Research Article

Isolation and Screening of Protease Producing Soil Fungi

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Abstract

Background: Proteases are enzymes, whose function is to cleave peptide bonds of proteins and split them down into small polypeptides or free amino acids. Fungi are commercially imperative organisms for their capability to secret enzymes including proteases into the surrounding environment. Proteases are currently used in various industries. **Objectives:** In the present investigation we have isolated and identified certain extracellular protease producing fungi among the other soil fungi. **Materials and methods:** Fungi were isolated by serial dilution method from soil samples collected from 3 different sites. Isolated fungi were inoculated on skim milk, casein and gelatin agar plate for identification of extracellular protease producers. **Results:** A total of 31 fungal isolates were screened on agar medium. Among these, 6 isolates exhibited zone of hydrolysis on casein agar plates, 3 isolates exhibited clear zone on skim milk agar plates and 2 isolates were found positive on gelatin agar plates. **Conclusion:** Based on primary screening, we have identified 11 fungal isolates as protease producers. Proteolytic potential of these fungi can be further confirmed by secondary screening in submerged fermentation.

Keywords: Protease, fungi, soil, casein, gelatin, skim milk, agar plates

Introduction

Proteases are one of the most significant groups of industrial enzymes, contributing for approximately 60% of the global enzyme sell (Nunes and Martins, 2001). Proteases are industrially valuable enzymes and they are broadly used in number of industries including detergent, pharmaceutical, leather, textile, food, silver recovery (Abidi et al., 2008). The use of alkaline proteases for hydrolytic cleavage of fibrous proteins of feather, hair and horn has been well reported. Alkaline proteases are also used for resolution of the racemic mixture of amino acids, peptide synthesis, hydrolytic cleavage of gelatin layers in X-ray films (Gupta et al., 2002). Proteases can be obtained from animals, plants and microbes. But microbial proteases are preferred over animal and plant sources. Various microbes such as prokaryotes (bacteria and actinomycetes) and eukaryotes (fungi and yeast) are well known to produce protease enzymes (Madan et al., 2002). Potential utilization of fungal proteases is being more and more realized because downstream processing become easier with fungal proteases (Joo et al., 2001). Among the fungi, filamentous fungi are well known to produce a variety of hydrolytic enzymes and proteases are one of main group of enzymes. The different species of the genera (*Penicillium*, *Aspergillus*, *Rhizopus*, *Alternaria*, *Trichoderma*) are regarded as safe for the production of proteases and easy recovery from fermentation broth (Sandhya et al., 2005).

Proteases are complex enzymes that vary from each other in characteristics for instance specificity to substrate, specificity to active site and mode of action (Rao et al., 1998). Soil is an ideal habitat for a variety of microbes associated with the production of number of hydrolytic enzymes. Numerous techniques can be utilized for screening of protease producers based on the detection of the occurrence of extracellular proteases. The utilization of a solid agar medium containing casein, gelatin, skim milk protein has been broadly explained in the literature (Rodarte et al., 2011). In view of this background we have collected soil samples from 3 different sites and used them for isolation of fungi followed by their screening on skim milk agar plate, gelatin agar plate and casein agar plate for identification of protease producers.

Materials and methods

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Collection of soil samples

Soil samples were collected using sterile spatula from 3 diverse areas (mustard crop field, wheat crop field and medicinal garden of Banasthali University) and transferred in sterile Petri plates. Petri plates were labeled with the site of collection, brought to laboratory and stored in refrigerator till use.

Isolation of soil fungi

Fungi were obtained by serial dilution agar plate method as explained earlier by Waksman (1922). One gram of each of the soil sample was separately added in test tube containing 9 ml of sterile distilled water followed by vortexing for 10 minutes to make soil suspension of 10^{-1} dilution. One ml of soil suspension from 10^{-1} dilution was transferred in another test tube containing 9 ml of sterile distilled water to prepare 10^{-2} dilution. This step (withdrawal and transfer of soil suspension) was repeated until the preparation of 10^{-6} dilution. 0.1 ml of each of the soil dilution (10^{-1} to 10^{-6}) was aseptically inoculated and uniformly spreaded in sterile Petri plate containing the potato dextrose agar (PDA) medium (Sinha et al., 2013). Streptomycin antibiotic was added in PDA medium just before pouring of PDA medium into Petri plates to avoid growth of bacteria in PDA plates. The inoculated PDA plates were kept at 28ºC for 5 days in an incubator. Different fungi appeared on PDA plates were further purified by point inoculation in PDA plates followed by incubation.

Screening

Fungal isolates were assessed for detection of lipase production on skim milk agar plate and casein agar plate. The composition of skim milk agar medium (g l^{-1}) is as follows: skim milk powder, 28; casein enzymatic hydrolysate, 5; yeast extract, 2.5; dextrose, 1; agar, 15 and pH was adjusted to 7.2. The composition of gelatin agar medium (g l^{-1}) is as follows: gelatin, 30; casein enzymatic hydrolysate, 10; sodium chloride, 10; agar, 15 and pH was adjusted to 7.2. The composition of casein agar medium (g l^{-1}) is as follows: KH_2PO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1; glucose, 10; 15% of casein, 25; agar, 13 and pH was adjusted to 7.2. 15% casein was made by dissolving 3.75 g casein in 25 ml of distilled water. The isolates were individually point inoculated on skim milk and casein agar plate and kept at 28˚C for 6-8 days. Inoculated plates were observed for clear zone around fungal colonies. Zone of hydrolysis in gelatin agar plate was observed only after flooding the plate with mercury chloride solution.

Results and discussions

Isolation of soil fungi

The results of isolation are presented in Table 1 and Figure 1 to 3. Diverse fungi with different colony morphology and characteristics (texture, sporulation, zonation, shape, size, surface and reverse colouration) were appeared on PDA plates from different dilutions of soil samples (Figure 1 to 3). The texture of the isolated fungi was velvety, floccose and powdery. The zonation of isolated fungi was slightly radially furrowed to heavily furrowed on reverse side. Poor, moderate and heavy sporulation pattern was observed. The colour of isolated fungi was black, white, brown, orange, creamish, chocolate and green. Some of the fungi were growing all around Petri plate. Most of the fungi were circular in shape and remaining fungi were irregular in shape.

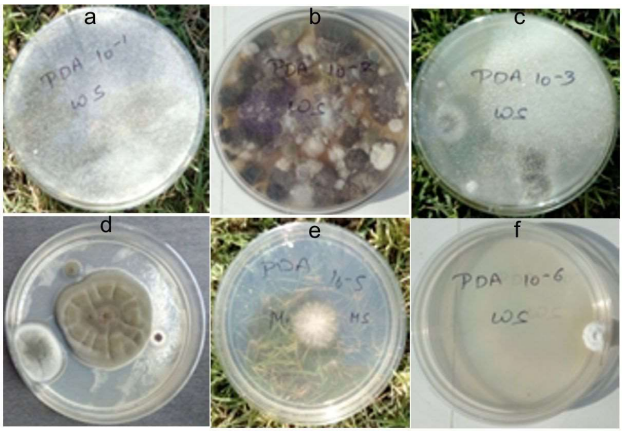

Figure 1. PDA plates showing different fungal colonies in different dilutions of soil sample (wheat crop field) **(a):** Dilution 10^{-1}; **(b):** Dilution 10^{-2}; **(c):** Dilution 10^{-3}; **(d):** Dilution 10^{-4}; **(e):** Dilution 10^{-5} **(f):** Dilution 10^{-6}.

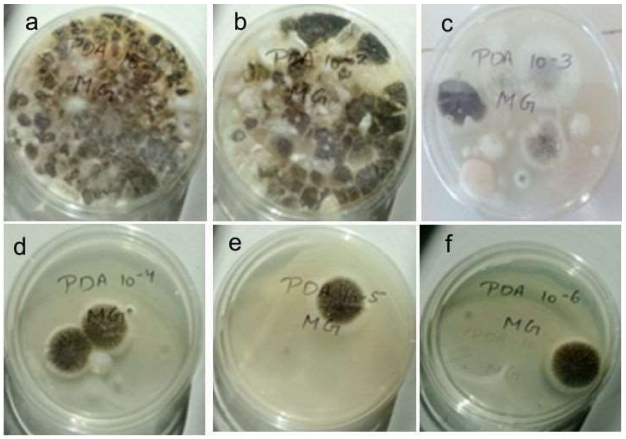

Figure 2. PDA plates showing different fungal colonies in different dilutions of soil sample (medicinal garden) **(a):** Dilution 10^{-1}; **(b):** Dilution 10^{-2}; **(c):** Dilution 10^{-3}; **(d):** Dilution 10^{-4}; **(e):** Dilution 10^{-5} **(f):** Dilution 10^{-6}.

Figure 2 represents that most of the fungi isolated from medicinal garden soil were black-green in colour and their circular colony was surrounded by white peripheral ring whereas Figure 3 represents that most of the fungi isolated from mustard crop field soil were chocolate in colour and

their circular colony was surrounded by white peripheral ring. Table 1 represents that maximum load of fungi were obtained from soil sample of medicinal garden whereas least number of fungi were obtained from the soil sample of mustard crop field.

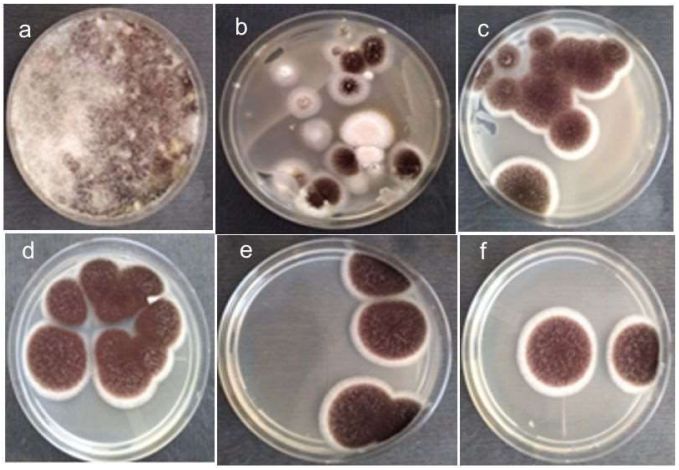

Figure 3. PDA plates showing different fungal colonies in different dilutions of soil sample (mustard crop field) **(a):** Dilution 10^{-1}; **(b):** Dilution 10^{-2}; **(c):** Dilution 10^{-3}; **(d):** Dilution 10^{-4}; **(e):** Dilution 10^{-5} **(f):** Dilution 10^{-6}.

Soni and Sharma (2014) reported isolation of mycoflora by serial dilution agar plate technique from soil samples of different regions of Bhopal. Total 14 species were obtained from 20 soil samples. Among all the species, *Aspergillus niger* was dominant. Choudhary and Jain (2012) collected soil samples from 3 different sites (garden soil, crop field and poultry farm) and used for isolation of fungi followed by screening for identification of protease producers on Reese agar medium. Geethanjali and Reshma (2014) reported isolation and screening of protease producing fungi from soil samples of forest region.

Table 1. Total number of fungi appeared on PDA plates from different dilutions of 3 different soil samples

Dilutions	Medicinal garden soil	Mustard crop soil	Wheat crop soil
10^{-1}	49	TNTC	TNTC
10^{-2}	30	16	43
10^{-3}	12	11	4
10^{-4}	6	8	4
10^{-5}	1	4	1
10^{-6}	1	2	1

TNTC: Too numerous to count.

Screening

Based on the difference in colony morphology and characteristics different fungal isolates were used for screening in skim milk, casein and gelatin agar plates. Protease producing fungi on were identified by the appearance of zone of hydrolysis (clear zone/halo zone) around their colonies (Figure 4). Clear zone was developed due to hydrolysis of milk protein/casein/gelatin by extracellular protease into small polypeptides and free amino acids, therefore opacity of the medium around fungal colony was not retained. Presence of opacity around fungal colony indicated that fungal colony did not produce extracellular protease in its vicinity, therefore milk protein/casein was not degraded, hence opacity of the medium was retained.

We have tested 31 fungal isolates on skim, gelatin and casein agar plate. Among these, 6 isolates were found

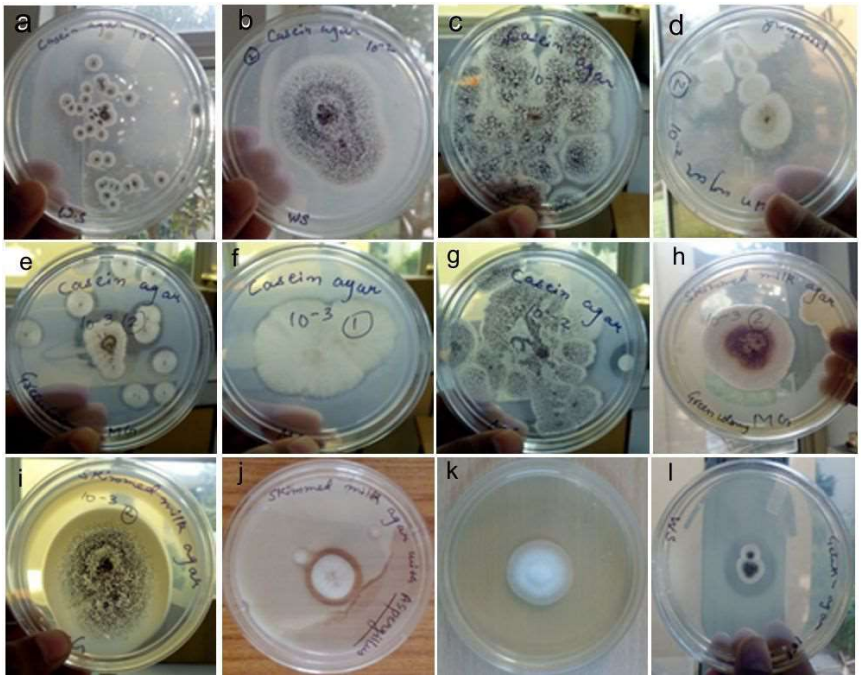

Figure 4. Zone of hydrolysis around fungal colony: casein agar plate (**a-g**); skim milk agar plate (**h-j**) and gelatin agar plate (**k-l**).

positive on casein agar plate, 3 isolates were found positive on skim milk agar plate and 2 isolates were found positive on gelatin agar plate.

Alnahdi (2012) reported screening of bacterial isolates of sea water on gelatin agar plates. Protease producers were identified by appearance of clear zone (developed due to hydrolysis of gelatin by the action of extracellular protease) around their colonies. Clear zones were visible upon flooding the plates with mercury chloride solution. Choudhary and Jain (2012) reported screening of 141 fungal isolates on casein agar plates for the detection of alkaline protease producers. Among the all isolates, 108 fungal isolates demonstrated zone of hydrolysis around their colonies. Palsaniya et al. (2012) reported isolation and screening of soil bacterial isolates on gelatin agar medium. At the end of incubation plates were flooded with 1% tannic acid for visualization of clear zone around bacterial streak line. Similarly gelatin agar medium was also used for screening of protease producers by Josephine et al. (2012).

Conclusion

The demand of proteases is continuously increasing in the industries, therefore our aim was to isolate and identify the extracellular protease secreting fungi from soil samples of different sites. In this context, fungi were isolated on PDA plates by serial dilution method followed by their screening. Among the all (31) tested fungi, 6 isolates were found positive on casein agar plate, 3 isolates demonstrated clear zone on skim milk agar plate and 2 isolates were found positive on gelatin agar plate. Further studies on these 11 fungal isolates and their product (proteases) can be carried out to find out the protease of unique property and stability.

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Declaration of interest

The authors report no declarations of interest.

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