ISOLATION AND SCREENING OF MOULDS FOR EXTRACELLULAR PROTEASE PRODUCTION

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ABSTRACT

Moulds were isolated from samples obtained from soil, food and Winogradsky column, and screened for extracellular protease production. A total of forty seven moulds were isolated and made pure. The spore forming unit (SFU) of the moulds per gram of the sample ranged from 1.0 × 10^2 (Log_{10} 4.00) for onion, to 5 × 10^4 (Log_{10} 5.70) for raw cow meat. The pure isolates were subjected to protease screening on skim milk agar (SMA) to determine their proteolytic activity. Aspergillus niger BM-1 isolated from beef 'suya', produced the highest zone of proteolysis (73 ± 10 SD), when screened qualitatively and semi-quantitatively on skim milk agar and incubated at 28 ^{o} C for 4 days. The mould isolate with the least activity (12 ± 0.0 SD) was identified as Mucor spp. A few other isolates, which showed appreciable proteolytic activity, were species of Penicillium and Rhizopus. Protease production showed significant statistical differences (P = 0.05), between the mould isolates. The findings of this study indicate that A. niger BM-1 produces protease and has a wide range of applications for industrial and domestic purposes.

Keywords: Extracellular protease, Screening, Food samples, Skim milk agar, Moulds and Aspergillus *niger*.

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 (Encyclopaedia Britannica, 2018; Ahmed *et al*., 2017; Oyeleke *et al*., 2010; Mitchell *et al*., 2007). 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 (Souza *et al*., 2015; Kuberan *et al*., 2010).

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 (Mukhtar and Ul-Haq, 2009). Enzymes have dragged 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 Chakrabory, 1991).

Fungal genera such as *Aspergillus*, *Mucor*, *Trichosporon* and *Rhizopus* and bacteria in the genera *Clostridium, Bacillus, Pseudomonas, Aeromonas, Alkaligenes, Serratia, Arthobacter* and *Streptomyces*, produce extracellular proteases (Ajayi *et al*.; 2014 Pant *et al*., 2015). The current supply volume of protease does not meet up the global demand due to high cost of production, 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 environmemtal factors (Choi *et al*., 2015). The search for proteases with novel characteristics for diverse industrial applications continues to increase by the day.

Different medium and proccess factors such as temperature, incubation period, inoculum size, medium pH, salt (NaCl) concentration and substrate

composition, are known to influence the activity of industrial proteases. 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 cell cultivation and the ease of enzyme recovery and genetic manipulation to generate novel enzymes for various applications (Rayda *et al*., 2012).

The present study has the potential for discovering hyperactive protease-producing moulds that may overcome cost –intensive nature as well as the climatic and ethical limitations associated with protease from plant, animal and other sources. In this study, mould isolates were obtained from various environmental samples and screened for extracellular protease production.

MATERIALS AND METHODS

Isolation of Fungi from Winogradsky Column

Some of the fungal 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 (28 ^{o} C, 5 d). The fungal 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.

Isolation of Fungi from Food Samples

Fresh food samples and those undergoing deterioration, including bread, biscuit, cooked meat, *suya* meat, onion, cassava, foofoo and yam peel, were used for the isolation of proteolytic moulds on PDA. The sample (25 g) was marcerated and transferred aseptically into 225 mL of 1 % 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}. 1 mL aliquot of the required dilutions (10^{-1} and 10^{-2}) was aseptically transferred into duplicate Petri dishes. Thereafter 20 mL of sterile, molten PDA (45 ^{o} C), was poured into the plates aseptically (under the Bunsen burner flame), and swirled back and forth to mix the inoculums with the medium and allowed to solidify.

The plates were incubated in upright position under sterile conditions at room temperature for five days. Thereafter, fungal colonies that developed were sub-cultured unto fresh sterile PDA plates, and the pure isolates were maintained on PDA slants in the refrigerator.

Isolation of Moulds from Soil Samples

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

Qualitative and Semi-Quantitative Screening of Mould Isolates for Protease Production

The fungal isolates were screened for their ability to produce protease using the method of Oyeleke *et al*. (2010). Point inoculation of the pure mould isolates was carried out at the centre of sterile skim milk agar (SMA) plates in duplicates, followed by incubation (28 ^{o} C, 4 d). The plates were then flooded with trichloroacetic acid (TCA), to make the zones of hydrolysis more visible. The

clear zones of proteolysis were measured (mm) and recorded. The most proteolytic mould was selected as the working strain and maintained on PDA slants at 4 ^{o}C.

Colonial (cultural) Morphology

The standard colonial features of each of the pure mould isolates on PDA plates, were recorded.

Wet-mount Microscopic Examination

The mycelium of the young growing pure culture of each mould isolate was placed in a drop of freshly prepared lacto-phenol cotton blue stain on a clean glass slide, using sterilized mycological needle. Microscopic features such as the arrangement of conidia, conidiophores, branching and septation were noted. The results were compared with the Atlas of fungi and fungi identification (Raper and Fennel, 1965).

RESULTS

Isolation of Moulds from Winogradsky

Column, Soil and Deteriorating Food Samples

Moulds were isolated and counted from Winogradsky column, soil and food samples (SFU/g): ranging from 1.0×10^2 (Log_{10} 4.00) for onion, to 5×10^4 (Log_{10} 5.70) for both raw cow meat and MRH (Table 1).

Qualitative and Semi-Qualitative Screening of Moulds for Extracellular Protease Production On SMA Petri Plates

A total of forty seven mould isolates were screened in duplicate Petri dishes containing SMA (pH 7) for their proteolysis activity (Table 2). *A. niger* BM -1 showed the highest activity (73 ± 10), while that with the least activity (12 ± 0.0) was a *Mucor* spp. A few other isolates which showed proteolytic activity were species of *Penicillium* and *Rhizopus*, but *A. niger* BM -1 was selected as the production strain and subjected to molecular characterization due to its being generally regarded as safe (GRAS) status. These zones of clearance around each mould colony are indicative of proteolytic activity due to the organism's ability to elaborate extracellular protease enzyme, as well as the extent of proteolysis (Figure 1).

Table 1. Total mould counts from various local sources on potato dextrose agar

Samples	Mould counts (SFU/g) $\times 10^2$	Log_{10}(SFU/g)	Isolates
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

SFU/g; Spore forming unit per gram.

Cultural and Morphological (Macroscopic and Microscopic) Identification of Filamentous Fungal isolates

The moulds isolated from various sources were sub-cultured on fresh PDA plates to obtain pure cultures, followed by morphological and microscopic identification in lacto-phenol cotton blue stain. Their unique characteristics were confirmed using standard atlas. The identification features of the various mould isolates are shown in (Table 3). A. niger showed white mycelia, turning dark-brown to black, and the reverse is white to yellow on PDA (Figure 2). The wet-mount procedure showed globose, ellipsoidal conidia and flask-shaped phialides under the microscope.

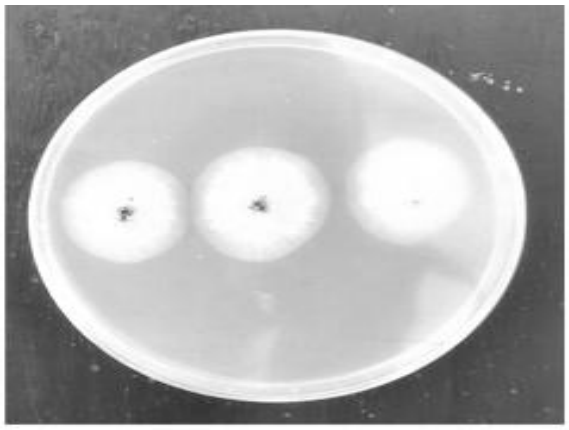

Figure 1. Zones of proteolysis of *A. niger* BM-1 on SMA.

Table 2. Qualitative screening of fungal isolates showing the top fifteen most proteolytic strains

Isolates/code	Zones of proteolysis (mm)		Mean (mm)
	X_1	X_2	$\bar{X} \pm SD$
C1	28.0	31.0	30 ± 1.5
O	32.0	29.0	31 ± 1.0
E1	30.0	31.0	31 ± 0.5
H1	30.0	30.0	30 ± 0.0
H2	24	36	30 ± 4.0
G2	35.0	30.0	33 ± 2.5
F1	37.0	??	37 ± 0.0
J	31.0	30.0	31 ± 0.5
K	30.0	30.0	30 ± 0.0
L	32.0	30.0	31 ± 1.0
n1	34.0	29.0	32 ± 2.5
hi	47.0	31.0	39 ± 8.0
M	63.0	83.0	73 ± 10
Tt	54.0	53.0	54 ± 0.5
HH	30.0	35.0	33 ± 2.5
DD	34.0	32.0	33 ± 1.0

X_1 and X_2 = Duplicate zones of proteolysis; X = mean zone of proteolysis.

Table 3: Colonial and Morphological Identification of Fungi Isolates

Cultural Characteristics	Microscopic properties	Isolates
White yellow mycelia, turning dark brown to black Reverse, white to light Yellow.	globose, ellipsoidal conidia; flask-shaped phialides	*A niger*
White to grey to black colour; Wooly texture; filling Entire dish.	long unbranched sporangiophores Stolons found, no rhizoids/septa	*Rhizopus* spp.
Dull-green; reverse white. Brownish with age.	Uniseriate, radiate Conidia head	*A. Clavatus*
Yellow-green; reverse goldish to red brown or pale yellow.	conidia ellipsoidal pyriform; smooth Stipes.	*A. Flavus*
Cinnamon to brown. Reverse white to brown.	Ellipsoid, smooth Conidia, stipe smooth, finely roughened	*A. terreus*
Blue green to grey. Reverse white to tan or Pale yellow.	Sub-globose, smooth conidia; smooth stipe, Flask-shaped	*A. fumigatus*
White at beginning, turns Yellow, tan, pale green, Reverse white to yellowish Red.	smooth conidiophores conidial heads support biseriate vesicles	*A. versicolor*
Velvety powdery, green, blue-green, gray-green white, yellow or pinkish on the surface. Reverse white to yellowish, atimes red or brown, edge white.	Hefty phialides single-celled conidia. Phialides may have brush-like appearance.	*Penicillium* spp.

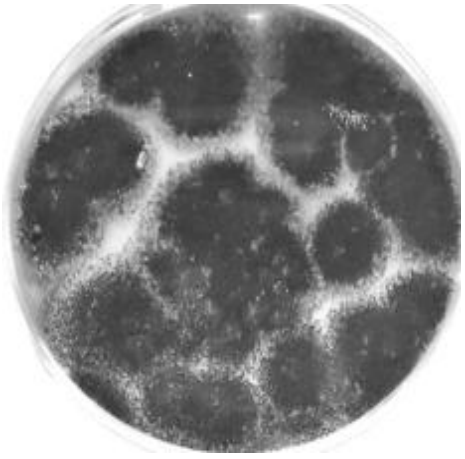

Figure 2. Colonial features of *A. niger* BM-1 on PDA

DISCUSSION

Extracellular protease production by *Aspergillus* spp. and other moulds has been reported by several authors; *Aspergillus tamari, A. dimorphicus, A. ochraceus, A. niger, Fusarium solani, F. morniliforme, Penicillum feuutanum, P. watsmanii,* and *Trichosporon japonicum* (Muthukrishnan and Mukilarasi, 2016). In this study, a total of forty seven species of filamentous fungi were isolated from local sources such as Winogradsky column, milled rice husk, soil samples as well as fresh and deteriorating common food samples like onion, cow meat *suya*, bread, white yam peel, cassava peel, cake, foofoo and biscuit. The total mould counts (SFU/g) from the samples ranged from 1.0×10^2 (Log_{10} 2.00) for the lowest in cassava peel to 5.0×10^4 (Log_{10} 4.70) for the highest in MRH and '*suya'* meat (Table 1). Aspergilli grow abundantly as saprophytes on decaying vegetation and have been found in large numbers in mouldy hay, organic compost piles, leaf litter and the like. Most species are adapted for the degradation of complex plant polymers, but they can also dine on substrates as diverse as dung, human tissues, and antique parchments. There is even a report of an unidentified *Aspergillus* species being capable of the solubilization of low rank coal (Picot *et al*., 2018; Hageskal *et al*., 2011).

All the forty seven isolates were qualitatively and semi-quantitatively screened on skim milk agar for extracellular protease production under 5 days incubation room temperature (28 ^{o} C). *A. niger* BM-1 which was isolated from cow meat '*suya'* produced the highest protease activity in mm (73 ± 10) as indicated by a halo zone of proteolysis around the growing mould colony. This is followed by another strain, *A. niger* Tt which produced a zone of clearance of (54±0.5) in mm. The least proteolytic isolate was *Mucor* spp. which gave an enzyme activity of (12±0.00) in mm. Choudhary and Jain (2012), have reported proteolytic zones of 41.0 mm and 30.0 mm for *A. niger* and *A. flavus* respectively using agar plate diffusion technique. In another research, *A. niger* has been reported producing a zone of about 34.0 mm on skim milk agar qualitatively (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 produced an extracellular protease enzyme.

CONCLUSIONS

In this study, moulds have been isolated from various environmental samples (food, soil and Winogradsky column), identified and screened on SMA for their proteolytic potential. The highest extracellular protease producer, *A. niger* BM -1 (isolated from 'suya' meat), was followed by other *Aspergillus* species, *Penicillium*, *Rhizopus*, *Mucor* etc.

In all, the results obtained during this investigation and those reported by other workers, indicated that *Aspergillus niger* (e.g *A. niger* BM - 1) is a potential mould for the industrial production and commercial exploitation of extracellular protease.

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