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Exploration of Moulds for Extracellular Protease Production

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Abstract
Protease enzymes carry a domineering status because of their wide scale applications in the fabric upgrading, food processing, dairy, leather, waste management, detergent and allied industries. They account for more than 60 % of total global enzyme sales. Mould isolates from samples of soil, food and Winogradsky column were screened for extracellular protease production. The filamentous fungi were isolated and purified by sub-culturing on potato dextrose agar. The spore forming unit (SFU) of the moulds per gram of the sample ranged from 1.0 × 10^2 (log 4.00) to 5 × 10^4 (log 5.70) for raw onion and raw meat, respectively. The moulds recovered were Aspergillus niger, A. fumigatus, as well as species of Mucor, Penicillium and Rhizopus. The pure isolates were subjected to protease screening on skim milk agar to determine their proteolytic activity and the enzyme appears to be ubiquitous in all the isolates. Aspergillus niger strain BM-1which was isolated from beef 'suya', produced the highest zone of proteolysis (73 ± 10 SED), on SMA at 28^o C for four days. A species of Rhizopus was the least proteolytic isolate (30 ± 0.0 SED) while other proteolytic isolates were species of Penicillium and Mucor. Filamentous fungi could serve as principal producers of protease enzymes for large scale industrial production and commercial exploitation.

Keywords: *Aspergillus niger*, moulds, protease, enzymes, skim milk agar,

INTRODUCTION
A protease (synonym for proteolytic enzyme or proteinase or peptidase) is an enzyme that breaks down the long chains of other proteins or polypeptides by catalyzing the hydrolysis of peptide bonds (Oyeleke *et al*., 2010; Ahmed *et al*., 2017; Encyclopaedia Britannica, 2020). The enzyme performs proteolysis, i.e. catalyzes protein degradation by hydrolyzing the peptide bonds that link amino acid monomers together in the polypeptide chain forming the protein (Kuberan *et al*., 2010; Souza *et al*., 2015). Proteases refer not to a single enzyme but a group of enzymes, including proteinases, peptidases and amidases. The proteinases hydrolyze intact protein molecules to proteoses, peptones and some amino acids. Peptidases hydrolyze peptones to amino acids while amidases hydrolyze amino acids and release ammonia (Solanki *et al*., 2021).

Enzymes have dragged the world's attention due to their wide range of applications and numerous competitive advantages, replacing the use of harsh chemicals in various industrial catalytic processes (Malathi and Chakraborty, 1991). Various researchers have reported the isolation of proteases from diverse bacterial and fungal sources (Sharma *et al*., 2019). The

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current supply volume of protease does not meet the demand and the cost of production is exorbitant coupled with the problem of environmental degradation and depletion of natural resources. There are also drawbacks in using enzymes in healthcare and other industries because of sensitivity to temperature, pH and other parameters (Choi *et al*., 2015; Solanki *et al*., 2021).

Different medium and process parameters such as temperature, incubation period, inoculum size, medium pH, salt (NaCl) concentration and substrate composition are known to influence the activity of industrial proteases. The search for proteases with novel characteristics for diverse industrial applications continues to increase by the day. Some of the advantages of microbial proteases over plants and animal sources include wide biochemical diversity, rapid rate of microbial growth, little space required for fermentation and the ease of enzyme recovery and genetic manipulation to generate novel enzymes for various applications (Rayda *et al*., 2012). Microbial proteases hold a pivotal position in various industries such as textile, detergent, leather, feed and waste treatment (Sharma *et al*., 2019).The high cost of enzyme production is a major barrier for the successful application of proteases in industries. Protease yields have been improved by screening for hyper-producing strains and/or optimization of submerged fermentation medium (Surywanshi and Pandya, 2017). With the rising demand for proteases globally, there is the need to explore novel sources of protease and increase the volume of production and supply. This study was designed to isolate filamentous fungi from various environmental sources and qualitatively screen them for extracellular protease production.

METHODOLOGY

Some of the mould isolates employed in this research were isolated from Winogradsky column. About 0.1 mL of the column culture taken from the aerobic zone was aseptically inoculated onto the surface of sterile potato dextrose agar (PDA) using the spread plate procedure and incubated at 28 ^{o}C for 5 days. The mould colonies that developed were sub-cultured severally on PDA plates in order to obtain pure cultures, which were then maintained on PDA slants in the refrigerator.

Fresh food samples and those undergoing deterioration, including bread, biscuit, cooked meat, *suya* meat, onion, cassava peel, foofoo and yam peel, were also used as sample sources for the isolation of moulds on PDA. Each sample (25 g) was macerated and transferred aseptically into 225 mL of 1% of sterile buffered peptone water contained in a 250 mL Erlenmeyer flask to obtain the sample homogenate (10^{-1}). Ten-fold serial dilutions were carried out to obtain dilutions: 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. About 1 mL aliquot each of the required dilutions (10^{-1} and 10^{-2}) was aseptically transferred into duplicate Petri dishes. Thereafter, 20 mL of sterile and molten PDA (45 ^{o}C), was poured into the plates aseptically, while swirling back and forth. The plates were incubated in upright position under sterile conditions at room temperature for five days. Thereafter, fungal colonies which developed were sub-cultured unto fresh sterile PDA plates, and the pure isolates were maintained on PDA slants in the refrigerator (FAO, 1979).

Each properly mixed garden soil sample (1 g) was aseptically transferred into 9 mL of 1 % sterile of BPW in a test tube to obtain 10^{-1} dilution. The soil suspension was serially diluted to obtain dilutions 10^{-2} to10^{-5}. Dilutions 10^{-4} and 10^{-5} were used for the count and isolation of moulds on PDA and incubated at 28 ^{o}C for 5 days. Pure filamentous fungi were isolated and maintained on PDA slants in the refrigerator (Simonovicova *et al*., 2021).

The colonial characteristics of each of the pure mould isolates on PDA plates were observed and recorded using standard procedures (FAO, 1979; Onuorah *et al*. 2015; Raja *et al*., 2017).The moulds were sub-cultured on fresh PDA plates to obtain pure cultures, followed by morphological and microscopic identification in lactophenol cotton blue stain (Sigma Aldrich, Germany). The mycelium of the young growing pure culture isolate was placed in a drop of freshly prepared lacto-phenol cotton blue stain on a clean glass slide, using sterilized mycological needle. Their unique characteristics were confirmed using standard atlas. Microscopic features such as the arrangement of conidia, conidiophores, branching and septation were noted (Watanabe, 2010; Raja *et al*., 2017).

Screening of Moulds for Protease Production

The mould isolates were screened for their ability to produce extracellular protease using the method of Oyeleke *et al*. (2010). Point inoculation of the isolates was carried out at the center of sterile skim milk agar (SMA) plates in duplicates, followed by incubation at 28 ^{o}C for four days in upright position in the dark. The plates were then flooded with trichloroacetic acid (TCA) to make the zones of hydrolysis more visible. The diameters (mm) of the clear zones of proteolysis were measured and recorded. The most proteolytic mould was selected as the working strain and maintained on PDA slants at 4 ^{o}C.

Statistical Analysis

The results obtained from this study were subjected to one-way ANOVA and student's t test to compare significant differences of means ($p<0.05$) between the mould counts from various sources and the zones of proteolysis recorded for the selected isolates using statistical software, SPSS version 17.0.

RESULTS AND DISCUSSION

Moulds were isolated and counted from Winogradsky column, soil samples and food samples (SFU/g): ranging from 1.0×10^2 (log 4.00) for onion, to 5×10^4 (log 5.70) for raw cow meat (Table 1).The mould counts (SFU/g): from cassava peel (1.0×10^2), rice husk (5.0×10^4) and beef *suya* meat (5.0×10^4) are presented in Table 1. The count from fresh cassava peel is lower probably due to its higher water activity compared to the other samples. Xerophilic moulds require a lower water activity (0.65) than yeast (0.88) and bacteria and archaea (0.91) (Chap *et al*., 2018). A higher mould count of 3.9×10^5 CFU/g from soil samples has been reported by other authors (Ameh and Kawo, 2017). The mould isolates from this study, included *Aspergillus niger*, BM-1, A. niger Tt, *A. fumigatus* and species of *Penicillium*, *Rhizopus* and *Mucor*. *A. niger* can be isolated from a wide range of environmental samples and substrates (Mohammed, 2019). Sharma *et al*. (2019) has reported the isolation of proteolytic bacteria and moulds from soil, food and other sources. Extracellular protease production by *Aspergillus* spp. and other moulds has been and other moulds has been reported by several authors; *Aspergillus tamari, A. dimorphicus,*

Table 1. Total mould counts from various foods and environmental samples

Samples	Mould counts (SFU/g) $\times 10^2$	Log SFU/g	Isolates selected
Wino column	90	5.00	2
Cake	5	3.70	5
Rice husk	500	5.70	3
Bread I	7	3.85	
Locust bean	30	4.48	3
Onion	10	4.00	3
Yam peel	23	4.36	4
Cassava peel	1	4.84	3
Suya meat	500	5.70	5
Bread II	45	4.65	6
Soil I	350	5.54	5
Soil II	55	4.74	4

Table 2. Screening of mould isolates for the top-ten most proteolytic strains

Code	Isolate	Zones of proteolysis (mm)		
		$\times_1$	$\times_2$	$\times \pm$ SED
BM 1	*Aspergillus niger*	63.0	83.0	73 ± 21.5
C1	*Mucor* sp.	28.0	31.0	30 ± 1.5
C1	*A. fumigatus*	32.0	29.0	31 ± 0.5
A1	*Penicillium* sp.	30.0	31.0	31 ± 0.5
A2	*Rhizopus* sp.	30.0	30.0	30 = 0.0
G2	*Rhizopus* sp.	24.0	36.0	30 ± 4.0
Tt	*A. niger*	54.0	54.0	54 ± 0.0
J	*Penicillium* sp.	37.5	8.00	37 ± 0.0
K	*Mucor* sp.	31.00	30.0	31 ± 0.5
L	*Mucor* sp.	30.0	29.0	30 ± 0.5

Plate 1.

Zones of Proteolysis on skim milk agar

Plate 2.

A. niger on PDA

A ochraceus, A. niger, Fusarium solani, F. morniliforme, Penicillum feuutanum, P. watsmanii, Mucor sp*, Rhizopus* sp and *Trichosporon japonicum* (Muthukrishnan and Mukilarasi, 2016; Ameh and Kawo, 2017; Sharma *et al*., 2019). The top-ten most proteolytic isolates which were screened for extracellular protease activity are as shown (Table 2). *A. niger* strain BM-1 showed the highest activity (73 ± 10 SED), while that with the least activity (30 ± 0.0 SED) was a *Rhizopus* spp.

A few other isolates which showed proteolytic activity were species of *Mucor*, *Penicilluim*, *Rhizopus* and *A. fumigatus*. *A. niger* strain BM -1 was selected as the production strain and subjected to molecular characterization due to its status of generally recognized as safe (GRAS). A microorganism or microbial substance may be GRAS if its recognition of safety is based on the views of experts qualified to evaluate its safety (U.S. FDA, 2018). This permits the use of *Aspergillus* protease in various food applications.

After incubation on skim milk agar (SMA), the formation of a transparent zone which surrounded the mould colony, indicates that the skim milk in that area has been hydrolyzed by extracellular protease excreted by the moulds (Plate 1). The amylolytic, cellulolytic, lipolytic, proteolytic, and xylolytic activities of fungal isolates, including *A. flavus*, *A. gracilis*, *A. penicillioides*, *A. restrictus*, A. *fumigatus*, *Rhizopus* sp. *Sterigmatomyces halophilus*, and *Gymnoascus halophilus* have been investigated (Ali *et al*., 2014; Ameh and Kawo, 2017; Chamekh*etal*., 2019; Chung *et al*., 2022). Among the halophilic *Aspergillus* species, *A. gracilis* (Ali *etal*., 2014), *A. caesiellus* (Bartista-Garcia *et al*., 2014), *A. flavus*, and *A. restrictus* (Ali *et al*., 2014; Annapurna *et al*., 2012) have been reported to exhibit amylolytic, lignocellulolytic, and proteolytic activities, respectively. However, the protease characteristics of *A. reticulatus* remains largely unknown (Chung *etal*., 2022). Production, optimization and purification studies on proteases have been reported in *Bacillus* spp. by Hashmi *et al*. (2022). *A. niger* BM -1 was the most proteolytic (73 ± 10 SED) mm as indicated by a halo zone around the growing mould colony. The next was *A. niger* Tt (54 ± 0.5 SED) in mm and the least was *Rhizopus* sp. (30 ±0.00 SED) in mm. *A. niger* has been reported producing a zone of about 35 mm on skim milk agar (Surywanshi and Pandya, 2017). The zones of proteolysis on skim milk agar are indicated by a halo area of clearance around the mould colony, showing that *A. niger* BM -1 produces extracellular protease. The cultural characteristics of the test organism (*A.*

niger BM-1) are also shown on PDA in Plate 2. *Aspergillus* strains grown on PDA medium showed a colony diameter of 66-70 mm, with an initial growth of white color, which with time acquires a black coloration due to the production of spores (Simonovicova *et al*., 2021).

CONCLUSION

This work describes the isolation of moulds from environmental samples such as cassava peel, yam peel, bread, raw beef, *suya*, onion, soil and Winogradsky column and their screening for extracellular protease production. Extracellular protease appears to be ubiquitous in all the moulds: *Aspergillus niger*, *A. fumigatus* and species of *Penicillium*, *Rhizopus* and *Mucor* which were isolated to various degrees of activity. The filamentous fungi isolated during the study shows that appreciable amounts of protease can be produced from *Aspergillus niger* strain BM-1 isolated from *suya* meat.From this study it can be concluded that this working isolate could serve as a novel source of extracellular protease for possible industrial applications.

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