Kumar and Jain, 2017). Enzyme-producing microorganisms include bacteria, fungi, and actinomycetes (Agu *et al*., 2013; Agu *et al.*, 2014; Orji *et al*., 2014; Chidi-Onuorah *et al.,* 2015; Okafor *et al*., 2016; Agu *et al*., 2017; Ojiagu *et al*., 2018).

Fungal protease has attracted the attention of experts in environmental biotechnology since fungi can grow on low-cost substrates and secrete huge quantities of the enzyme into the culture medium. Production of fungal protease has advantages over bacterial protease as mycelium may be easily detached by filtration (Kumar and Jain, 2017; Souza *et al.,* 2015; Anitha and Palanivel, 2012; Vishwanatha et *al.,* 2010b; Anitha and Palanivelu, 2013). An early stage in the development of industrial fermentation processes isolated different organisms for the production of protease on a large scale (Germano et *al.,* 2003). In the current investigation, the fungal protease was isolated from soil samples at the surface and a depth of 15 cm at Nnamdi Azikiwe University, Awka.

By a process of fermentation, proteases can be isolated and purified in a relatively shorter period of time, exhibiting high substrate specificity and catalytic activity (Warcup, 1999; Mohanasrinivasan*et al.,* 2012; Kutateladze*et al.,* 2012). It is estimated that proteases account for 1–5% of the genome of infectious organisms and 2% of the human genome (Germano et *al.,* 2003). According to researchers, proteases control the activation, synthesis, and turnover of proteins to regulate physiological processes. Proteases are vital in the imitation and spread of infectious diseases, and because of their significant role in the life cycle, they are imperative for drug discovery (Aladdin *et al.,* 2017).

Proteases are involved in normal and pathophysiological processes or conditions. This involvement of proteases may lead them to produce a therapeutic agent against deadly diseases such as cancer and AIDS (Cappuccino and Sherman, 2008). Proteases similar in sequence and structure are grouped into clans and families, which are available in the MEROPS database. The proposed review highlights the proteolysis, function, and wide range of sources among different bacteria of microbial proteases. It also discusses the broad range of applications and upcoming advancements for the discovery of new and fresh proteases, especially alkaline proteases from bacteria (Dubey *et al.,* 2010).

The aim of this work is to isolate, characterise, and screen for proteolytic fungi from the soil and to determine the proteolytic activity of the isolates.

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Materials and Methods

Sample Collection

Soil samples were collected from Science Village (L1) and Social Sciences (L2), all at Nnamdi Azikiwe University Awka. The soil samples were collected from a depth of about 15cm and the surface; a sterilised hand trowel was used to collect about 100g each of the top soil and depths, according to Oyelekee et *al.* (2010). The samples were taken to the microbiology lab at Nnamdi Azikiwe University, Awka.

Isolation of fungi

The sample was processed under sterile conditions. Ten-fold serial dilution was carried out (10^{-1} to 10^{-5}) and 0.2 ml from each dilution of 10^{-1}, 10-3, and 10^{-5} was plated out in a petridish using the pour plate method. An appropriate volume of the medium was dispensed into the petridish. The plates were allowed to gel, then inverted and incubated at room temperature for up to 4 days. Pure cultures were isolated on Sabourand Dextrose Agar using the point inoculation method. Fungal identification was done on the basis of colony morphology (macroscopic characteristics) and microscopic characteristics using standard taxonomic references (Warcup, J. H. 1999).

Characterization of Fungus

This was done based on the description of the gross morphological appearance of fungal colonies on the SDA culture medium and the slide culture technique for microscopic evaluation with reference to the Manual of Fungal Atlases (Agu and Chidozie, 2021).

Screening for proteolytic fungi

Skim milk agar medium is used for screening protease production by fungi (Namasivayam and Nirmala, 2013; Ayob and Simarani, 2016; Vijayaraghavan and Vincent, 2013). The pure culture isolates were streaked on the skim milk agar plates, incubated at room temperature (27°C) for four days, and stained with iodine for the appearance of activity. Then the appearance of a clear zone in the medium around the colony indicates protease activity. The zones diameters were measured in mm, and results are recorded (Warcup, J. H., 1999; Abe *et al.*, 2015). The enzymatic index (EI) is expressed as R/r, where R is the degradation zone diameter and r is the colony diameter (Hankin and Anagnostakis, 1975; Abe *et al.*, 2015). The species that exhibits the maximum clear zone is selected for further identification.

Protease production

The fungal isolates were incubated in sterile 100 ml of protease-specific fermentation broth containing (%w/v): yeast extract 1.0, $MgSO_4$ 0.02, glucose 2.0, K_2HPO_4 0.1, pH 7.0. Flasks inoculated were incubated at 280 °C for 3–4 days in a rotary shaker.

At the end of the incubation, the contents of the flask were filtered through Whatmann filter paper No. 1, and then the filtrates were centrifuged at 8000 rpm at 40 °C for 10 minutes. Pellets were discarded after centrifugation, and the clear supernatant was used as a source of protease enzyme. The supernatant of crude enzymes was further used for subsequent studies.

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RESULTS

The fungal isolates were isolated using SDA agar plates incubated at room temperature (270 °C) for five days. Fourteen (14) fungi were isolated from the soil samples as shown in Table 2, and the isolates had different morphological characteristics, different colony colours, and different colony sizes. The surface of the soil showed a higher number of isolates in comparison with the 15cm depth of the soil. Table 3 shows the cultural and microscopic features of the fungal isolates from the soil. Fourteen isolates isolated from the soil samples were subjected to screening for protease enzymes. Based on the screening for the production of protease enzyme, only two (2) fungal organisms showed a great zone of clearance.

The identification of isolated fungal species that show the highest production of extracellular protease was done based on macroscopic and microscopic characteristics. The identified fungal samples are listed in Table 4.

Table 1: Different soil samples from Nnamdi Azikiwe University, Awka.

Site	code
Science village (surface)	L1S
Science village (15 depth)	L1D
Social sciences (surface)	L2S
Social science (15 depth)	L2D

S=Soil surface, D=15cm depth in soil, L=Location.

Table 2: Percentage frequency of the isolates

Sample	Number of isolates	Percentage of total isolate
L1S	5	37.5%
L1D	3	21.4%
L2S	4	28.6%
L2D	2	14.3%

S= Soil surface, D= 15cm depth in soil, L= Location.

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Table 3: Cultural and Microscopic features of the fungal isolates from the soil

Isolates	Cultural features	Microscopic features	Organism
1	On SDA, colonies had rapid growth rate. However, colonies were flat and compact with yellow basal felt covered by a dense layer of black conidial heads with powdery texture. The colour on the reverse side was pale yellow. Colonies were incubated at 30^0C for 5 days.	Septate hyphae with Conidiophores were hyaline or pale-brown to black, erect,simple, with foot cells basally, inflated at the apex forming globose vesicles, bearing conidial columns with over 4 fragments apically composed of catenulate conidia.	*Aspergillusniger*
2	On SDA, Colonies are usually fast growing, in shades of green, sometimes white.	Conidiophores are hyaline, smooth or rough walled. Phialides are usually flask shaped, consisting of a cylindrical basal part and a distinct neck. Conidia are in long dry chains, divergent or in columns, are globiose, ellipsoidal, cylindrical or fusiform, hyaline or greenish, smooth or rough-walled.	*Penicilliumsp*
3	On SDA, Colonies are granular, flat, often with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age	Conidial heads are typically radiate, later splitting to form loose column. Conidiophores atipes are hyaline and coarsely roughened, often more noticeable near the vesicle. Conidia are globose to subglobose.	*Aspergillusflavus*
4	On SDA, Colonies are floccose, pale grayish brown	Sporangiophores are hyaline and mostly sympodially branched with long branches erect and shorter branches becoming circinate (recurved). Sporangia are spherical, varying from 20-80µm in diameter, with small sporangia often having a Persistent sporangial wall.	*Mucorsp*
5	Colonies are usually fast growing, pale or white, becoming purple, colored with or without a cottony aerial mycelium.	Conidiophorea are short, single,lateral monophialides in the aerial mycelium, later arranged I densely branched clusters. Macroconidia are hyaline, two to several celled, fusiform to sickle- cell and pedicellate basal, cell.	*Fusariumspp*

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Table 4: Zone Diameter ± S.D of proteolytic fungi

Isolate code	Mean Protease activity (Diameter of the zone clearance (mm))		Organism
	72hrs	96hrs	
2L1S	2.7 ± 0.10	5.1 ± 0.10	*Aspergillus sp*
3L1D	1.5± 0.10	3.6 ±0.10	*Pencillium sp*

DISCUSSION

In the current study, a total of 14 fungi were isolated from 4 soil samples, and the focus is to choose fungi that have different characteristics. This result is apparent to Tarman*et al.,* (2011)who isolated a total of 24 fungi from 6 samples. Table 3 above shows the result of different species of fungal isolates from this study which includes *Aspergillus spp., Penicillium sp., Mucor sp., Fusarium spp.,* this result agrees with the observed fungal organisms obtained from Mohanasrinvasan*et al.,* (2012) on Isolation and Screening of Protease from fungal isolates of soil.Fungal strains belonging to different genera and exhibited variation in protease activity. Isolates identified from their morphological characteristics, *Aspergillus sp.* colonies are green to gray and small, *Penicillium sp.* has a large white settlement. Results of the clear zone around isolates showed that *Aspergillus sp.* shows highest protease activity compared with other isolates in this study,this was also seen in the work of Brandner *et al.,* (2012) where there was production of protease enzyme at different time, which shows the highest production by *Aspergillus sp.* at 96 hours. The area formed by the digestion of proteins with multiple protease enzymes produced by the isolates, sources of protein are from skimmed milk added to it to determine the production of protease by strains, zone showed greater digestibility higher activity (Cappuccino and Sherman, 2008). The protease enzymes activity increased from 72hrs to 96hrs of incubation and after the point that the production of enzyme was forced to stop because there is no increase in the emission zone is observed. This may be caused by nutrient stress, aging culture, accumulation of toxic end products, and moderate protein degradation by protease.

Fungal isolates belonging to different genera and variation exhibited in protease activity. Protease which has been produced from the isolated fungi, *Aspergillus sp.* isolated from the surface using room temperature (27^0C) provided high production in a relatively short incubation period.

This research revealed that the fungi isolated from the soil in two different locations has protease activity determined by skim milk agar method. Based on the microscopic observations, the 1L1S fungi isolate was identified as *Aspergillus sp*., then 3L1D fungi isolate

was identified as *Penicillium sp*. *Aspergillus sp*. resulted in the highest production of the active protease as indicated by the halo hydrolysis zone formed around the colony. This study has given a hint that microbial wealth of protease producing fungi isolated from soil can harness for biotechnological processes.

CONCLUSION

Protease extracted from fungi has potential applications in a number of industrial processes and constitute a class of industrial enzymes representing approximately 25-33% of the world enzyme bank. Demand and selection of the right organism play a vital role in high yield of desirable protease enzyme. A large number of protease are available commercially which are very costly, but microbial proteases have successfully replaced chemical hydrolysis of starch in starch processing industries which ultimately will save our billions of naira and will meet the rising industrial demands.

Nigeria is a country blessed with abundant resources needed for the production of these enzymes. On this basis I recommend that Scale-up strategies should be developed for the extraction of enzyme from microorganisms. Strain development strategies should be looked into for detection of high fermenting and hydrolyzing strains.

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