

RESEARCH ARTICLE

Isolation and identification of alkaline protease producing fungi from soils of different habitats of Sagar and Jabalpur District (M.P)

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Abstract

Proteolytic fungi from the soil samples were isolated on Reese agar plate containing casein as the protein substrate. The mycoflora of different localities of Sagar and Jabalpur districts of Madhya Pradesh were investigated at three different locations viz. Garden, Crop field and Poultry farms. These samples were labeled according to the site of collection as GS (Garden Soil), CFS (Crop Field Soil) and PFS (Poultry Farm Soil). Fifty soil samples were collected from the superficial layer of soil, 5-6 cm depth. Alkaline pH was observed across the sampling sites and a total of 38 samples were found positive for the occurrence of fungi. The total fungal counts of the soils were estimated by standard dilution plate technique. The isolated fungi were identified by their cultural and morphological characteristics. One hundred forty one fungal forms were obtained from the positive soil samples. These included 56 fungal forms from Poultry farm, 47 from Garden soil and 38 from Crop field soils. Greater numbers of species were isolated on dilution plate method as comparison to soil plate method. Using conventional media and techniques, all collection sites yielded populations of filamentous fungi belonging to the phylum Ascomycota (5), Deuteromycota (4), Zygomycota (4). *Aspergillus* and *Fusarium* were the most predominant genera.

Keywords: Proteolytic fungi, mycoflora, poultry farm, *Aspergillus*, *Fusarium*.

Introduction

The soil is a dynamic medium for microbial/biological activities (Griffin, 1972) and that the number and kind of microorganisms present in a particular soil depends on many environmental factors such as amount and type of available nutrients, moisture and the degree of aeration, pH, temperature etc (Prescott *et al.*, 1993). They play pivotal role in various biochemical processes and thus are responsible for recycling of organic compounds in nature. The beneficial effects of soil microorganisms are manifold and range from nitrogen fixation to break down of complex organic matter of plant and animal origin, production of industrial products such as primary metabolites (enzymes, amino acid and vitamins), production of secondary metabolites (antibiotics, alcohol and organic acids), and/or enhancing the bioavailability of nitrates, sulfates, phosphates etc in soils (Bridge and Spooner, 2001). Fungi are an important component of the microbiota typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions (Ainsworth and Bisby, 1995). The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem. They perform ecological services that strongly impact the quality of human life and have enormous potential for providing economic benefits. It is estimated that there are 1.5 million fungal species on earth, of which only 70,000 have been described up to now thus presents a potential source of novel organisms (Hawksworth and Rossman, 1997; Hawksworth, 2001).

Soil borne fungi are adopted to survive at a broad range of pH. As a result of pH range for vigor and growth, they are more destructive at acid, neutral and alkaline pH. Fungi make up to 5×10^3 to 9.0×10^6 counts per gram of soil (Atlas and Bartha, 1998). They grow and carry out active metabolism when conditions are favorable with adequate moisture, aeration and relatively high concentration of utilizable substrates (Miyamoto *et al.*, 2002). Early in 1922, Waksman isolated fungi from soil and counted the number of fungi present in soil and Warcup in 1950, isolated fungi from soil using soil-plate method. Using these methods a number of workers provided information indicating wide occurrence of fungi in soils of various habitats including rhizosphere soils (Manivannan and Kathiresan, 2007), poultry farm soils (Nehra *et al.*, 2002; Charles *et al.*, 2008), garden soils (Nehra *et al.*, 2002; Kalpana Devi *et al.*, 2008), sandy soils (Al-Falih, 2001), soil samples from butcherries (Usama, 2008) etc. Isolation of industrially important microorganisms possessing proteolytic activity has also been carried out by many workers. Nehra and co-workers (2002) isolated proteolytic fungi from diverse sources such as garden soil and alkaline soil. Kalpana Devi (2008) isolated proteolytic *Aspergillus niger* from soil a sample of Coimbatore, Tamil Nadu. Poultry farm soil was used for isolation of *Aspergillus nidulans* HA-10, a proteolytic fungus by Charles *et al.* (2008). Soil yeasts isolated from sandy soil obtained from Riyadh, Saudi Arabia were tested for their proteolytic activity by Al-Falih (2001).



Table 1. Details of soil samples collected from different habitats of district Sagar and Jabalpur, M.P.

Habitats	Locality	Number of samples	pH (range)
District Sagar, M.P.			
Garden soil	Microbiology Department (AMB Dept.)	2	6.9-7.0
	Zoology Department	3	7.0-7.5
	Botany Department	2	7.3-7.5
	Civil Lines	2	7.8-8.1
Crop field	Makronia	2	7.8-8.0
	Patharia	5	6.5-8.1
	Tikitoria	2	7.7-8.1
	Makronia	2	7.7-8.0
Poultry farm	Jhansi Road	1	7.9-8.0
	Bartuma	2	7.7-7.9
	Bamhori	2	7.9-8.0
District Jabalpur, M.P.			
Garden soil	Bhavartal	3	7.8-8.0
	Tagore udyan	2	7.9-8.1
	Vijay Nagar	2	7.3-7.5
	Sohagi	1	7.9-8.0
Crop field	Bergi	2	7.3-7.5
	Patan	2	6.8-6.9
	Vijay Nagar	3	7.3-7.5
	Panagarh	2	7.8-8.0
Poultry farm	Barella	3	7.3-7.5
	Snehnagar	2	7.3-7.5
	Katanga	3	7.9-8.1
Total		50	

Table 2. Distribution of positive samples in different habitats of district Sagar and Jabalpur, M.P.

Habitats	Place of sample collection	No. of samples tested	No. of positive Samples	No. of fungi isolated
District Sagar, M.P.				
Garden soil	Microbiology Dept. (AMB Dept.)	2	2	5
	Zoology Department	3	1	4
	Botany Department	2	2	5
	Civil lines	2	1	5
Crop field	Makronia	2	1	7
	Patharia	5	3	10
	Tikitoria	2	1	4
Poultry farm	Makronia	2	2	7
	Jhansi Road	1	1	6
	Bartuma	2	2	7
	Bamhori	2	2	9
District Jabalpur, M.P.				
Garden Soil	Bhavartal	3	2	11
	Tagore Udyan	2	2	9
	Vijay Nagar	2	2	8
	Sohagi	1	1	1
Crop field	Bergi	2	1	2
	Patan	2	1	5
	Vijay Nagar	3	3	9
	Panagarh	2	2	6
Poultry farm	Snehpura	3	1	7
	Barella	2	2	6
	Katanga	3	3	8
Total		50	38	

Table 3. Distribution of positive soil samples in different habitats of surveyed districts.

Sources of soil samples	No. of samples examined	No. of samples positive	Positive samples (%)
Garden Soil	16	12	75.00
Crop field soil	17	11	64.70
Poultry farm soil	17	15	88.23
Total	50	38	76.00

Manivannan and Kathiresan (2007) isolated a fungus *Penicillium fellutanum* from rhizosphere soil of a mangrove of the bank of Vellar estuary, Portonovo, South East coast of India. Back in 1993, Dixit and Verma isolated mesophilic proteolytic fungi from the forest soil from Bhopal, Madhya Pradesh. Monem and Omkoithoum (1992) isolated two proteolytic fungi *Aspergillus fumigatus* and *Penicillium* sp. from Thaqbah soil in Saudi Arabia. A thermotolerant mesophilic fungus *Aspergillus terreus* was isolated from butcheries soil sample at Zagazig, Sharkia, Egypt by Usama Ali (2008) and was tested for its proteolytic activity. A yeast strain, *Aureobasidium pullulans* was isolated from sediments of saltern in Qingdao, China that produced proteases (Chi *et al.*, 2007). Propagules of the proteolytic fungi are also found to be carried by soils of deep sea sediments and sediments of coral lagoon. Raghukumar *et al.* (2003) isolated *Aspergillus ustus*, a deep-sea fungus from the sediments of Central Indian Basin and tested it for its protease producing ability.

Ikram-ul-Haq *et al.* (2006) isolated *Penicillium chrysogenum* IHN₅ from soil samples of different places of Lahore. Negi and Banerjee (2006) isolated *Aspergillus awamori* an amylolytic and proteolytic fungus from soils of Kharagpur, West Bengal. A few mould cultures of *Rhizopus* spp. were isolated from soil samples of Lahore area by pour plate method by Ikram-ul-Haq (2003). A new *Aspergillus flavus* strain IMI 327634 was isolated from soils of Madras by Malathi and Chakraborty (1991). It was found to produce protease having depilatory properties. In addition to soils, proteolytic fungi have also been isolated from a wide variety of other substrates. Nehra *et al.* (2002) used decaying wheat straw, decomposing poultry waste and spoiled meat for isolation of proteolytic *Aspergilli* spp. An isolate of *Aspergillus fumigatus* B149 was isolated from degraded blood samples collected from the Government abattoir, Jabalpur (M.P) by Renu *et al.* (2001).

In Thailand, a yellow-green *Aspergillus flavus* var *columnaris* was isolated from the soy sauce industry and used in production of protease by Impoolsup *et al.* (1981). A strain of *Alternaria alternata* isolated from infected mandarin oranges (Kunte and Shastri, 1979) was found to produce proteolytic enzymes in wheat bran Czapek dox medium (Patil and Shastri, 1981). Shumi *et al.* (2004) tested different protein sources (i.e., pulses, fish, dry fish and meat) to enhance recovery of proteolytic fungi for their industrial applications.

Mulimani *et al.* (2002) isolated a bleach stable and alkali-tolerant protease producing fungus from spoiled casein. A strain of *Aspergillus flavus* (AP2) possessing proteolytic properties was isolated from pulse sample by Flora (2004). Review of literature indicates a wide occurrence of proteolytic fungi in soils of various countries, sediments, spoiled casein, pulses, fish, meat etc. It is also evident from several studies that from soils these fungi can be easily isolated using pour plate method or serial dilution methods as suggested by Warcup (1950) and Waksman (1927). These techniques also allow growth of a number of bacteria and actinomycetes in plates and posing certain problems in isolation of fungi in pure form. However, the methods are available to avoid bacterial growth by supplementing an antibiotic Chloramphenicol (0.1 g/L) in the medium (Boukhout and Robert, 2003).

Materials and methods

Sample collection: The soil samples used for the isolation of proteolytic fungi were collected from three sites viz. gardens, crop fields and poultry farms of different localities of Sagar (23°50 N latitude and 78°43 E longitude) and Jabalpur (23°10 N latitude and 79°59 E longitude) districts of Madhya Pradesh. The samples from the above habitats were collected randomly from the superficial layer of soil not exceeding 5-6 cm depth using pre-sterilized spatula and were transferred into sterilized polythene bags. The samples were then brought to the laboratory and kept at 15°C until processed.

Soil samples

Samples of garden soils rich in organic matter were collected from different localities of Sagar and Jabalpur districts. The areas surveyed are given in Table 1 and 2. The soils rich in nitrogenous/proteinaceous waste materials/fertilizers etc were given preference during collection. The sites of collection in two districts are given in Table 1 and 2. Samples of poultry farm soils rich in proteinaceous organic waste were given preference. The details of collection sites are shown in Table 1 and 2.

Determination of pH of soil samples

The pH of all the samples was taken using a suspension of the soil samples (Table 1). For these 10 g of each soil samples was suspended in 100 mL of double distilled water and shaken vigorously for 30 min. The pH of the sample was then determined using pH meter following Foster (1995).

Isolation of fungi

Reese Agar medium (K₂HPO₄-2.0 g, (NH₄)₂SO₄-1.5 g, MgSO₄.7H₂O-0.3 g, CaCl₂-0.3 g, Urea-0.3 g, Yeast Extract-0.5 g, Glucose-2.5 g, Casein-5.0 g, Agar-20 g, Distilled Water-1000 mL) was chosen as growth medium. The initial pH of the medium was adjusted to 9.0 with solution of 1 N NaOH.

Table 4. Distribution of alkaline protease producing fungi in different habitats of District Sagar and Jabalpur (M.P).

Fungi Isolated	District Sagar (M.P)			District Jabalpur (M.P)			Total No. of Isolates
	GS	CF	PF	GS	CF	PF	
<i>Absidia</i> sp.	-	-	1	1	1	1	4
<i>Alternaria alternata</i>	-	-	2	-	-	1	3
<i>Alternaria</i> sp.	-	-	1	-	1	1	3
<i>Aspergillus flavipes</i>	-	1	-	2	-	-	3
<i>Aspergillus flavus</i>	2	1	3	2	-	3	11
<i>Aspergillus fumigatus</i>	1	2	2	2	1	1	9
<i>Aspergillus niger</i>	4	3	2	2	3	3	17
<i>Aspergillus nidulans</i>	-	1	2	2	1	1	7
<i>Aspergillus ochraceous</i>	1	-	1	-	-	1	3
<i>Aspergillus oryzae</i>	-	-	-	1	-	1	2
<i>Aspergillus penicillioides</i>	-	-	-	1	-	-	1
<i>Aspergillus</i> sp.	2	1	2	-	2	2	9
<i>Aspergillus restrictus</i>	-	1	-	-	2	-	3
<i>Aspergillus terreus</i>	-	-	-	-	-	2	2
<i>Aspergillus ustus</i>	-	1	1	1	-	-	3
<i>Aspergillus versicolor</i>	1	1	1	-	-	-	3
<i>Chrysosporium indicum</i>	-	-	-	-	-	1	1
<i>Curvularia lunata</i>	1	1	1	-	-	-	3
<i>Curvularia</i> sp.	-	2	-	-	-	1	3
<i>Fusarium chlamydosporum</i>	-	-	-	-	1	-	1
<i>Fusarium moniliforme</i>	-	-	-	2	-	3	5
<i>Fusarium oxysporum</i>	-	-	-	-	1	1	2
<i>Fusarium solani</i>	-	-	1	-	-	1	2
<i>Fusarium</i> sp.	1	1	-	1	-	-	3
<i>Helminthosporium</i> sp.	1	1	-	3	-	-	5
<i>Humicola</i> sp.	-	-	1	-	-	-	1
<i>Malbranchea</i> sp.	1	-	2	1	2	1	7
<i>Mucor racemosus</i>	-	-	-	1	1	-	2
<i>Mucor</i> sp.	-	1	1	-	-	-	2
<i>Paecilomyces lilacinus</i>	-	-	-	1	-	-	1
<i>Paecilomyces</i> sp.	-	1	1	2	-	-	4
<i>Penicillium</i> sp.	3	2	2	-	-	1	8
<i>Rhizopus</i> sp.	-	-	1	1	1	1	4
<i>Rhizopus stolonifer</i>	1	1	1	1	-	-	4
Total	19	21	29	28	17	27	141

The fungal strains from the soil samples were isolated by serial dilution method (Waksman, 1927) on Reese Agar media (Reese *et al.*, 1950). The soil sample was mixed and a suspension of 1 g (dry weight equivalent) in 10 mL of sterile distilled water was prepared. One ml of the soil suspension was then diluted serially (six fold) and used in the estimation of fungal population. The plates were incubated at $\pm 28^{\circ}\text{C}$ for 4-5 d.

Purification of isolated fungi and identification

The isolated fungi were purified by point inoculating them on plates containing PDA (Potato dextrose agar) medium. The fungi were purified by repeated point inoculation. The purity of the isolated fungus was confirmed by microscopic examination of the culture at 400X magnification using light microscope. After ensuring purity, the cultures were subcultured on PDA slants and allowed to grow for a period of 5-7 d and subsequently stored at 4°C as stock cultures.

Usually, working as well as stock cultures are maintained and the working cultures were transferred to fresh PDA slants at regular intervals of 3 months. The isolated fungi were sub cultured on potato dextrose agar and allowed to grow and sporulate. On the basis of their colony and morphological characteristics, the fungi were identified. Lacto phenol cotton blue stain was used as mounting fluid. The slides were observed under microscope and fungi were identified by following the mycological literature. The following morphological characteristics were evaluated: colony growth (length and width), presence or absence of aerial mycelium, colony color, presence of wrinkles and furrows, pigment production etc. The characteristics were compared with the standard description of, 'A manual of Soil Fungi', by Gilman, (1957), 'Industrial Mycology' by Onions *et al.* (1981) and 'Compendium of Soil Fungi' by Domsch and Gams (1980).

Results and discussion

Fungi occurring in natural habitats with changing environmental conditions are important from the industrial point of views. They can be found producing novel metabolites or enzymes with hyper catalytic properties and hence, in the present study an attempt has been made to isolate the proteolytic fungi from soils collected from different habitats. Fifty soil samples collected from different habitats have been used for the isolation of fungi. These include 9 samples of garden soil, 9 of crop fields and 7 samples of poultry farm soils of district Sagar, M.P. and 7 of garden soil, 8 of crop field and 10 samples of poultry farm soils of district Jabalpur, M.P (Table 1). Out of these, a total of 38 samples were found positive for the occurrence of fungi (Table 2). The percentage distribution of positive samples in different habitats was found to be 64.70, 75 and 88.23% in samples collected from crop fields, gardens and poultry farm soils, respectively (Table 3).

In the present study, a total of 141 fungal forms were obtained from the positive samples. These include 56 fungal forms from poultry farm soils, 47 from garden soil and 38 from crop field soils (Table 4). The soil samples of poultry farms yielded maximum number of fungi. These were identified and found to belong to 16 species of 11 genera including *Absidia*, *Alternaria*, *Aspergillus*, *Chrysosporium*, *Curvularia*, *Fusarium*., *Humicola*, *Malbranchea*, *Mucor*, *Penicillium* and *Rhizopus*. In the present study, the samples belonging to garden soil yielded a total of 10 different fungal genera i.e., *Absidia*, *Aspergillus*, *Curvularia*, *Fusarium*, *Helminthosporium*, *Malbranchea*, *Mucor*, *Paecilomyces*, *Penicillium* and *Rhizopus* while 11 fungal genera i.e., *Absidia*, *Alternaria*, *Aspergillus*, *Curvularia*, *Fusarium*, *Helminthosporium*., *Malbranchea*, *Mucor*, *Paecilomyces*, *Penicillium* and *Rhizopus* were isolated from samples of crop field soils. A total of 73 fungal forms belonging to *Aspergillus* were isolated from samples of district Sagar and Jabalpur. Among 73 isolates of *Aspergilli*, 37 were obtained from Jabalpur district, while 36 were isolated from Sagar district. A total of 14 isolates were belonging to genus *Fusarium*. Among these, 4 were collected from soil samples of Sagar district while 10 were isolated from samples of district Jabalpur. Out of 8 species of *Penicillium*, 7 were isolated from soils of Sagar district.

The data given in Table 4 indicated the dominance of genus *Aspergillus* in the soils of surveyed habitats. Members of *Aspergillus* have been reported widely in soil since their nature allows them to have nutrients and moisture for all different studies of life cycle. The published work on global distribution of *Aspergilli* in soil has been documented by Domesch *et al.* (1980), Raper and Fennel (1965) and Christensen and Tuthill (1985). However, Klich (2002) gave an account on distribution of *Aspergilli* and diversity based on data from 62 soil surveys and found that they occur in abundance in tropical and subtropical latitudes and that many rare species occur only in these areas.

In present study, a total of 73 isolates belonging to 13 different species have been recorded (Table 4). Among these maximum number of isolates belonging to *Aspergillus niger* have been collected. *Aspergillus niger*, *A. fumigatus* have been isolated from all habitats during present study. The second most dominant genus was found to be *Fusarium* in the present study. Five different species have been recorded during present investigations. Isolates of *Fusarium moniliforme* were recorded only from soils of gardens and poultry farms of district Jabalpur only. Two isolates of *F. oxysporum* were recorded from soils of district Jabalpur only. The distribution and record of isolation of all other isolated fungi is presented in Table 4. Soils of habitats such as gardens, crop fields and poultry farms etc. are considered as rich habitats for fungi with varied metabolic potential. *Aspergillus* species have been of interest for centuries because of their positive impact as fermentation agents and because of their negative and their toxicity impact as degraders of stored agriculture products (Jain *et al.*, 1994; Jain, 1999). Microbial proteases replaced traditional proteases from animal and plant resources and have found various applications in recent era. Proteases are widely distributed among microorganisms including fungi, bacteria and actinomycetes (Jain *et al.*, 2010). Among fungi species of certain genera such as *Aspergillus*, *Penicillium*, *Paecilomyces*, *Rhizopus* and *Rhizomucor* are well-know producers of proteases (Prudlov *et al.*, 1973; Kundu and Manna, 1975; Mukherjee and Chaudhuri, 1977; Anglo and Orillo, 1977; Baranova *et al.*, 1979; Tomod *et al.*, 1979; Impoolsup *et al.*, 1981; Khan *et al.*, 1983; Dahot, 1987; Abbas *et al.*, 1989; Thakur *et al.*, 1990; Malathi and Chakraborty, 1991; Banerjee and Bhattacharyya, 1992; Dahot, 1993; Hanzi *et al.*, 1993; Iksari and Mitchell, 1994; Suseela, 1998; Aikat and Bhattacharyya, 2001; Marzan *et al.*, 2001; Mulimanni *et al.*, 2002; Sandrogermano *et al.*, 2003; Shumi *et al.*, 2004; Agrawal *et al.*, 2004; Haq *et al.*, 2004; Oliveria *et al.*, 2006; Srinubabu *et al.*, 2007; Manivannan and Kathiresan, 2007; Kalpana Devi *et al.*, 2008; Sindhu *et al.*, 2009; Vamsi Krishna *et al.*, 2009) and are reported active over a wide pH range (Rao *et al.*, 1998).

Conclusion

In the present study, a number of fungi belonging to *Paecilomyces*, *Rhizopus*, *Malbranchea*, *Mucor*, *Humicola*, *Curvularia* and *Alternaria* have been isolated using Reese medium having pH 9.0. Their growth on Reese medium is although high indicating their pH tolerance but may be found with greater protease producing potential.

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