



## SCREENING OF BIOTECHNOLOGICALLY IMPORTANT FUNGI FROM THE SOILS OF CHITTOOR DISTRICT, ANDHRA PRADESH, INDIA

P. Lakshmi Narasimha Reddy\*

Department of Botany, S.V. University, Tirupati-517 502, Andhra Pradesh, India.

\*Author for Correspondence: Narasimha Reddy

Department of Botany, S.V. University, Tirupati-517 502, Andhra Pradesh, India.

Article Received on 13/01/2016

Article Revised on /03/2016

Article Accepted on 24/02/2016

### ABSTRACT

The present study is an effort to isolate the fungi from the cultivated and uncultivated soils of Chittoor district and screen them for the production of some important hydrolytic enzymes such as amylase, cellulase, pectinase, phosphatase and pigment production. In this work, more than 44 fungal isolates were isolated from the cultivated and uncultivated soils of Chittoor district, Andhra Pradesh, were screened for enzymes, identification of the screened isolates and secondary screening for enzyme production was done. A total of 20 isolates were characterized, up to the species level. Interestingly species of *Aspergillus* were predominated. A total of six isolates were selected for the secondary screening for amylase, cellulase, pectinase and phosphatase enzyme production.

**KEYWORDS:** fungal enzymes, abiotic factors, Chittoor district, fungal pigment.

### INTRODUCTION

Soil biology refers to the many kinds of organisms, in addition to plant roots, that live in soil, including soil bacteria, fungi, and various fauna such as insects, nematodes, protozoan's and earthworms. Fungi constitute a major group of microorganisms that are widely distributed in environment especially in soil (Boer et al., 2005). Since they produce wide variety of hydrolytic enzymes and hence exist in nature in saprophytic mode (Ng TB, 2004). Fungi are one of the dominant groups present in soil which strongly influence ecosystem structure and functioning and thus playing a key role in many ecological services. (Orgiaz A et al., 2012) as antagonists, nutrient cyclers, and pathogens. Therefore, there is a growing interest in assessing soil biodiversity and its biological functioning (Barrios E, 2007). At the ecosystem scale, extracellular enzyme activity is influenced by organic matter abundance and composition (Sinsabaugh RL et al., 2008).

The role of many enzymes has been known for a long time. Their existence was associated with the history of ancient Greece, where they were using enzymes from microorganisms in bakery, brewing, alcohol production, cheese making etc., (Haki and Rakshith, 2003). Dr. Takamine (1914) was the first person to realize the technical possibility of cultivated enzymes and to introduce them to industry. Nowadays enzymes are used in large scale textile, detergents, food, paper, and leather industries. The major classes of enzyme offering immediate application are the hydrolytic enzymes (Kirk

et al 2002). Among all industrial enzymes, hydrolytic enzymes account for 85%. Microbial enzymes are preferred due to cost effective, controllable and reliable (Oyeleke and Oduwale, 2009) and also because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. Today, the new 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 (Bakri et al., 2009). The enzymes of fungal origin are used in the industrial process, which amount to billions of dollars of revenue annually (Arunyasi et al., 2010). Due to their diversity, fungi have been recognized as a major source of new enzymes with useful and/or novel characteristics (Bakri et al., 2009).

Soil provides a varied and complex environment for all soil inhabitants. Soil is also known to harbor different microorganisms including diverse group of fungi. Hence the soils from Chittoor district can be a source of fungi of Biotechnologically importance. Screening of microorganisms with higher  $\alpha$ -amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Gupta et al., 2003)

In the present work, we have isolated, identified and screened the fungal isolates for the production of industrially important enzymes and pigments from soil samples of Chittoor district, Andhra Pradesh, India.

## MATERIALS AND METHODS

### Collection of soil samples

Soil samples were collected from the cultivated and uncultivated soils of different areas of the Chittoor district. Collection of soil samples were made at a depth of 15 cm from six different points within the area. The samples were then air-dried, powdered and mixed well to represent a single sample. The soil samples were sieved and processed aseptically and used for the isolation of fungi.

### Isolation of soil fungi

#### Soil dilution plate method

The soil samples were inoculated to the PDA, SA, CZA medium by spread plate technique following serial dilution, and incubated at 25°C for 3 days. The media were amended with Chloramphenicol to prevent the growth of bacteria (Srinivasan, 2004). The fungal species were grown on the Czapek Dox agar plates, identified based on cultural and morphological characteristics using standard manuals (McGinnis et al., 1982).

### Screening for Enzymes and Pigment Production

#### Screening of fungal enzymes

The fungal strains were screened for amylase, cellulase, pectinase, and phosphatase as follows.

#### Amylolytic activity

Cultures were spot inoculated on starch agar medium, starch as a sole source of carbon. Clear zone of hydrolysis around the colonies after 5 days of incubation at 30°C were noted either visually or after staining with iodine solution. (Kathiresan and Manivannan, 2006)

#### Cellulolytic activity

Cellulase activity was detected by growing fungi in a Petri plate containing Czapeck mineral salt agar medium. Using Cellulose (0.5%) as a sole source of carbon. The plates were incubated for 7 days at 25 - 30°C, stained with Congo red solution for 15 minutes. Then de-stain with salt solution for 10 - 15 minutes. Unstained areas indicated where the cellulose has been broken down and the diameter of the clear zone was measured to provide a quantitative comparison of cellulolytic activity. (Samira et al., 2011).

#### Pectinolytic activity

Pectinase activity was detected by growing fungi in a Petri plate on mineral salt agar medium containing 1% (Mandel and Weber) citrus pectin as sole carbon source. After incubation (at 30°C for 5 days) the plates were stained with ruthenium red and purple halos around the colonies were observed for pectin.

#### Phosphate solubilising activity

Phosphatase activity was detected by growing fungi on Pikovskayas agar medium. Using tri calcium phosphate (3%) was used as sole carbon source. The halos around the fungal colonies. After 5 - 7 days at 28 - 30°C indicated the presence of phosphatase activity.

### Pigment Production

The isolates producing diffusible extracellular pigments were selected and preserved.

### Enzyme extraction

22 ml of 0.1 M phosphate buffer (pH 6.5) was added to the cultures, the mixtures were shaken for 30 min at 19°C and 140 rpm on a rotary shaker. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4°C for 15 min. The supernatant was filtered through Watmann no.1 filter paper and the filtrate was used as the crude enzyme preparation.

### Enzyme assay

Cell-free culture filtrate was used as crude enzyme preparation to assay amylase, cellulase, pectinase and phosphatase activities. Crude enzyme preparation was incubated in the presence of 500 µl (1% w/v) substrates: starch for amylase, cellulose for cellulase, citrus pectin for pectinase and tri-calcium phosphate for phosphatase.

### Assay of amylase, cellulase and pectinase enzyme activities

#### Dinitrosalicylic acid (DNSA) method

Assay system for amylase activity was carried out by measuring the amount of reducing sugar according to the DNSA method (Murao et al., 1979). Amylase activity was determined by incubating a mixture of 1 ml of aliquot of each enzyme source and 1% soluble starch dissolved in 0.1 M phosphate buffer, at pH 7, at 50°C for 30 min. The reaction was stopped by adding 1 ml of 3, 5 Dinitrosalicylic acid, and then followed by boiling for 10 min. The final volume was made up to 12 ml with distilled water and the reducing sugar released was measured at 540 nm (Miller, 1959). One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol glucose equivalent per minute under the assay conditions. Reducing sugar (Glucose or maltose) concentration was determined from a standard curve under same condition using glucose. Same method is followed to analyse cellulase and pectinase activities using respective substrates.

#### Estimation of Phosphatase

Acid and alkaline phosphatases were assayed by adopting the standard procedure of Tabatabai and Bremner (1969) using acetate buffer (pH 9.4), respectively. The enzyme substrate (p-nitrophenyl phosphate) mixture was incubated at 37°C for 1 h and the enzyme activity was expressed as enzyme unit (EU). One unit is the amount of enzyme, which hydrolyzes 1.0 µkat of p-nitrophenyl phosphate min<sup>-1</sup> G at pH 5.4 (acid phosphatase) or pH 9.4 (alkaline phosphatase) at 37°C.

The reaction was terminated. Proteins precipitated by TCA were removed by centrifugation at 10,000 rpm for 10 min and supernatant was analyzed for liberated Pi (Ames 1966). One unit of phytase activity was defined as the amount of enzyme, which liberates 1 µkat Pi min<sup>-1</sup>.

**RESULT AND DISCUSSION**

More than 44 fungal isolate were isolated from Different soils by different techniques and 11 isolates were characterized based on colony morphology and microscopic mount of the isolates. About 33 isolates were not identified. The isolates are *Aspergillus niger*, *A. flavus*, *Aspergillus japonicus* *A. terreus*,

*Chaetomiumglobozun* *Penicillium*, *Cladosporiumcladosporioides* and *Nigrosporasphaerica*. Some *Aspergillus* and *Penicillium* isolates were characterized only up to Genus level. The members of *Aspergillus*, were dominant among fungal isolates. Table 1 depicts production of hydrolytic enzymes and pigment by the isolated fungi.

**Table: 1 Enzyme and pigment production by fungal isolates.**

S.No.	Isolate	Amylase Production	Cellulase Production	Pectinase Production	Phosphatase Production	Pigment
1	<i>Aspergillus niger</i>	+++	++	++	++	----
2	<i>A. flavus</i>	+++	+++	+++	+++	+++
3	<i>A. japonicas</i>	+++	-----	++	++++	----
4	<i>A. terreus,</i>	++	++	-----	-----	+++
5	<i>Chaetomiumglobozun</i>	+++	++	++	+	++
6	<i>Penicillium,sp</i>	-----	+++	+++	+++	++
7	<i>Cladosporiumcladosporioides</i>	+++	+	+	++++	++
8	<i>Nigrosporasphaerica</i>	-----	-----	-----	-----	----
9	SS 7	-----	-----	-----	++++	----
10	B 12	-----	-----	-----	+++	----
11	B 14	-----	-----	-----	+++	----

The sign (+) indicates the positive screening test and (-) indicates a negative screening test.

The number of (+) mark ranging from 1+ to 3+ indicates the extent of hydrolysis as indicated by the zone of clearation surrounding colony.

More than 85% of the isolates showed the amylase production, followed by Cellulase (58%), pectinase (36%), phosphatase (37%) and water soluble pigments (40%). Of these starch hydrolysis positive isolates, based on the extent of zone of hydrolysis formed on starch agar plates, 14 isolates belonging to different genera were selected for secondary screening by submerged fermentation. The amount of extracellular protein synthesized and the specific amylase activity of the isolates is shown in the table 2.

In the secondary screening, the production of extracellular proteins was found to be more in *Cladosporium cladosporioides* and *Aspergillus niger* compared to the isolates of other fungal genera. In

*Aspergillus. sp* produced highest concentrations of protein. The Specific activities of amylases of respective isolates were also calculated. The *Cladosporium cladosporioides* and *Aspergillus niger* showed more specific activity.

Different soil sample was collected and isolates the fungal sp by using the PDA (potato dextrose agar) medium. The isolates are identified such as *Aspergillus niger*, *A. flavus*, *A. japonicus* *A. terreus*, *Chaetomium globozun* and *Cladosporium cladosporioides* isolated cultures were subjected used to screened the enzyme activity of amylase all the fungal sp shows the enzymatic activity there was clear zone formation on the specific agar medium. According to the results these fungal sp are used to the production of amylase. Amylase production medium was used to assay the particular enzyme activity by the specific methods. According to the Protein content of culture filtrate and specific activity of crude amylase, cellulase, Pectinase, and Phosphatase.

Name of the fungi	Amylase	
	Extracellular Protein $\mu\text{g}/\text{ml}$	Specific activity $\mu\text{mol}/\text{ml}/\text{min}$
<i>Aspergillus niger</i>	132 $\pm$ 08	3.02 $\pm$ 0.06
<i>A. flavus</i>	116 $\pm$ 05	2.01 $\pm$ 0.04
<i>A. japonicas</i>	105 $\pm$ 06	3.31 $\pm$ 0.05
<i>A. terreus,</i>	115 $\pm$ 11	2.51 $\pm$ 0.03
<i>Chaetomium globozun</i>	135 $\pm$ 04	3.25 $\pm$ 0.10
<i>Penicillium,sp</i>	86 $\pm$ 13	2.58 $\pm$ 0.09
<i>Cladosporium cladosporioides</i>	156 $\pm$ 06	4.63 $\pm$ 0.05
<i>Nigrospora sphaerica</i>	134 $\pm$ 10	3.25 $\pm$ 0.02
SS 7	75 $\pm$ 08	2.29 $\pm$ 0.08
B 12	55 $\pm$ 12	2.21 $\pm$ 0.07
B 14	128 $\pm$ 04	3.20 $\pm$ 0.12

results (fig. 1.1), the fungal sp., of *Cladosporium cladosporoides* and *Aspergillus niger* shows the high activity than the other fungal sp, *Aspergillus sp. K-27*, produced extracellular glucoamylase and  $\alpha$ -amylase using wheat starch as a carbon source, and its productivity was double by the addition of  $\alpha$ -methyl-D-glucose to the medium (Abe et al., 2004). *Aspergillus sp. JGI 12*, can be industrially exploited for the synthesis of  $\alpha$ -amylase and glucoamylase, and strain improvement studies can be carried out to enhance enzyme production (Alva, et al., 2007). The strategy to produce amylase from a single bioreactor was successful as it resulted in a considerably good amount of enzymes produced by *Cladosporium cladosporoides* and *Aspergillus niger* in laboratory conditions.

Bankar et al. (2012) worked on the isolation and screening of forest soil of Bhadra Wild Life Sanctuary, for potent amylolytic fungi. The isolated fungi were mainly belonged to *Penicillium chrysogenum*, *Aspergillus candidus*, *Aspergillus fumigatus*. It was found that *Penicillium sp* showed more amylase activity for both 3rd day and 7th day incubation. *Penicillium chrysogenum* was found to produce more soluble crude protein.

#### Amylase producing fungi

S.No	Name of the fungi	Clear zone (mm)
1	<i>Cladosporium cladosporoides</i>	20
2	<i>Aspergillus niger</i>	20
3	<i>Aspergillus japonicus</i>	15
4	<i>Chaetomium globosum</i>	15
5	<i>Aspergillus terreus</i>	10
6	<i>Aspergillus flavus</i>	15

Arunsasi et al. (2010), isolated 15 fungal species namely, *A. fumigatus*, *A. oryzae*, *A.niger*, *A. flavus*, *A. nidulans*, *A. sulphurus*, *A. terreus*, *Trichoderma vessei*, *T. viridae*, *Penicillium citrinum*, *P. oxalicum*, *Fusarium moniliformis*, *F.oxalicum*, *F. oxysporum*, *Rhizopus oryzae* from the soil samples of the coastal region of Neendakara, along the West cost of Kerala, India and screened them for amylase production and found that *A. flavus* produced maximum zone of hydrolysis on Starch agar media. Sathyaprabha et al. (2011) reported the isolation of fungi namely, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus nidulans* and *Aspergillus niger* from soil samples obtained from crude petroleum oil contaminated soil. They screened the isolates for amylase and cellulose production.

**Table. 4: Cellulase producing soil fungi**

S.No	Name of the fungal strain	Clear zone (mm)
1	<i>Aspergillus niger</i>	10
2	<i>Aspergillus flavus</i>	15
3	<i>Nigrospora sphaerica</i>	12
4	<i>Chaetomium globosum</i>	12
5	<i>Cladosporium cladosporoides</i>	06
6	<i>Penicillium sp.</i>	15

#### Amylase assay (DNS method) Cellulolytic activity

Gautam et al. (2010) studied on the isolation and screening of cellulolytic fungi from municipal solid waste. Out of 20 fungal culture from environmental sources including 8 different zones, 16 fungi were found to possess cellulose degrading ability. Pothiraj et al. (2006) isolated *Rhizopus stolonifer*, *Aspergillus niger* and *Aspergillus terreus* by primary selection from a naturally contaminated cassava waste by serial dilution plate technique and reported the production of cellulase by solid state fermentation. Cellulolytic fungi belonging to *Aspergillus fumigatus*, *Trichoderma sp.I* and *Chaetomium sp.* Results obtained during this study clearly indicate that cellulase activity of *Aspergillus fumigatus* and *Trichoderma sp.I* were found relatively towards the higher side and *A. niger*, *A. flavus*, *A. nidulans*, *Alermaria sp.*, *Penicillium sp.* moderate range while *Fusarium sp.*, *Humicola sp.* and *Torula sp.* showed low cellulase activity.

Microbial Pectinase can be stated as the most important enzyme for the juice industry. Although pectinase production is an inherent property of most all organisms, only those microbes that produce a substantial amount of extracellular pectinase are of industrial importance and have been exploited commercially. Of these, strains of *Aspergillus* species dominate the industrial sector. *Aspergillus niger* showed maximum growth at pH 6.8 as compared its growth at other various respective pH range varying from pH 6.8 to pH 7.0. During growth studies, the maximum biomass or cell mass was obtained in between 24 hrs to 48 hrs. The production of Pectinase from *Aspergillus niger* is greatly influenced by initial culture pH. The optimum pH for production of pectinase by *A. niger* was 6.8. In the present study, however, the most significant level of growth and production of pectinase were supported by pH ranging between 6.8 to 7.0. The entire fermentation process was carried out at room temperature (30°C). Earlier studies have stated that the optimum temperature for the production of Pectinase from *A.niger* was found out to be 28°C (Rombouts et al., 1988) and 30°C (Sayem et al., 2006).

**Table 3: Pectinolytic Fungi Phosphate solubilising activity**

S.No.	Name of the fungi	Clear zone(mm)
1	<i>Aspergillus niger</i>	10
2	<i>SS-7(Un identified)</i>	20
3	<i>Cladosporium cladosporides</i>	20
4	<i>B-12(Un identified)</i>	15
5	<i>Aspergillus japonicus</i>	20
6	<i>B-14 (Un identified)</i>	10
7	<i>Aspergillus flavus</i>	10
8	<i>Penicillium sp</i>	10
9	<i>Chaetomium globosum</i>	5

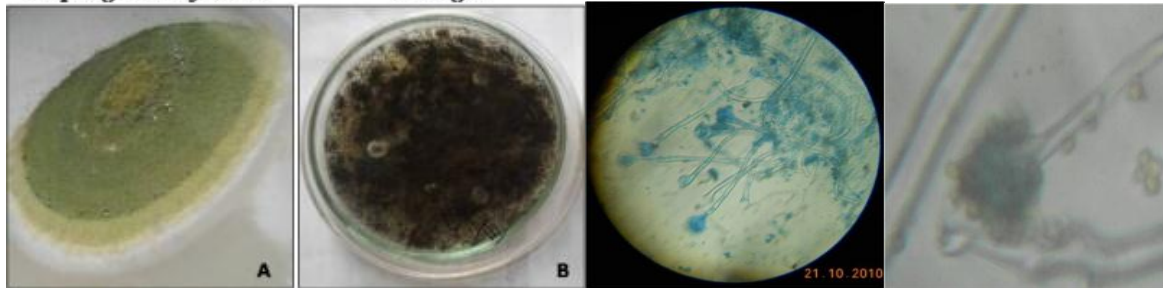
S. No	Name of the Fungi	Clear Zone (mm)
1	<i>Aspergillus nige r</i>	10
2	<i>Aspergillus flavus</i>	15
3	<i>Chaetomium globosum</i>	12
4	<i>Aspergillus japonicus</i>	10

**Phosphate solubilising activity**



*Aspergillus flavus*

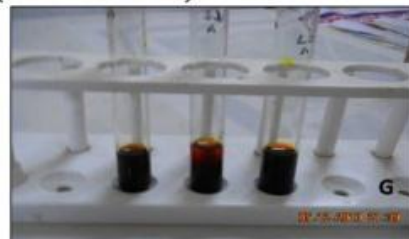
*A. niger*

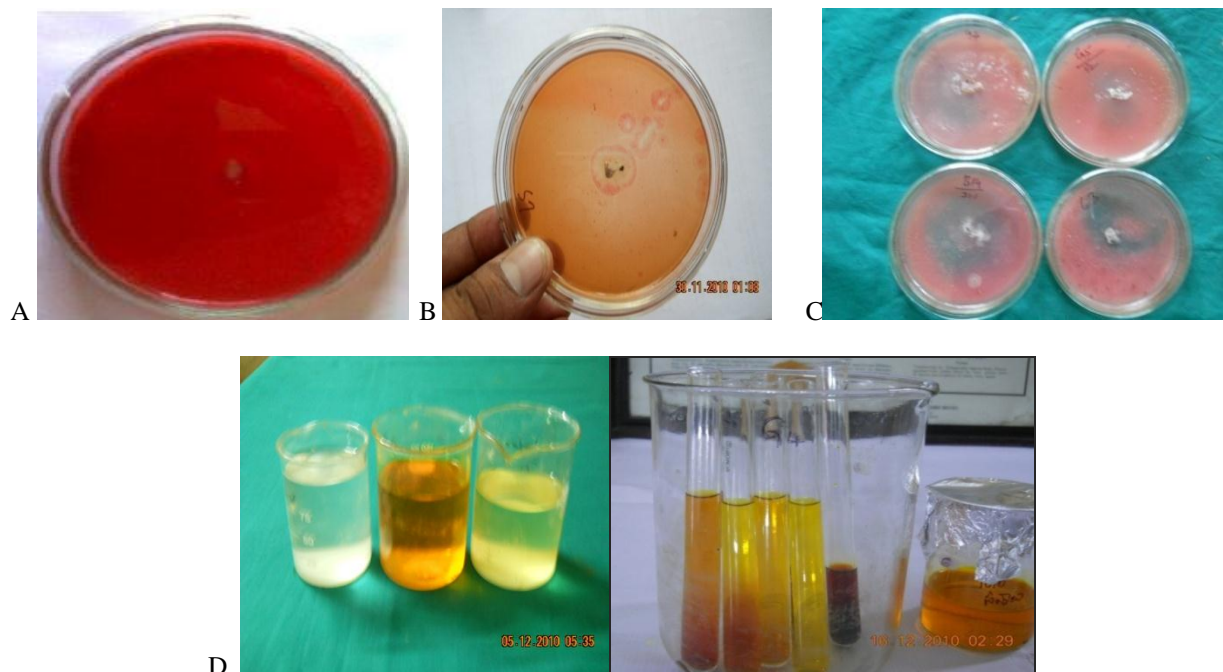


**Amylase enzyme**



**Amylase assay (DNS method)**





D Control Plate B) *Aspergillus niger* C) *Chaetomium globosum* D) Cellulase assay

## CONCLUSION

The present study showed that the soils of Chittoor district can be a very good source of industrially important enzymes. The secondary screening for the production of amylase, cellulase, pectinase, and phosphatase was also done for six selected isolates. Among the fungal isolates, *Aspergillus*, *Penicillium*, *Chaetomium globosum* and *Cladosporium* predominated. In the present study, the screening for only the main groups of hydrolytic enzyme producers was made. The

vast microbial biodiversity of the Chittoor district is yet to be exploited so that the indigenous soils can be screened for the isolation of other novel fungi with the ability of production of some other important enzymes, antibiotics and other bioactive compounds. Since each application of enzymes in industry requires different specificities, the hydrolytic enzymes with different properties have to be isolated. Further studies with regard to the optimization of production parameters and the catalytic properties are required to be done.

## REFERENCES

- Kathiresan, K and Manivannan, S. Alpha amylase production by *Penicillium fellutanun* isolated from Mangroove rhizosphere soil. *African Journal of Biotechnology*, 2006; 3(10): 829-832.
- Samira, M., Mohammad, R and Gholamreza, G. Carboxymethyl cellulose and filter paperase activity of new strains isolated from Persian Gulf. *Microbiology Journal*, 2011; 1(1): 8-16.
- Gautam, S.P., Bundela, P.S., Pandey, A.K., Awasthi, M.K and Sarsaiya, S. Screening of cellulolytic fungi for management of municipal solidwaste. *Journal of Applied Sciences in Environmental Sanitation*, 2010; 5(4): 391-395.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L and Randall, R.J. Protein measurement with the folinphenol reagent. *Journal of General Microbiology*, 1951; 131: 3017-3027.
- Oyeleke, S. B and Oduwale, A.A. Production of amylase by bacteria isolated from a cassava waste dumpsite in Minna, Niger state, Nigeria. *African Journal of Microbiology Research*, 2009; 3(4): 143-146.
- Bakri, Y., Mangali, M and Thonart, P. Isolation and Identification of a New Fungal Strain for Amylase Biosynthesis. *Polish Journal of Microbiology*, 2009; 58(3): 269-273.
- Haki, G.D and Rakshith, S.K. Developments in industrially important thermostable enzymes; a review. *Bioresource Technology*, 2003; 89: 17-34.
- Kirk, O., Borchert, T.V and Fuglsang, C.C. Industrial enzyme applications. *Current Opinion in Biotechnology*, 2002; 13: 345-351.
- Arunsasi., Kani, M., Panneerselvam, A., Jegadeesh, G., Muthu, K and Kumar, M.R. Optimizing the conditions of  $\alpha$ - amylase by an Estuarine strain of *Aspergillus* spp. *African Journal of Microbiology Research*, 2010; 4(8): 581-586.
- McGinnis, M.R., D'Amato, R.F and Land, G.A. *Pictorial Handbook of Medically Important Fungi and Aerobic Actinomycetes*. Praeger Publishers, New York, 1982; 160.
- Banakar, S.P., Thippeswamy, B., Thirumalesh, B.V and Naveenkumar, K.J. Isolation, production and partial purification of fungal amylase from forest soils of Bhadra Wildlife Sanctuary, Western Ghats. *Inventi Rapid: Pharmacy, Biotechnology and Microbiology*, 2012; 3: 1-7.
- Alva S, Anupama J, Salva J, Chiu YY, Vyshali P, Shruthi M, Yogeetha BS, Bhavya D, Purvi J, Ruchi

- K, Kumudini BS and Varalakshmi KN. Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. *African journal of Biotechnology*, 2007; 6: 576–581.
13. Sathyaprabha, G., Panneerselvam, A and Muthukumarasamy, S. Production of Cellulase and Amylase from wild and mutated fungal isolates. *E-Journal of Life Sciences*, 2011; 1(1): 39-45.
  14. Srinivasan, M.C. *Practical Mycology for Industrial Biotechnologists*. Tata McGraw-Hill Publishing Company Limited, New Delhi, 2004; 243.
  15. Miller GL Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 1959; 31; 426-428
  16. Tabatabai, M.A., and Bremner, J.M. Use of p-nitrophenyl phosphate for assay of soil phosphate activity. *Soil Biol. Biochem*, 1969; 1: 301-307.
  17. Alva S, Anupama J, Salva J, Chiu YY, Vyshali P, Shruthi M, Yogeetha BS, Bhavya D, Purvi J, Ruchi K, Kumudini BS and Varalakshmi KN. Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. *African journal of Biotechnology*, 2007; 6: 576–581.
  18. Pothiraj, C., Balaji, P and Eyini, M. Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste. *African Journal of Biotechnology*, 2006; 5(20): 1882-1885.