ISOLATION AND IDENTIFICATION OF PROTEOLYTIC FUNGI FROM BEAN CROP SOIL AND PADDY MILL SOIL

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Abstract

The present study, eleven fungi were isolated from bean crop soil in Vegetable and Fruit Resources Development Centre, Hlegu Township and twelve fungi were isolated from paddy mill soil in Daik-U Township. Twenty-three fungi were screened by visible plate technique using Potato Dextrose Agar medium (Supplement with 1% Casein). Among all isolated fungi, eight proteolytic fungi were showed clear zone around the fungal colony on Skim Milk Agar (SMA) medium for the preliminary study of proteolytic fungi. Although four proteolytic fungi (V2,V5, D4 and D12) exhibited prominent enzyme activity about (2.5cm for five days, 3.5cm for four days, 2.2cm for four days and 2cm for six days) respectively based on the ratio of diameter of the clearing zone and colony. These four fungi were selected for identification of their colony morphology and spore formation. In the identification of four selected fungi, two *Penicillium* sp. V2 and V5 were isolated from bean crop soil and one *Penicillium* sp. D4 and one *Cladospordium* sp. D12 were isolated from paddy mill soil.

Keyword *Penicillium* sp. and *Cladospordium* sp., Protease fungi

Introduction

Proteolytic enzymes are also termed as peptidases, proteases and proteinases, which are able to hydrolyze peptide bonds in protein molecules. Proteases are generally classified as exopeptidases and endopeptidases. Exopeptidases cut the peptide bond proximal to the amino or carboxy termini of the protein substrate and endopeptidases cut peptide bonds distant from the termini of the protein substrate (Amer Ali Mahdi., 2019). Cells of every living organism consist of a chemical substance that possesses the ability to catalyze or speed up a biochemical reaction and acts as biocatalysts which are known as enzymes. Enzymes have better catalytic efficiency, adjustable activity and high specificity when compared to catalysts of chemical or synthetic origin. Proteases are obtained from diverse groups of organisms such as plants, animals and microorganisms, but commercially viable proteases are obtained from microorganisms, especially bacterial and fungal species.

Fungi are considered GRAS (Generally Regarded as Safe) organisms as compared to other microorganisms because they fulfill the criteria of industrial demands such as efficient growth on culture media in short duration and continuous supply of desired products. Fungi also secreted a large variety of proteases, lipases and amylases that play an important role in physiological processes such as germination, as defensins against other pathogens or for nutritional requirements for development. Secretions of fungal enzymes occur from the cells present at the top of hyphae. These secreted enzymes can be used for industrial preparations of valuable products. Fungal proteases have been widely studied due to their wide diversity. Proteases have been isolated from different fungi such as *Aspergillus flavus, Penicillium janthinellum, Neurospora crassa*, etc. Fungal proteases can be isolated through the fermentation process exhibiting high catalytic and specificity for the substrate Salazar-Cerezo *et al*., 2020.

Proteases are considered the most useful and powerful enzymes as they break down complex protein compounds into amino acids and peptides. Around 60% of global enzyme usage is accounted for by protease enzymes. Alkaline protease are the most industrially used or exploited enzymes. More than 3000 different enzymes have been identified and a lot of them

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being utilized in biotechnological and industrial applications. The protease enzyme constitutes two-thirds of the total enzymes used in various industries including meat tenderization, detergents, cheese making, dehairing, baking, contact lens cleaners, waste management and silver recovery Gupta *et al*., 2002 and Amer Ali Mahdi., 2019.

Materials and Methods

Collection of soil samples

The soil samples were collected from bean crop soil in Vegetable and Fruit Resources Development Centre, Hlegu Township and paddy mill soil in Daik-U Township. The dust and dead matter on the upper layer of the solid were removed and it was dug down to 6 inches deep. Then the soil sample was put into the laboratory using sterilize plastic bags for further analysis.

Isolation of fungal strains (Atlas, 1993)

The fungal strains were isolated on Potato Dextrose Agar (PDA) medium (potatoes - 200g, casein - 100g, dextrose - 15g and agar - 20g at pH 7.0) was chosen as growth medium used then the media were boiled until the agar well dissolved. The pH of the media was adjusted before autoclaving and then the media were sterilized by autoclaving at 121ºC (15psi) for 15minutes. After cooling down, chloramphenicol were added to the medium and then the medium was poured into sterilized petridishes.

Preparation of samples by serial dilution methods (Waksman, 1927)

The soil samples were used to isolate the different colonies of fungi by serial dilution method. 1g of soil samples was suspended in 100mL and through mixed well for 15minutes with the vortex to obtain 10^{-2} dilution (1:100) and 1mL of sample solution was mixed with 9mL of sterilize distilled water in a sterilize test tube to make 10^{-3}, 10^{-4}, 10^{-5} respectively.

Isolation of pure culture from plate (Collins, 1964 and Atlas, 1993)

After incubation period by pour plate method, the different types of fungal colonies were stored into respectively separate agar slants. For further study, the slant were repeatedly sub-cultured by streaking method to obtain pure culture and then pure cultured fungi were kept at room temperature for further studies of colony morphological characters and spore formation.

Preliminary screening of protease producing fungi (Pelczar and Chan, 1972)

The isolated strains were screened for preliminary protease enzyme production on Skim Milk Agar (SMA) medium by using streak plate method. Skim Milk Agar (SMA) medium were used for screening of protease production by fungi (Namasivayam and Nirmala, 2013, Ayob and Simarani,2016). The isolates pure culture isolates were streaked on the Skim Milk Agar (SMA) medium and were incubated at room temperature for 1 to 7days. Then the appearance of clear zone in the medium around the colony were indicates protease activity. The zone diameter were measured in cm and results are recorded (Warcup, 1950 and Abe *et al*., 2015). The enzyme index (EI) expressed as R/r, which R is the degradation zone diameter and r is the colony diameter according to the method of (Hankin and Anagnostakis, 1975, Abe *et al*., 2015). The species that exhibits maximum clear zone selected for further identification.

Identification and characterization of protease enzyme

The four selected fungi were identified with their colony morphological and spore formation according to the method of Barnett (1960) and Dube (1983).

Results

Isolation of fungi

In the present investigation, the bean crop soil in Vegetable and Fruit Resources Development Centre, Hlegu Township and paddy mill soil in Daik-U Township were used for the isolation of fungi by using serial dilution of pour plate method at room temperature for five days. Protease producing fungi eleven and twelve strains isolated from the bean crop soil and paddy mill soil respectively on Potato Dextrose Agar (Added 1% Casein) medium. All isolated fungi were designated as shown in following Table (1).

Table 1 Designated of all isolated fungi by using Potato Dextrose Agar (Supplement with 1% Casein) medium

No.	Sources	Isolated Strains
1.	Bean crop soil	V1 to V11
2.	Paddy mill soil	D1 to D12

Preliminary production of proteolytic enzyme on Skim Milk Agar (SMA) Medium

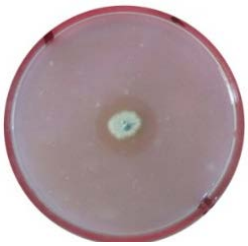
In this investigation, all isolated strains were screened for protease production ability on Skim Milk Agar (SMA) medium by streak plate method. The clear zone formation around the fungal growth was identified as the positive protease produce which may be due to hydrolysis of casein. Among all isolated fungi 4 strains from the bean crop soil and 4 strains from paddy mill soil were showed clear zone on Skim Milk Agar (SMA) medium for the studied of preliminary protease producing fungi as shown in Figure (1 to 8). These result were calculated based on the method of (Hankin and Anagnostakis,1975, and Abe *et al*.,2015). Among 8 proteases positive activity, more prominent of the 4 strains were selected for further investigation. In the bean crop soil, V5 exhibited the high of enzyme activity about 3.5cm for four days and also V8 were showed the lower of enzyme activity about 1.2cm for four days as shown in Table (2). In paddy mill soil, D12 showed the prominent of enzyme activity about 2cm for six days and also D11 shown the poor enzyme activity about 1.9cm for three days based on the ratio of diameter of clearing zone and colony respectively were selected for further investigation.

Selection of most potent proteolytic activity of fungi

In this study, 8 proteolytic fungi 2 strains from bean crop soil and 2 strains from paddy mill soil were selected for further investigation based on their prominent clear zone than the other strains. And then, the four selected fungi were identified based on colony morphology and spore formation according to the method of Barnett, 1960 and Dube, 1933.

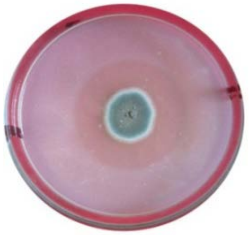
Table 2 Enzyme index of the 8 positive isolated from bean crop soil and paddy mill soil according to the (Hankin and Anagnostakis, 1975, and Abe *et al*., 2015)

Isolated fungi	Clear zone diameter (cm)							Colony diameter (cm)							Enzyme index (EI) (cm) = Clear zone diameter / Colony diameter						
	Incubation hours																				
	24	48	72	96	120	144	168	24	48	72	96	120	144	168	24	48	72	96	120	144	168
V2	1.3	2	2.7	3	3.7	-	-	0.6	0.9	1.1	1.3	1.5	-	-	2.2	2.2	2.4	2.3	2.5	-	-
V5	0.7	2	2.7	2.8	-	-	-	0.5	0.6	0.7	0.8	-	-	-	1.4	3.3	3.8	3.5	-	-	-
V8	1.3	2	2.2	2.3	2.5	3	3.5	0.8	1.2	1.6	1.8	2	2.5	3	1.6	1.7	1.4	1.3	1.2	1.2	1.2
V11	1	1.2	2	-	-	-	-	0.7	0.7	0.8	-	-	-	-	1.4	1.7	2.5	-	-	-	-
D4	1.5	2	2.3	2.7	-	-	-	0.8	1	1.1	1.2	-	-	-	1.9	2	2.1	2.2	-	-	-
D10	1	1.5	2	2.6	-	-	-	0.6	0.8	1	1.2	-	-	-	1.6	1.8	2	2.1	-	-	-
D11	0.8	1.3	1.7	-	-	-	-	0.5	0.7	0.9	-	-	-	-	1.6	1.8	1.9	-	-	-	-
D12	1	1.5	2	2.5	3	3.2	-	0.5	0.7	0.9	1.2	1.5	1.6	-	2	2.1	2.2	2.1	2	2	-

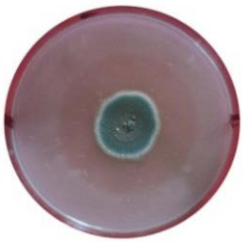


One day old culture

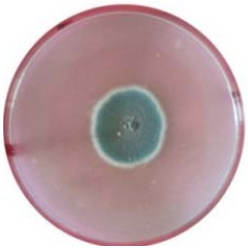
Two days old culture



Three days old culture



Four days old culture



Five days old culture

Figure 1 Proteolytic activity of isolated V2 on Skim Milk Agar medium for one to five days old culture

One day old culture Two days old culture Three days old culture Four days old culture

Figure 2 Proteolytic activity of isolated V5 on Skim Milk Agar medium for one to four days old culture

Oneday old culture Two days old culture Three days old culture Four days old culture

Five days old culture Six days old culture Seven days old culture

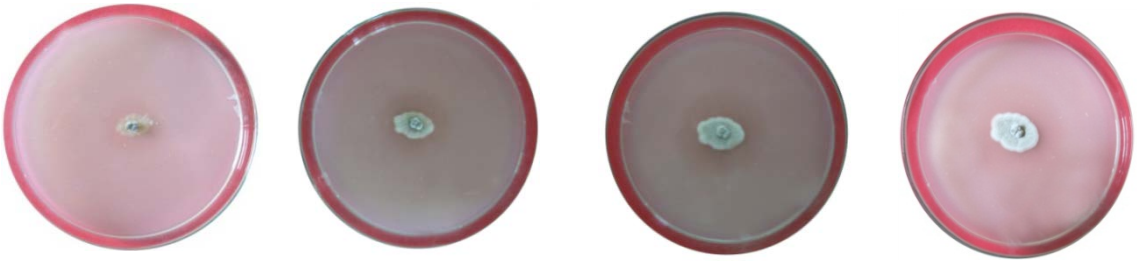
Figure 3 Proteolytic activity of isolated V8 on Skim Milk Agar medium for one to seven days old culture

One day old culture Two days old culture Three days old culture

Figure 4 Proteolytic activity of isolated V11 on Skim Milk Agar medium for one to three days old culture

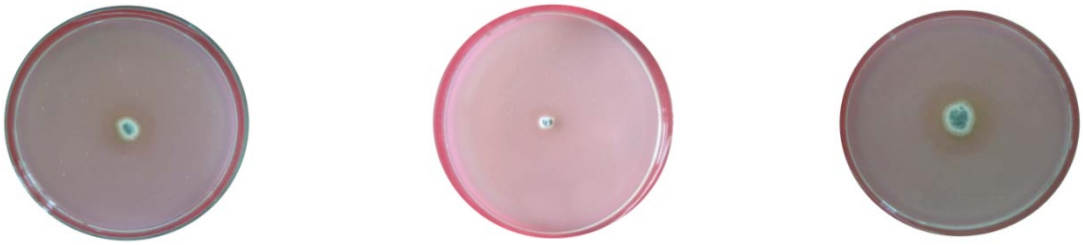
One day old culture Two days old culture Three days old culture Four days old culture

Figure 5 Proteolytic activity of isolated D4 on Skim Milk Agar medium for one to four days old culture



One day old culture **Two days old culture** **Three days old culture** **Four days old culture**

Figure 6 Proteolytic activity of isolated D10 on Skim Milk Agar medium for one to four days old culture



One day old culture **Two days old culture** **Three days old culture**

Figure 7 Proteolytic activity of isolated D11 on Skim Milk Agar medium for one to three days old culture

One day old culture **Two days old culture** **Three days old culture**

Four days old culture **Five days old culture** **Six days old culture**

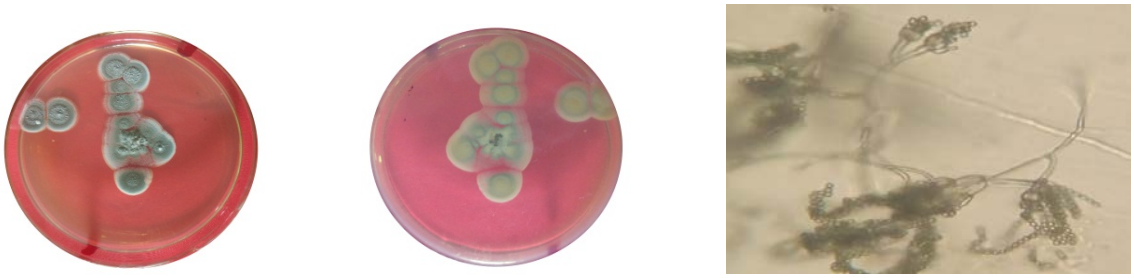
Figure 8 Proteolytic activity of isolated D12 on Skim Milk Agar medium for one to six days old culture

Morphological characteristics of genus level on prominent fungi

In this investigation, morphological characters of 4 selected strains were carried out for identification. In this study of investigation, isolated V2 belong to *Penicillium* sp., isolated V5 belong to *Penicillium* sp., isolated D4 belong to *Penicillium* sp. and D12 belong to *Cladosporium* sp. according to the method of Barnett,1960 and Dube,1933as shown in Figure (9 to 12).

Microscopical characters of V2

The mycelium color is green color inside and white color periphery in surface view and yellow color on reverse view. Conidiospores arising from the mycelium singly, branched near the apex to form a brush-like, conidia-bearing apparatus, conidia brightly colored in mass, 1-celled, ovoid, produced basipetally. According to these microscopical characteristics, V2 may be *Penicillium* sp**.** as shown in Figure (9).

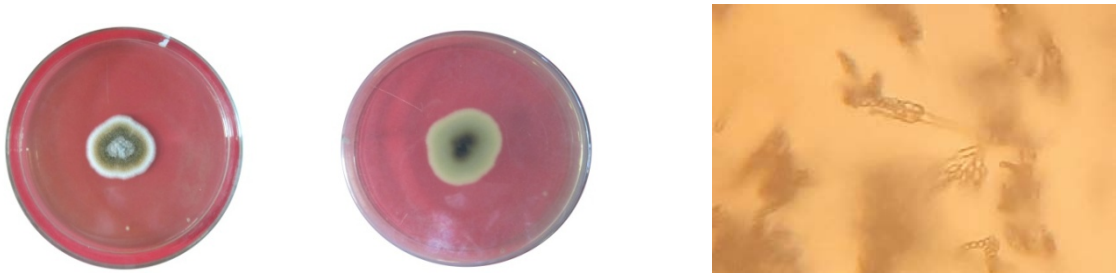


Surface view **Reverse view** **Micrograph of *Penicillium* sp. (X400)**

Figure 9 Cultural characters and morphological character of isolated fungi V2 from bean crop soil

Microscopical characters of V5

The mycelium color is yellow brwon color inside and white color periphery in surface view and pale yellow color on reverse view. Conidiospores arising from the mycelium singly, branched near the apex to form a brush-like,conidia-bearing apparatus, conidia brightly colored in mass, 1-celled, ovoid, produced basipetally. According to these microscopical characteristics, V5 may be *Penicillium* sp**.** as shown in Figure (10).

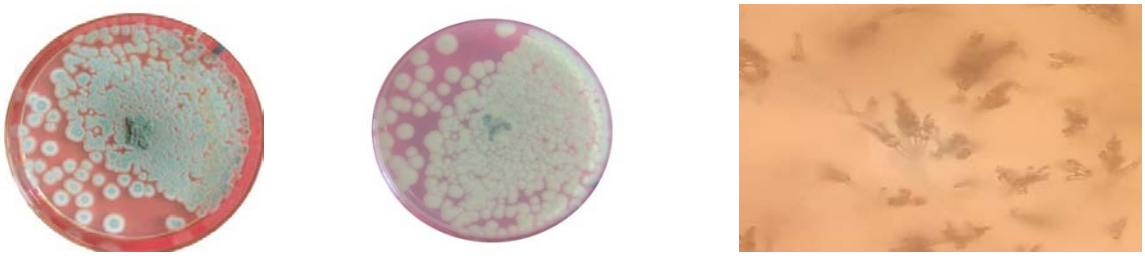


Surface view **Reverse view** **Micrograph of *Penicillium* sp. (X400)**

Figure 10 Cultural characters and morphological character of isolated fungi V5 from bean crop soil

Microscopical characters of D4

The mycelium color is pale green color inside and white color periphery in surface view and pale yellow color on reverse view. Conidiospores arising from the mycelium singly, branched near the apex to form a brush-like,conidia-bearing apparatus, conidia brightly colored in mass, 1-celled, ovoid, produced basipetally. According to these microscopical characteristics, D4 may be *Penicillium*sp**.**as shown in Figure (11).

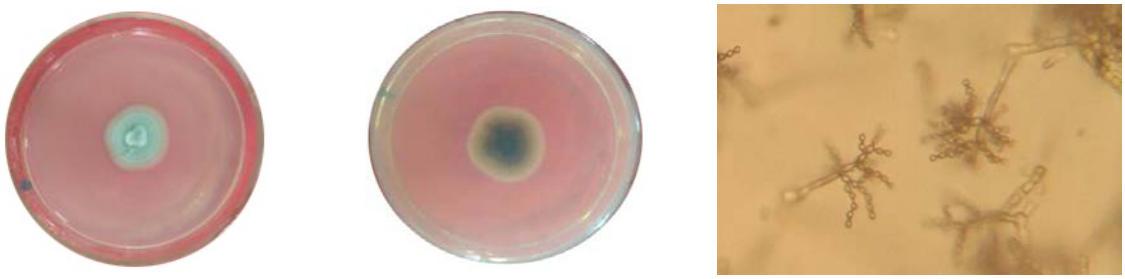


Surface view **Reverse view** **Micrograph of *Penicillium* sp. (X400)**

Figure 11 Cultural characters and morphological character of isolated fungi D4from paddy mill soil

Microscopical characters of D12

The mycelium color is green color inside in surface view and pale brown color on reverse view. Pigment present. Conidiophores dark, branched variously near the apex, clustered, conidia dark, 1 or 2 celled, ovoid to cylindrical, some typically lemon-shaped. According to these microscopical characteristics, D12 may be *Cladosporium*sp. as shown in Figure (12).



Surface view **Reverse view** **Micrograph of *Cladosporium* sp. (X400)**

Figure 12 Cultural characters and morphological character of isolated fungi D12 from paddy mill soil

Discussion and Conclusion

A total of twenty-three fungi were isolated from bean crop soil (Vegetable and Fruit Resources Development Centre, Hlegu Township) and paddy mill soil (Daik-U) Township on Potato Dextrose Agar medium (supplement with 1% Casein). Among all isolated fungi, eleven fungi from bean crop soil and twelve fungi from paddy mill soil were study for preliminary protease producing fungi on Skim Milk Agar (SMA) medium. In the 8 proteolytic fungi, V2 (2.5cm) for four days, V5 (3.5cm) for four days from bean crop soil and D4 (2.2cm) for four days, D12 (2cm) for six days from paddy mill soil were selected based on larger clear zone of hydrolysis (halo) as shown in Table (2) and Figure (1 to 8).

The maximum protease activity of four selected fungi were subjected to colony morphology and spores formation to identify probably fungi species and our fungi isolate were found out to be of three *Penicillium* sp. (V2, V5 and D4) and one *Cladosporium* sp. (D12) as shown in Figure (9 to12). Vaishali Choudhary and P.C. Jain, 2012 were reported that the valuable for protease production observed in the garden soil, crop field soil and poultry farm soil. Vamsi Krishna *et al*., 2009 and Benluvankar *et al*., 2016 were stated that a high protease activities secreted by soil fungi observed in *Penicillum*sp. Sawao *et al*., 1971 were also reported that the acid protease of *Cladosporium* sp. as a good strains for the producing of protease

enzyme. The investigation of present research was focused on isolation; screening and identification of protease producing fungi were showed significant ability to degrade the proteolytic material in present plantation site. The selected proteolytic strains from this study were be continuing for further study to examine the optimal experimental conditions for production of protease enzyme.

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