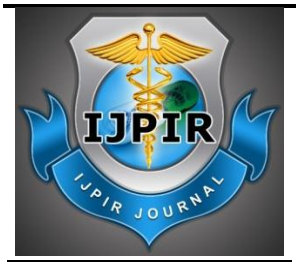
Research Article



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Screening of industrial important enzymes from microfungal isolates of agricultural soil and litter samples from Sivakasi, Tamil Nadu

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ABSTRACT

Fungi colonization and subsequent decomposition of plant substrate depended as much on the ability of the fungi to produce enzymes necessary to degrade particular plant polymers. The degradation of the substrate is achieved only through a range of enzymes produced by the fungi. In recent year importance is given to our understanding of the diversity of fungi and has great scope for industrial application. Seven micro-fungal species were isolated from three samples such as agricultural soil, leaf litter soil, and wood debris soil. Fungal species were isolated through pure culture techniques and their pH tolerant studies between 6 and 8. The growth characteristic was studied in relation to alkalinity and pH. The fungal species were tested for amylase, protease, cellulose, lipase enzyme through plate assays. The maximum fungal enzyme activities were screened in *Aspergillus flavus* for amylase, cellulase for *Aspergillus niger*, protease for *Aspergillus ochraceus*, lipase for *Aspergillus terreus.*

Keywords: Micro-fungal diversity, Fungal growth, Industrial enzymes, Plate assay.

INTRODUCTION

Fungi are among the most numerically abundant organisms in the terrestrial ecosystem and are the primary decomposers of organic residues in soil. They account for as much as 70 to 80% by weight of the soil microbial biomass. Fungi are unique organisms due to their morphological, physiological, and genetic features, and they are ubiquitous, and able to colonize all matrices such as soil, water, air, in natural environments, in which they play key roles in maintaining the ecosystems equilibrium. The air is an important vehicle for the dissemination of fungal propagules (conidia, spores, hyphae and sclerotia), which represent the main component of the bioaerosol [1].

In terms of biodiversity, fungi are probably the second most common group of organisms on our planet, with an estimated 1.5 million species,

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second only to arthropods [20]. All fungi are heterotrophic and obtain the organic substance necessary for their growth through three different ways, saprophytism, parasitism (pathosistic symbiosis), and mutualistic symbiosis. Saprotrophic fungi play a key role in the decomposition of organic matter and, therefore, in the circulation of the elements, both in natural and anthropic environments [3]. Depending on the soil depth and nutrient conditions the fungi play an important component on the microbiota and typically constituting more of the soil biomass than bacteria [2]. Soil microfungi play an important role in the degradation of organic debris [7]. The species of *Aspergillus, Penicillium* and *Paecilomyces Bainer* are among the most abundant and widely distributed microfungi in nature [10]. Microfungi play a focal role in nutrient cycling in regulating soil biological activity [4]. High sporulation capability of fungi as mitosporic fungi such as Deuteromycetes, Zygomycetes, can easily.

Role of decomposition of organic matter is determined by the fungi depend on the ability to utilize the plant substrate. Besides the chemical composition, environmental factors such as temperature, moisture content, availability of nutrients and energy-source, regulate the process of litter decomposition. It is known that no single species of fungi are able to use all components of plant litter completely and different fungi appear in succession on the substrate over a period of time in order to decompose the organic matter and release the bound nitrogen back to nature [19, 14]. The colonization and subsequent decomposition of plant substrate depended as much upon on the ability of the fungi to produce enzymes necessary to degrade particular plant polymers. The degradation of the substrate is achieved only through a range of enzymes produced by the fungi and other microorganisms. There is a growing realization that fungi inhabiting plants, living tissues and fallen leaf litter produce enzymes and secondary metabolites which are besides have many uses and application in industry and human welfare [24].

The potential of using microorganisms as biotechnological sources of industrially relevant enzymes have stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms [8]. Selection of the right organism plays a key role in high yield of desirable enzymes [25]. When the environment is unsuitable for growth in the condition of low nutrients, presence of metabolites, they able to grow with the help of their asexual spores, called conidia, show an exogenously imposed dormancy induced by the phenomenon termed fungi stasis, allowing the fungus to survive [11]. Fungi are microorganisms which are well known for their wide range of novelty of enzymes they produce and enzymes of fungal origin are used in the industrial process for which, amount to billions of dollars of revenue annually [5]. Due to their diversity, fungi have been recognized as a source of new enzymes with useful and novel characteristics [6].

MATERIALS AND METHODS

Sampling site

The soil sample was collected from the agricultural field, wood debris, leaf debris, in the area of Sivakasi, Virudhunagar district. The sample collecting site has located in the region of sivakasi is located in the Bypass road of long trees sub locality, sivakasi locality, Virudhunagar district, Tamilnadu state 626123.

The leaf litter soil was collected in the area of bypass road of long tree is situated latitude is 9”45’ and longitude is 77”79’. The agricultural soil samples were collected in the area of Katalipatti Sivakasi. kattalaipatti in is located in kaliappa nagar sub-locality, Sivakasi locality, Virudhunagar district, tamilnadu state 626123. The latitude of kattalaipatti in area is 9”,45’ and longitude of kattalaipatti 77”,78’.

The wood debris soil samples were collected in the area of Alangulam area, sivakasi. The Alangulam area is located in sithurajapuram sub locality, sivakasi locality, virudhunagar district, Tamilnadu 6126123. The latitude of alangulam area, 9”.43’ and longitude of Alangulam area is 77”, 79’.

Sample collection

Agricultural soil

Soil samples from the agricultural field was collected from slightly moisture content in the

depth of 2-3cm. They were immediately transferred to sterile polyethylene zip cover and taken to the laboratory for fungal analysis.

Wood debris soil

The wood debris soil was collected with the help of sterile spatula and transferred to sterile polyethylene zip cover for laboratory for fungal analysis.

Leaf litter soil

The litter sample contains three layers, they differentiated as L-upper, most layer consisting of recently fallen senescent leaves lying loosely on the surface of the soil. F1 layer immediately below the L layer with dark brown leaves having high moisture content, usually compacted. F2 layer-lower most layer below F1 layer and the soil surface in an advanced stage of decomposition. The semi-decomposed leaf litter soil was collected in the region of the F2 layer. The *Aegle marmelos* leaf was used for the isolation of fungi in the leaf litter site.

Fungal isolation from soil samples

One gram soil was mixed thoroughly in 10ml of sterile water in a glass tube and shaken thoroughly. From this initial suspension, serial dilutions were prepared .One ml of the required dilution (1/1000) was pipetted into five replicate plates containing potato dextrose agar medium with antibiotic. The plates were incubated at room temperature in glass chambers under aseptic conditions for 4 days and then examined for fungal growth. All fungal colonies developed were recorded**.**

Slide preparation and identification

Lactophenol and Lactophenol cotton blue stain (Hi Media laboratories, private limited) were used as the staining solution. Slides prepared were sealed with DPX mountant. Identification of the fungal species was done using "The Genera of Hyphomycetes from soil" [15] and Compendium of soil fungi [12].

Percentage of Frequency of Fungal species (PF)

Percentage frequency of individual fungal species from five replicated plated was calculated using the formula:

$$PF = \frac{\text{Total number of colonies of individual fungal species}}{\text{Total number of colonies of all fungi in 5 replicate plates}} \times 100$$

SCREENING FOR FUNGAL ENZYME ACTIVITIES

Amylase production

The fungal isolates were screened for amylase activity was carried out on agar media in Petri plates containing the respective substrates. The following substrate was used, for amylase production -1% starch and supplemented with 1% yeast extract and malt extract, agar 2g.The cultures were spot inoculated at the centre of the plate and incubated at 28º C for a period of 5 days to get a good growth of the cultures. For amylase activity, the plates were flooded with 1% iodine in 0.2% KI solution. A zone of clearance around the culture indicated that the culture had the ability to digest starch [27].

Cellulase production

The fungal isolates were screened for cellulase activity was carried out on agar media in Petri plates containing the respective substrates. The following substrate was used, for cellulase production 1% carboxy methyl cellulose (CMC) and supplemented with 1% yeast extract and malt extract, agar-2g. The cultures were spot inoculated at the center of the plate and incubated at 28º C for a period of 5 days to get good growth of the cultures. For cellulase, the plates were flooded with an aqueous solution of 0.1% Congo red. After 30 minutes, the plates were repeatedly washed

with 1 M sodium chloride and zone of clearance around their growing margin indicated cellulase activity [27].

Protease production

The fungal isolates were screened for protease activity on agar media in petri plates containing 1% skimmed milk and supplemented with 1% yeast extract and malt extract, agar-2g. The cultures were spot inoculated at the center of the plate and incubated at 28º C for a period of 5 days to get a good growth of the cultures. For protease activity the zone of clearance around the culture indicate protease activity [27].

Lipase production

The fungal isolates were screened lipase activity was carried out on agar media in Petri plates containing 10ml tween 20, 15g peptone, 5g NaCl, 1g $CaCl_2$, 15g agar at pH 7.0 was adopted as per [27]. The lipase activity indicates as a zone of clearance around the colony and subsequent formation of a white precipitate of calcium monolaurate around the colony [9, 16].

Growth rate of the fungi at different pH (mass weight measurement)

The growth rates of fungal species were studied at pH values of 6, 6.5, 7, 7.5 and 8 using Potato Dextrose broth medium (PDA). Equal amount of inoculums was added to the flasks maintained at different pH after Culture was incubated at 28°c with continuous shaking at 180-200 rpm. The cells were harvested by centrifugation after 5th days. The pellet was suspended in distilled water and filtered through Whatman No.1filter paper and then pellets were dried to dryness and weighed to obtain the dry weight using a weighing balance [21].

RESULTS

Fungi being generally ubiquitous, their diversity was studied in three different environmental samples, namely the agricultural soil, leaf litter soil, wood debris soil. However, this study was broadly investigated on microfungal species and formed colony in the laboratory culture. Totally 7 microfungal species were isolated from three different environmental samples. The fungal species *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus terreus*, *Penicillium citrinum*, *Fusarium oxysporum*, *Curvularia lunata* were isolated from agricultural soil, leaf litter soil, wood debris soil. *Curvularia lunata* only recorded in agricultural soil sample. *Penicillium citrinum* recorded in both agricultural soil, leaf litter soil samples. *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus terreus*, *Fusarium oxysporum* recorded in all the three soil samples (Fig-1,2,3).

Fig: 1- Isolate of fungal colony from different samples

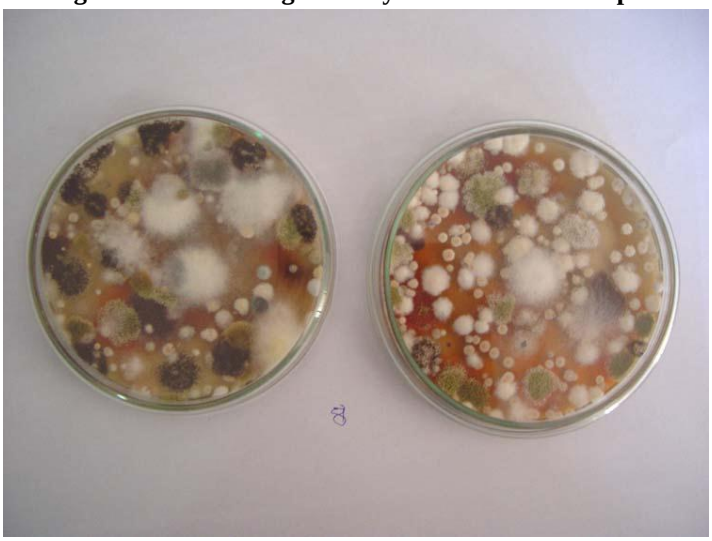


Fig:2-Pure culture of Fungal Isolate

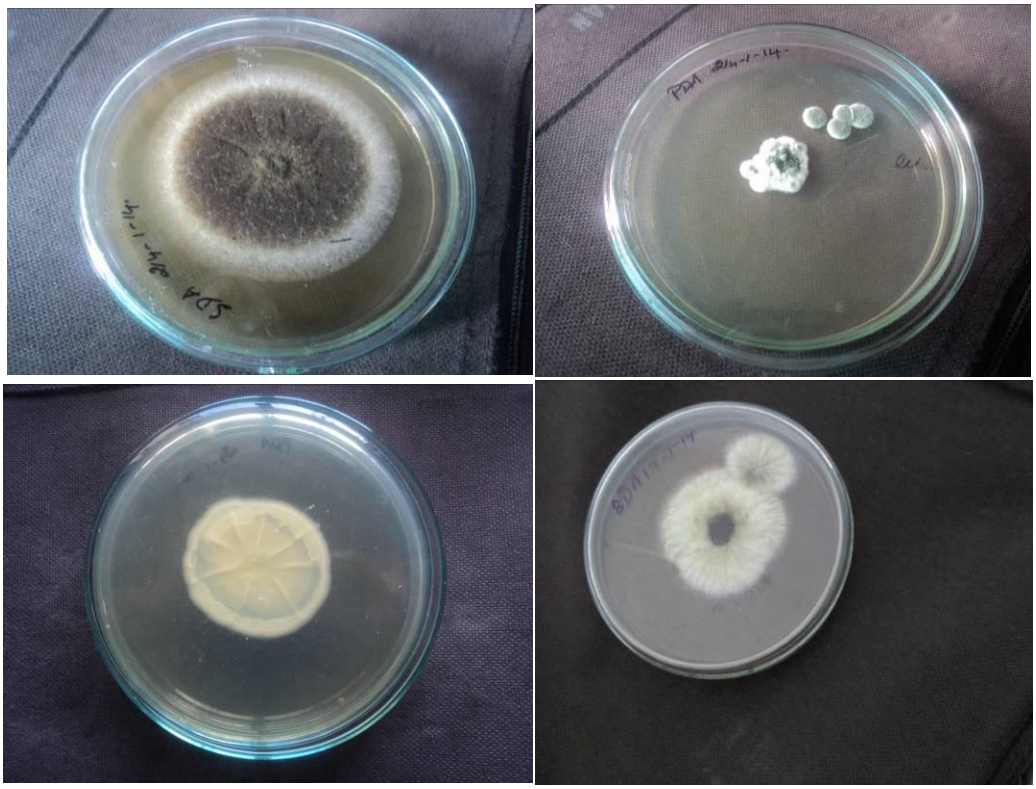
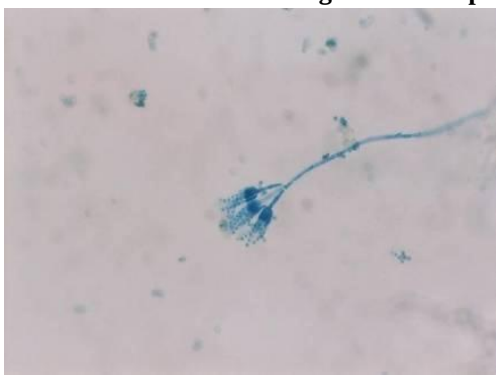
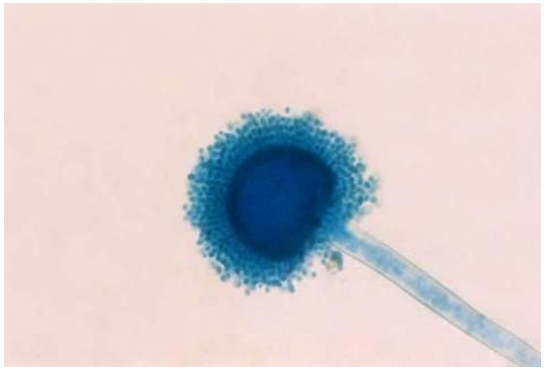


Fig:3-Microscopic identification of fungal species

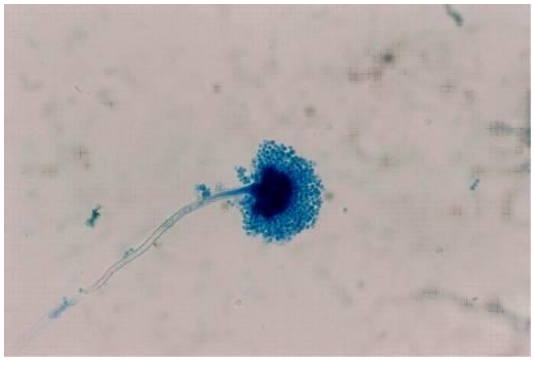


Penicillium citrinum

Curvularia lunata



Aspergillus flavous



Aspergillus terrus

Micro-Fungal frequency percentage

The fungal species *Aspergillus flavus* (21.43%) showed the maximum percentage frequency of occurrence in Agricultural soil followed by *Fusarium oxysporum*, *Aspergillus terrus*, *pencillium citrinum*, *Aspergillus niger*, *Aspergillus ochraceus*, *Curvularia lunata* (Table -1).

Table – 1. Percentage Frequency of fungal species in different Soil

S.No	Fungal species Hyphomycetes	Frequency percentage (PF %)		
		Agricultural soil	Leaf litter soil	Wood debris soil
1	*Aspergillus niger*	14.28	24.22	28.57
2	*Aspergillus flavus*	21.43	12.11	23.81
3	*Aspergillus ochraceus*	10.72	15.16	9.52
4	*Aspergillus terreus*	17.85	18.17	14.29
5	*Penicillim citrinum*	14.29	18.21	--
6	*Fusarium oxysporum*	17.87	12.13	23.81
7	*Curvularia lunata*	3.57	--	--

Growth of fungal dry mass weight at different pH

Growth rates of 7 fungal species were studied at different pH ranges of 6, 6.5, 7, 7.5 and 8 at the end of the 5th day growth. The maximum dry mass weight was recorded for the species *Aspergillus flavus* (0.65mg), *Aspergillus ochraceus* (0.53mg) and *Fusarium oxysporum* (0.50mg) at pH 7.5 followed by *Aspergillus terreus* (0.58mg), *Aspergillus niger* (0.54mg) and *Penicillium citrinum* (0.53mg) at pH 6.5. The fungal species *Aspergillus terreus* (0.55mg), *Penicillium citrinum* (0.49mg), *Curverlaria lunata* (0.48mg), *Aspergillus niger* (0.44mg), *Aspergillus ochraceus* (0.45mg) at pH 6 followed by *Aspergillus flavus* (0.52mg), *Penicillium citrinum* (0.50mg) and *Aspergillus niger* (0.48mg) maximum mass dry weight recorded at pH 7. The fungal species *Aspergillus flavus*(0.60mg), *Aspergillus terreus* (0.46mg), *Fusarium oxysporum* (0.44mg) at pH 8. (Table - 2).

Table 2: Growth of fungal species at different pH

S.No	Fungal Species	Dry weight of fungi in Different pH				
		6	6.5	7	7.5	8
1	*Aspergillus niger*	0.44	0.54	0.48	0.46	0.42
2	*Aspergillus flavus*	0.34	0.56	0.52	0.65	0.60
3	*Aspergillus ochraceus*	0.45	0.50	0.14	0.53	0.32
4	*Aspergillus terreus*	0.55	0.58	0.17	0.52	0.46
5	*Penicillium citrinum*	0.49	0.53	0.50	0.48	0.35
6	*Fusarium oxysporum*	0.36	0.39	0.36	0.50	0.44
7	*Curvularia lunata*	0.48	0.49	0.29	0.47	0.38

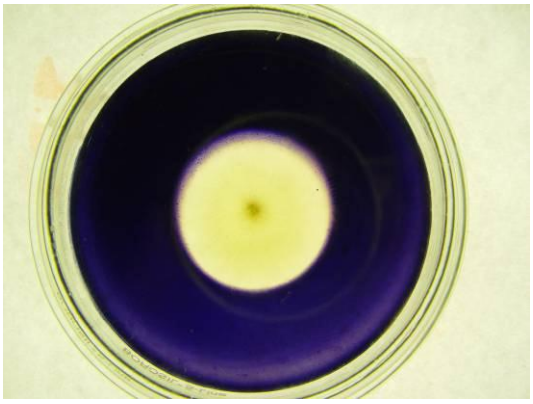
Screening of fungal enzymes

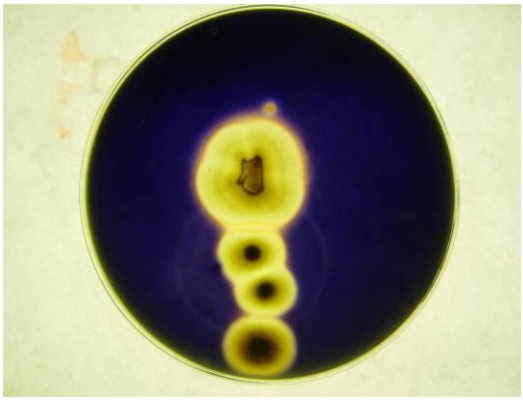
Four fungal species showed amylase activity from a total screening of 7 fungal strains. The maximum zone of activity was observed in *Aspergillus flavus* (15mm), followed by *Aspergillus ochraceus* (13mm), *Aspergillus niger* (12mm), *Aspergillus terreus* (10mm). The cellulase zone of activity was observed in *Aspergillus niger* (13mm). The protease zone of clearance activity was recorded in *Aspergillus ochraceus* (11mm). In lipase activity white precipitin around the colony was observed in *Aspergillus terreus* (9mm) (Table – 3 Fig. – 4)

Table 3: Screening of various enzyme activity in plate assay

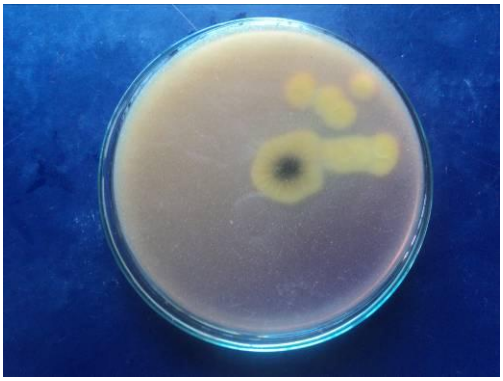
S.No	Micro-fungal species	Amylase activity(mm)	Cellulase activity(mm)	Protease activity(mm)	Lipase activity (mm)
1	*Aspergillus niger*	12	13	10	8
2	*A. flavus*	15	10	8	7
3	*A. ochraceus*	13	14	11	5
4	*A. terrus*	10	9	6	9

Fig:4. Screening of micro-fungal enzyme

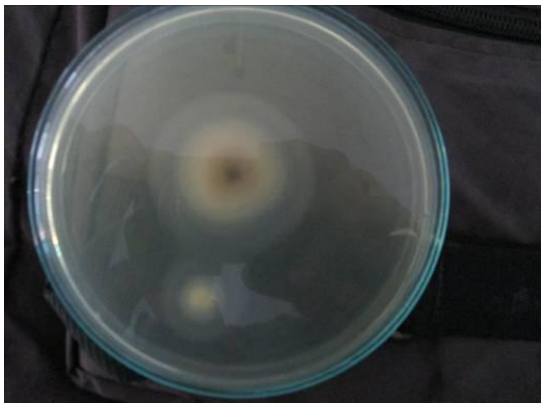
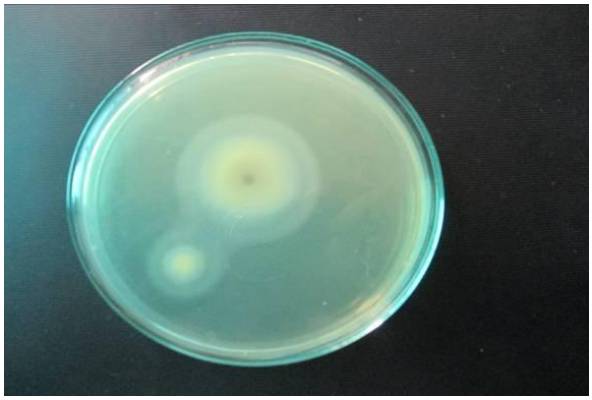




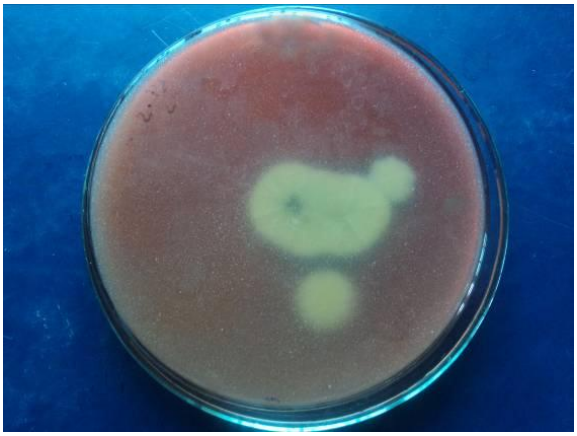
Zone of clearnce acivity of fungal Amylase from *Aspergillus niger*



Halo of clearance around the colony of fungal protease activity in *Aspergillus ochraceus*

Zone of clearance around the colony indicate Lipase activity in *Aspergillus ochraceus*



Zone of clearance acivity of fungal cellulase from *Aspergillus niger*

DISCUSSION

High sporulation capability of fungi as mitosporic fungi such as Deuteromycetes, Zygomycetes, can easily straggle to reach the suitable environmental condition and take the nutrients for their growth. When the environment is unsuitable for growth in the condition of low nutrients, presence of metabolites, they able to grow with the help of their asexual spores, called conidia, show an exogenously imposed dormancy induced by the phenomenon termed fungistasis, allowing the fungus to survive [27]. The present study has analyzed the fungal species capable of growth at acidic and alkaline pH. Totally 7 species of fungi were recorded from the agricultural soil, leaf litter soil, and wood debris soil and capable of forming colonies in neutral, acidic and alkaline pH. The recorded all fungal species classification under hyphomycetes.

Senthilkumar [26] reported that *Acremonium* sp., *Alterneria laternata*, *Aspergillus flavus*, *A.nidulance*, *A.niger*, *A.terrus*, *Curvularia lunata*, *Fusarium solani*, species were major Mycoflora associated with litter degradation at tropical grass land of south India. Similarly fungal species *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus terreus*, *Penicillium citrinum*, *Fusarium oxysporum* and *Curverlaria lunata* were encountered in the present study. The 7 fungal species belongs to *Curverlaria lunata* only recorded in agricultural soil sample. Fungal species *Penicillium citrinum* recorded in both agricultural soils and leaf litter soil samples. Fungal species *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus terreus*, Fusarium oxysporum recorded in all the three samples.

Mikami [21] reported that fungal species recorded between pH 6 to11 and most of them were isolated at pH 8. Among the genus

Aspergillus, several species such as *Aspergillus niger, A. sidowii, A. terreus* and *A. ustus*, showed the highest frequency at pH 11. In present study seven fungal isolates formed colonies and maximum growth rate recorded at pH 7.5. The maximum dry weight was recorded in pH 7.5 *Aspergillus flavus* (0.65mg), followed by *Aspergillus ochraceus* (0.53mg), *Fusarium oxysporum* (0.50mg). In at pH 6.5 maximum fungal growth was observed *Aspergillus terreus* (0.58mg) followed by *Aspergillus niger* (0.54mg), *Penicillium citrinum* (0.53mg).

Screening of fungal enzymes

Totally seven fungal species belong to four fungal enzymes studied, amylase activity was recorded to be maximum in all the species. Kathiresan and Manivannan [18] also recorded good amylase activity in *Penicillium fellutanum* and *Aspergillus flavus*. Similarly in present studies the maximum zone of activity was observed for amylase *Aspergillus flavus* (15mm), followed by *Aspergillus ochraceus* (13mm), *Aspergillus niger* (12mm), *Aspergillus terreus* (10mm). Jahangeer [17] reported that majority of *Aspergillus* and *Penicillium* sps. were found to possess cellulolytic activity. The most active cellulolytic enzymes were reported in *A. terreus, P. tigrinus, P. ostreatus F. fomentarus* and *A.niger* from agro-industrial wastes [22, 23]. In present studies cellulase zone of activity was observed in *Aspergillus ochraceus* (14mm) followed by *Aspergillus niger* (13mm), *Aspergillus flavus* (10mm), *Aspergillus terreus Aspergillus terreus* (9mm).

Several microfungal species of strains including *Aspergillus flavus*, *Aspergillus melleu*, *Aspergillus niger*, *Chrysosporium keratinophilum*, *Fusarium graminarum*, *Penicillium griseofulvin*, *Scedosporium apiosermum* isolated from soil of sugarcane field are reported to produce proteases [13]. In present study also Protease activity was recorded maximum in *Aspergillus ochraceus* (11mm) followed by minimum in *Aspergillus niger*(10mm), and *Aspergillus flavus* (8mm), *Aspergillus terreus* (6mm). The lipase activity was detected as a zone of clearance around the colony and subsequent formation of a white precipitate of calcium monolaurate the colony [9, 16]. Similarly lipase activity white precipitation around the colony was observed in *Aspergillus terreus* (9mm) followed by *Aspergillus ochraceus* (5mm), *Aspergillus niger* (8mm), *Aspergillus terreus*, and *Aspergillus flavus* (7mm).

CONCLUSION

Micro-fungal diversity differs from site of sampling and composition of nutrient in sampling. The micro-fungal species enzymes production depends on species specific and isolation site specific variation. This study focused on the different micro-fungal species screening of different industrial important enzymes such as amylase, cellulase, protease and lipase. This study is very useful for the screening of industrial important enzyme production species specific variation of enzyme production.

Acknowledgement

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