Original Article

Screening of Soil Fungi for production of Lytic enzymes

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Abstract

Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential. These enzymes are applied in the industrialization of detergents, starch, drinks, food, textile, animal feed, baking, pulp and paper, leather, chemical and biomedical products. Filamentous fungi are good producers of cellulolytic extracellular enzymes. The application of different techniques have allowed to identify and understand the functions of amylases, proteases and lipases in fungi infective process. Production of amylase and protease enzyme by different strains of soil fungi was the aim of this study. In the study, soil from different areas was taken and fungi was isolated from it on SDA media plates. Strains obtained were purified on SDA slants. 40 fungal isolates were obtained, of which six isolates (TS-01 to TS-06) exhibited enzymatic potential and were found to be good producers of Amylase and Protease. Remarkable production was verified for the fungal isolate *TS - 01*, but *TS- 06* also was excellent producer of the enzymes studied.

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Introduction

Biodiversity refers to the variability of life on Earth, all the living species of animals, plants and micro-organisms. According to Hawksworth (2002) [1], fungi are a major component of biodiversity, essential for the survival of other organisms and are crucial in global ecological processes. It has been found that more number of genera and species of fungi exist in soil than in any other environment [2]. They form the major group of organotrophic organisms responsible for the decomposition of organic compounds. Their activity participates in the bio-deterioration and biodegradation of toxic substances in the soil [3]. Contributing to the nutrient cycle and maintenance of ecosystem, fungi play an important role in soil formation, soil fertility, soil structure and soil improvement, decompose organic matter from humus, release nutrients, assimilate soil carbon and fix organic nutrients [4]. Fungi are well characterized due to their capacity to produce different extracellular compounds; which include a range of cuticle degrading enzymes corresponding to different polymers of the insect cuticle [5]. The function of this compounds, in pathogenicity, include insect cuticle dissolution, suppression of the immune system, interference with ion channels and other cellular functions in the host cell [6,7], thus giving them the ability to penetrate directly through the insect's cuticle by enzymatic hydrolysis. Some fungi produce enzymes to convert the insect's tissues in nutrients for their growth. Pathogenesis in fungi requires secretion of hydrolytic enzymes like protease, amylase, lipase, for penetration through these barriers [8]. Proteases are enzymes that are considered as the most important within the infective process [9], which degrades the proteinaceous material. The solubilized proteins are degraded by amino peptidases and exopeptidases until amino acids, serving as nutrients for entomopathogenic fungi [10]. The objectives of the present investigation were to isolate and purify different strains of soil fungi from different soil samples, to screen out the amylase and protease producing strains of soil fungi and to analyze quantitative production of amylase and protease enzymes.

Materials and Methods

Collection of Soil Samples:

Soil samples were collected from different areas of Lucknow (U.P.) which included the dry soil sample (A) and the wet soil sample (B). Samples were collected from 10 – 15 cm deep pits dug in the area to be sampled with a surface sterilized trowel.

Isolation and maintenance of Fungi from soil samples:

Fungi were isolated from soil by Serial dilution technique. The dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion. A tenfold serial dilution could be 1 M, 0.1 M, 0.01 M, 0.001 M serial dilutions. 1 ml of each dilution of each soil sample were spread onto the Sabouraud's Dextrose Agar media and plates (fortified with 0.1 mg/ml tetracycline) with a glass spreader and incubated at 28 ± 1°C in incubator for about 2 days. The strains obtained after incubation were purified and isolates were maintained by sub culturing in SDA slants.

Microscopic identification:

Slide culture technique was adopted to identify the isolates. A very fine and small colony of the isolate was stained with β-lactophenol / cotton blue and structural morphology was examined under compound microscope for identification.

Biochemical analysis:

Fungal enzymes play an important role in the growth of plant as well as enhancing pathogenicity. Hence, qualitative and quantitative assay was performed to estimate activities of protease and amylase enzymes produced by the isolates.

Estimation of proteolytic activity by isolates:

Qualitative Analysis: Plate assay method was used for the qualitative analysis of the enzymes [11]. Production of proteolytic enzymes by isolates was detected by using gelatin as protein source in growth medium. The fungal strain was spot inoculated in petridishes with nutrient agar medium supplemented with 1% gelatin. After inoculation, the petridishes were incubated at 28±1^{o}C for 2 days. Gelatin degradation was observed as clearing zone around fungal colonies. This zone of gelatinolysis was seen clearly upon flooding the plate with aqueous saturated solution of mercuric chloride reagent. Mercuric chloride solution reacted with gelatin to produce a white precipitate which made the clearing zone visible. The clearing zone was measured indicative of the extracellular protease activity of the fungal strain, with the help of vernier calipers. Enzymatic index was measured by the following formula:

$$E.I. = D - d$$

Where, E.I. – Enzymatic index D - Diameter of colony plus clearing zone d - Diameter of colony

Quantitative Analysis:

Spectrophotometric analysis for proteolytic enzymes:

The quantitative estimation of the enzyme produced by the isolates was measured by Spectrophotometric analysis [12]. Minimal broth supplemented with 1% gelatin was used as the growth medium . Erlenmeyer flasks with capacity 150 ml were dispensed with 25 ml broth each. These flasks were inoculated with spore suspension of fungal isolate and were incubated at 28 ± 1^{o}C in an incubator. The enzyme activity was measured at the end of incubation period. Aliquots were asceptically removed from the flasks and were centrifuged at 5000 rpm for 5 minutes. The supernatant was used as crude enzyme extract (CEE). To determine extracellular protease activity, reaction mixture was prepared by mixing Crude enzyme extract- 1 ml and Buffered casein stock solution (1g casein in 100 ml of 0.1 M PO_4 buffer)- 1 ml. This reaction mixture was incubated in water bath set at 36±1^{o} C for 60 minutes. At the end of one hour of incubation, the reaction was terminated by addition of 3 ml

of TCA which precipitated the unhydrolysed casein. Precipitate was removed by centrifugation and to the supernatant, 5 ml Na_2CO_3 (0.4 M) and 0.5 M Folin's reagent were added. These were incubated at room temperature for 20 minutes and enzyme activity was read as absorbance at 660 nm in UV- VIS Spectrophotometer. Control was prepared by taking 1 ml distilled water in place of crude enzyme extract.

Estimation of amylolytic activity by isolates:

Qualitative Analysis: Starch was employed as a carbohydrate source in the medium. The fungal strain was spot inoculated in petridishes with minimal media supplemented with 1% starch. These plates were incubated at 28±1°C for 2 days. At the end of incubation period, colony diameter was measured and plates were flooded with iodine reagent. A clearing zone developed immediately around the colony which was measured with the help of vernier calipers. Enzymatic index was measured by the following formula:

$$E.I. = D - d$$

Where, E.I. – Enzymatic index D - Diameter of colony plus clearing zone d - Diameter of colony

Spectrophotometric analysis for amylolytic enzymes:

Growth medium employed 1% starch supplemented in minimal broth. Erlenmeyer flasks with capacity 150 ml were dispensed with 25 ml broth each fortified with 0.1 mg/ml tetracycline. These flasks were inoculated with spore suspension of those isolates showing positive results in qualitative analysis for both enzymes, i.e. Amylase and Protease, and were incubated at 28 ±1°C in an incubator. The enzyme activity was measured at the end of 7th day. Aliquots were aseptically removed from the flasks and were centrifuged at 5000 rpm for 5 minutes. The supernatant was used as crude enzyme extract (CEE). To determine extracellular amylase activity, reaction mixture was prepared by mixing crude enzyme extract- 1 ml and 1% soluble starch in citrate phosphate buffer (0.1M, pH 6.5) - 1ml. This reaction mixture was incubated in water bath set at 40 ±1°C for 30 minutes. At the end of incubation period, reaction was stopped by addition of 6 ml water. Enzyme activity was read as absorbance at 540 nm and readings were recorded. Control tubes were prepared by addition of distilled water in place of CEE. Enzyme activity was read as absorbance at 540 nm in UV- VIS Spectrophotometer. Control was prepared by taking 2 ml of enzyme extract that has been boiled for 20 min (boiling inactivates enzyme), added to starch solution and treated with same reagent as experimental tubes.

Result & Discussion

Isolation of fungi from soil samples:

Pour plate method: Dilution of both soil samples i.e. dry soil and wet soil, were incubated in SDA and PDA media plates for 48 hrs by pour plate method. After incubation, total 15 strains were observed on plates (Table 1, Fig. 1). In the analysis, it was observed that SDA media was comparatively better than PDA media in the primary isolation of fungi from normal sterile sites, and more strains were obtained from wet soil than obtained from dry soil.

Table 1: Morphology of fungal Strains obtained from Soil Samples

Colony No.	Color of colony	Soil Sample	Dilution	Media
1	Whitish	A	10^{-1}	PDA
2	Black	A	10^{-1}	PDA
3	Light pink	A	10^{-2}	PDA
4	Light green	A	10^{-2}	PDA
5	Yellowish	A	10^{-3}	PDA
6	White	A	10^{-1}	SDA
7	Pale green	A	10^{-2}	SDA
8	Bluish green	A	10^{-2}	SDA
9	Dark green	A	10^{-2}	SDA
10	Light pink	A	10^{-2}	SDA
11	Light orange	A	10^{-2}	SDA
12	Light brown	B	10^{-1}	PDA
13	Dark green	B	10^{-2}	PDA
14	Yellowish green	B	10^{-2}	SDA
15	Whitish	B	10^{-3}	SDA

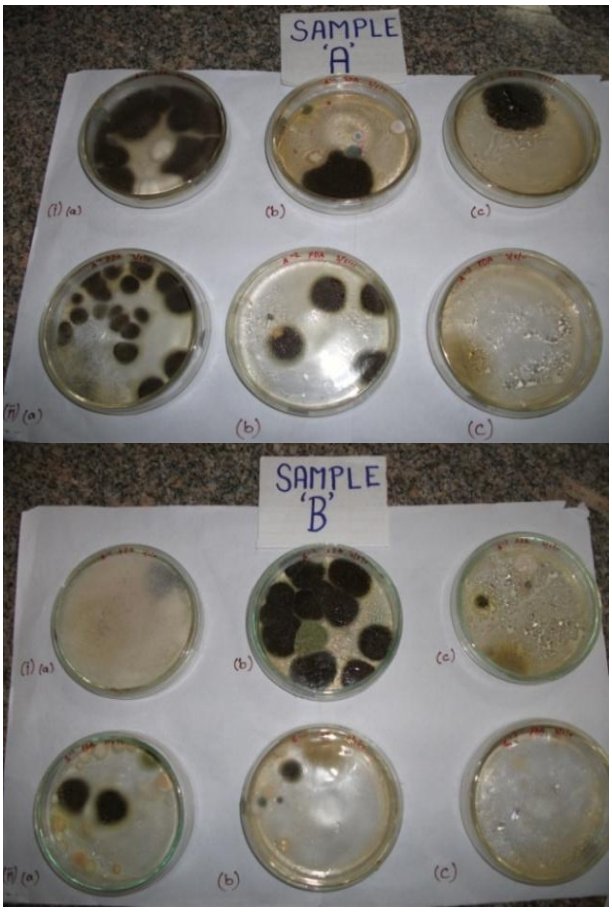


Fig 1: (A) Fungal Strains on media isolated from Soil (Wet – A) and (Dry-B)

Fig 1: (B) Slants with purified fungal isolates from soil

Table 2 : Activity of Amylase and Protease by six fungal isolates isolated from soil

Accession No	Amylase Production		Protease Production	
	Amylase (+/-)	Enzymatic Index	Protease (+/-)	Enzymatic Index
TS-02	-	0	+	4
TS-03	+	1	-	0
TS-04	+	2.16	+	1
TS-05	+	2.25	+	2.4
TS-06	+	2.4	+	2.28

Table 3: Strains with their optical density for amylase and protease production

Accession No.	Optical density at 540 nm			
	Starch		Gelatin	
	Absorbance	Transmittance	Absorbance	Transmittance
TS-01	0.955	11.00%	0.349	44.70%
TS-04	0.128	74.40%	0.321	47.70%
TS-05	0.595	25.40%	1.291	5.10%
TS-06	0.387	40.90%	0.444	35.90%

Maintenance of fungal isolates:

15 fungal strains so obtained were purified, of which Six isolates were selected and maintained in SDA slants (Fig. 1). Similar studies were conducted by Guimaraes *et al.*, 2006 [13] who isolated sixteen fungi and tested for production of amylase. They concluded that the IF-2, *Paecilomyces variotii*, *Rhizopus microsporus* var. *rhizopodiformis*, *Aspergillus phoenicis* and C-9A were the best amylase producers. The production of glucoamylase and α-amylase from *Rhizopus microspores* has also been reported [13].

Microscopic Identification of Strains:

The purified strains were identified by slide culture technique. A very fine and small colony of the isolate was stained with β-lactophenol cotton blue and examined under compound microscope.

Strain TS-01: Colonies consist of a compact white basal felt covered by a dense layer of dark-brown to black conidial heads. Conidial heads are biseriate with the phialides borne on brown head.

Strain TS-02: Colonies were white to pale yellow in colour, Conidiophores are well differentiated and erect, verticillately branched over most of their length, Conidia are hyaline or brightly coloured and one-celled.

Strain TS-03: Colonies were white with yellowish-green conidial heads, Conidiophores are hyaline, smooth-walled and bear terminal verticils of 3 to 5 metulae, each bearing 3 to 7 phialides.

Strain TS-04: Colonies were deep-yellow in colour, Microconidia are laterally along the hyphae.

Strain TS-05:Colonies were dark green to black in color, Phialides are flask-shaped or elliptical with distinctive funnel-shaped.

Strain TS-06:Colonies were white and cottony, Sporangiophores are distinctive and flask shaped, Non-septate hyphae.

According to studies conducted by Rohilla & Sarkar (2011) [14], twenty three soil samples were characterized for the incidence of fungal strains from pesticides contaminated agricultural soils. A total of 59 fungal strains were isolated and 33 fungi were characterized using various isolation and identification methods. In a similar study, a total of 15 species belonging to 6 genera of fungi were isolated from agricultural fields. The mycoflora were isolated by using soil dilution technique. Identification and characterization of the mycoflora were made with the help of authentic manuals of fungi (Fig. 2).

Biochemical Assay: Qualitative analysis:

Estimation of proteolytic activity by isolates: The purified isolates were spot inoculated on minimal gelatin media plate and were kept for incubation for 2 days at 28± 1 °C. After incubation, when plates were over flooded with mercuric chloride reagent, a clearing zone around fungal colonies were observed. Mercuric chloride reacts with gelatin to produce a white ppt. while the clearing zone indicates that gelatin around the colony has been degraded. Gelatin is degraded by protease enzyme. Plates with clearing zone indicate that protease enzyme was produced by the fungi. All strains, except

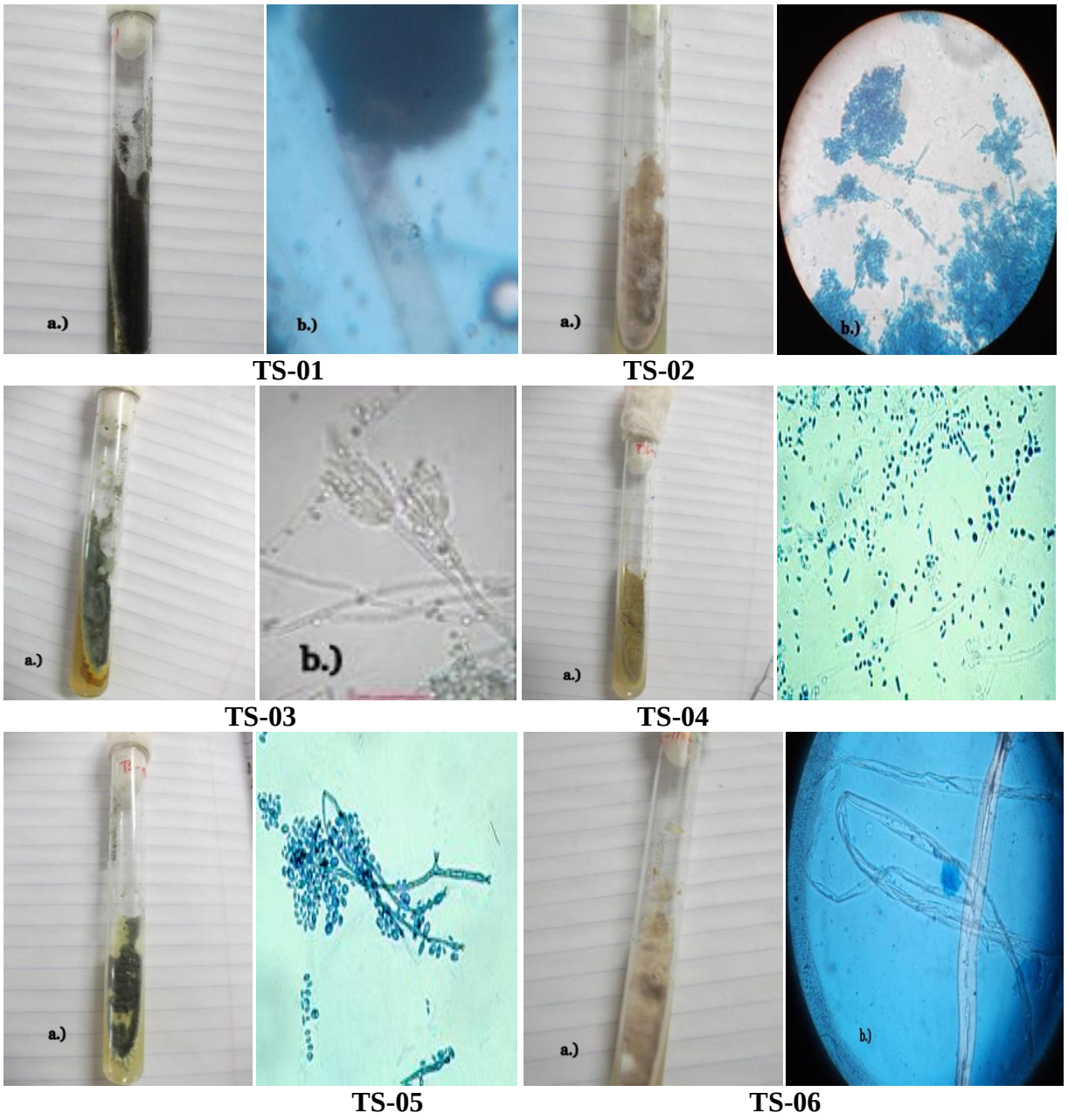


Fig 2: Purified fungal isolates and their microphotograph (TS-01 to TS-06)

TS-03, shows clearance zone, thereby, indicating production of protease enzyme by strain. Isolate TS-02 has maximum enzymatic index, which shows maximum protease production (Table 2, Fig. 3).

In a study by Oseni (2011) [15], Determination of protease activity from the fungal isolates showed that *Aspergillus flavus* had the highest activity at day 4 (7.5 x 10^{-4}) U/ml/min while it showed its lowest activity at Day 1 (3.2 x 10^{-4}) U/ml/min. Protease production was observed when the fungal strain *M. anisopliae* was grown in liquid media containing cuticle of various insects such as locusts *Schistocerca pallens*, *Rhammatocerus schistocercoides* and *Schistocerca gregaria* and *Manduca sexta* moth [16,17,18]. Murad *et al* (2006) [19] were able to isolate proteases from a culture where *M. anisopliae* grew in minimal medium containing *C. maculates* cuticle showing that this enzymes are produced in the presence of insect exoskeleton.

Estimation of amylolytic activity by isolates: The purified isolates were spot inoculated on minimal starch media plate and were kept for incubation for 2 days at 28± 1°C. After incubation, when plates were over flooded with iodine reagent, a clearing zone around fungal colonies were observed. Iodine reacts with starch to produce blue black color, while the clearing zone indicates that starch around the colony has been degraded. Starch is degraded by amylase enzyme. Plates with clearing zone indicate that amylase enzyme was produced by the fun-

gi. All strains, except TS-02, shows clearance zone, thereby, indicating production of amylase enzyme by strain. Isolate TS-01 has maximum enzymatic index, which shows maximum amylase production (Table 2, Fig. 3). As observed by Guimarães et al. (2006) [13], Significant levels of amylase were produced by *Paecilomyces variotii* and *A. phoenicis*. They also observed that a remarkable enzyme producer was *Rhizopus microsporus var. rhizopodiformis* that produced high levels of amylase, alkaline and acid phosphatases, and pectinase.

Quantitative analysis

Spectrophotometric analysis for proteolytic & amylolytic enzymes

Those strains which were showing positive results on both qualitative analysis, i.e. amylase and protease, were incubated for 7 days at 28 ± 1 °C in SDA broth. Then enzymatic activity was measured with the help of spectrophotometer. Results showed that TS-01 produces more amylase while TS-06 produces more protease than other strains, respectively (Table 3, Graph 1).

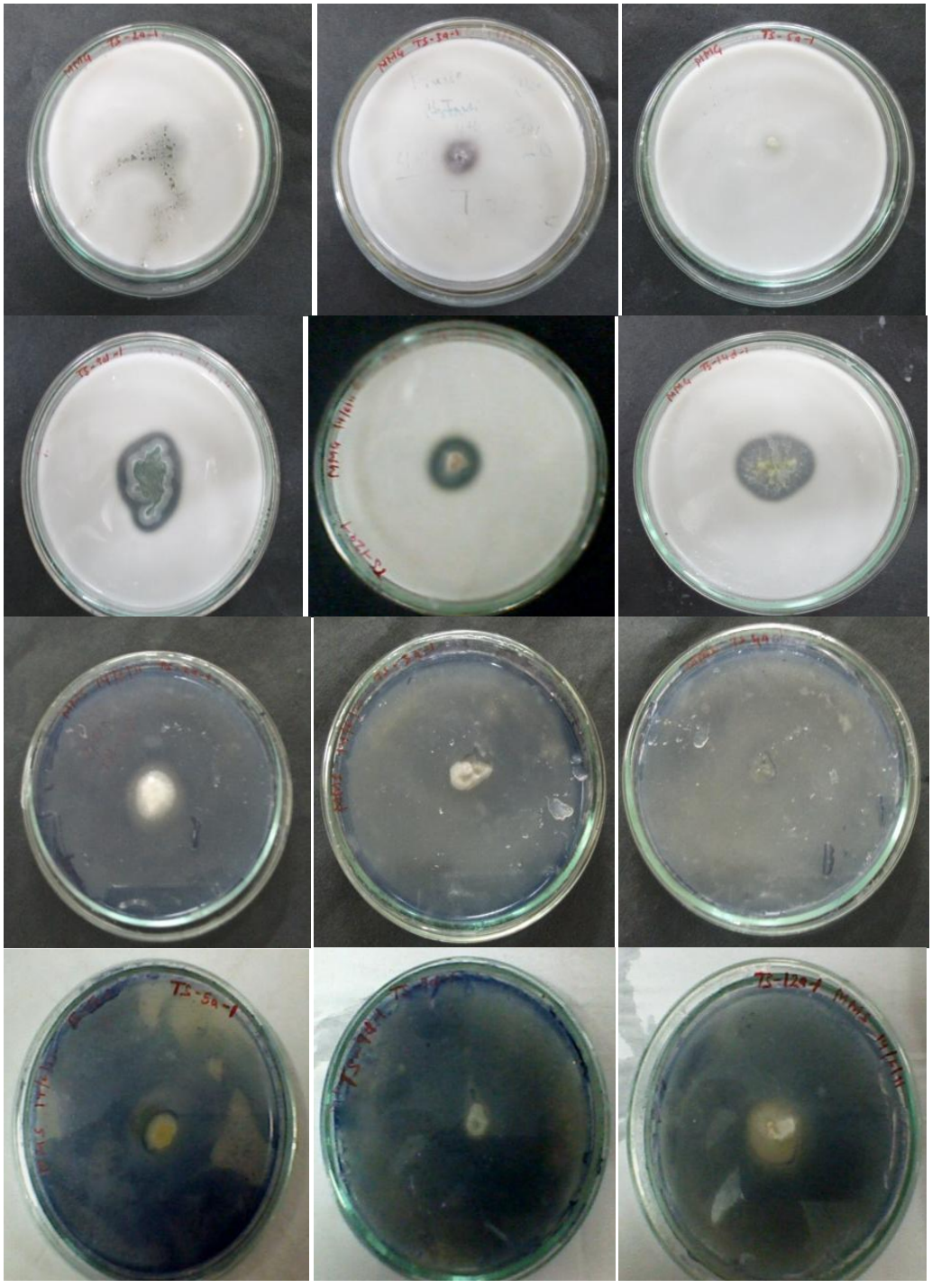
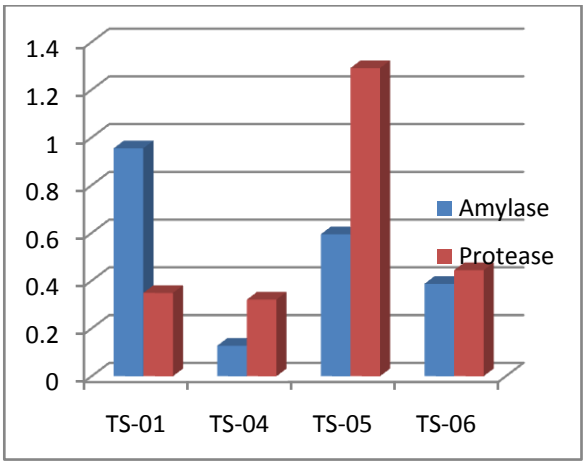


Fig. 3: Screening for Protease and Amylase production: TS-01, TS-02, TS-05, TS-06 shows positive results while TS-03 and TS-04 shows negative results.



Graph 1: Comparative Enzymatic activity (Amylase and Protease) of fungal isolates (TS-01, TS-04, TS-05 and TS-06)

Conclusion

In conclusion, forty isolates of filamentous fungi were obtained from soil. Among these fungi, six isolates exhibited enzymatic potential for industrial uses. Remarkable production was verified for *TS - 01*, *TS-05* and *TS-06* also was excellent producers of the enzymes studied. All these isolates can be subjected to studies in secondary metabolites to screen for anti- microbial and antiparasitic activities. The results involving the production of extracellular enzymes of fungi are very important because they reveal its biological and physiological apparatus, showing their specialization in the degradation of various substances found in different hosts or environments. This fact has opened a new research field in biotechnology with the intention of developing bioprocess that will be useful for a great scale production, along with strategies for establishment and simultaneous application of conidia and enzymatic extracts, as well as the possibility of its usage as fungicide for controlling plants diseases.

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Competing Interests

None declared.

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