

# ISOLATION, CHARACTERIZATION OF ASPERGILLUS FUMIGATUS AND OPTIMIZATION OF CULTURAL CONDITIONS FOR AMYLASE PRODUCTION

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## Abstract

The soil samples were collected from different depths of paddy and sugarcane fields. The samples were primarily screened for isolation of amylase producing fungi. Among the isolated fungi, amylase producing isolates were identified by growing on starch agar media. The isolate (15F) which form the maximum zone of clearance on starch agar media by iodine was identified and it was subcultured on potato dextrose agar (PDA). The isolate (15F) was morphologically characterized by performing cotton blue staining and scanning electron microscopic observations under required magnifications. Molecular characterization of isolate (15F) was performed by ITS/5.8S rRNA and  $\beta$ -tubulin gene sequence analysis and it was confirmed as *Aspergillus fumigatus* (MTCC Acc No 11399). Optimization of cultural conditions for maximum production of amylase was carried out by different cereal flours, incubation periods, temperatures, nitrogen sources and with different phosphate concentrations. *Aspergillus fumigatus* showed maximum amylase activity ( $230 \pm 0.7$ U/mg protein) when cultured in finger millet at  $35^{\circ}\text{C}$  for 72hrs of incubation period.

**Keywords:** Amylase, *Aspergillus fumigatus*, cereal flour, submerged fermentation

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## 1. INTRODUCTION

In modern times most important products of biological origin are enzymes, because they have numerous applications in various industrial processes. Among the industrial enzymes, starch degrading enzymes such as amylases gaining more importance in modern biotechnological era [1]. These enzymes are found in animals (saliva, pancreas), plants (malt), bacteria and fungi [2]. Being the most important sources for enzyme production the selection of suitable microorganisms plays a key role in high yield of desirable enzyme. The microbial amylases are the most important enzymes in present day biotechnology as they meet industrial demands. They have almost completely replaced chemical hydrolysis of starch in starch processing industries and have a great significant demand and they hold approximately 25% of the enzyme market [3]. Amylase is an amylolytic extracellular enzyme secreted by bacteria and fungi to carry out extracellular digestion. They break the insoluble starch into soluble end products such as glucose or maltose which are absorbed into their cells. Microbial amylases replace the chemical hydrolysis in starch saccharification [4]. Among the microbial production of amylases, fungal amylases are preferred over the bacterial sources mainly because of their more acceptable GRAS (Generally regarded as safe) status [1]. The fungal origin of amylases are more stable than the bacterial enzymes on a commercial scale [5, 2]. Molds are capable of producing high amounts of amylases [6]. Studies on fungal amylases,

especially the developing countries have concentrated mainly on *Rhizopus sp.* and *Aspergillus sp.*, probably because of their ubiquitous nature and non fastidious nutritional requirements of these organisms [7]. Amylases have potential applications in a number of industrial processes such as in food, baking, brewing, detergent, textile and paper industries. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medical and analytical chemistry [8]. The present work is undertaken for screening of amylase producing *Aspergillus sp.* from soil and optimization of cultural conditions for efficient production of amylases from cheaply available raw materials in the cost effective manner.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Soil Samples

The soil samples were collected under sterile conditions to avoid contamination from different depths viz. (10, 15, 20cms) of paddy and sugarcane fields after their harvesting in Pendurthi, in Vijayanagaram District of Andhra Pradesh, India.

#### 2.1.1 Characterization of Soil

Mineral matter of soil such as sand silt and clay contents were analyzed with the use of different sizes of sieves by the method of Alexander [9]. Soil pH was measured at 1:1.25 soil

to water ratio using pH meter. Organic carbon content of soil was determined by Walkely and Black method [10], and total nitrogen content in soil was measured by the Microkjeldhal method [11]. Phosphorous in soil was measured by the method of Chapman and Pratti [12].

## 2.2 Isolation of Fungal Isolates from Soil Samples

One gram of each soil sample was suspended in the 9.0ml of sterile water. This sample was serially diluted by dilution plating method [13]. They were poured into PDA (potato dextrose agar) (Hi-Media) with chloramphenicol, a broad spectrum antibiotic used in inhibition of bacterial growth. The plates were incubated at 28°C for 2-3 days.

## 2.3 Determination of Fungal Amylase Activity

The fungal isolates were tested for amylase production by starch hydrolysis. The modified starch agar media (Soluble starch-2g, Peptone-2g, Yeast extract -1g, Agar-2g, Distilled water -100ml at pH6) were inoculated with the isolates and incubated for 48 hrs at 28°C. After the completion of incubation period, the petridishes were flooded with the iodine solution, the zone of clearance formed around the microbial growth indicates the production of amylase.

## 2.4 Characterization of Fungal Isolates

### 2.4.1 Microscopic Identification of Fungal Isolates

Identification of fungal species was done as per the manuals of Domsch [14] and Barnett and Hunter [15]. After isolation of fungal isolate it was sub cultured on the PDA slants. Later it was primarily subjected to the Lacto phenol cotton blue staining, and then analyzed the morphology by Scanning Electron Microscope (JSM-6610 instrument model, JEOL/EO format) under required magnifications to observe morphology of mycelium and spore structures.

### 2.4.2 Molecular Characterization of Fungal Isolates

The molecular characterization of the fungal isolate was done by the gene sequence analysis of ITS/5.8S rRNA and  $\beta$ -tubulin gene, and construction of phylogenetic tree based on evolutionary relationship of taxa based on ITS sequence data and  $\beta$ -tubulin gene sequence data by Neighbor-Joining method [16]. The bootstrap consensus tree inferred from 100 replicates, which were taken to represent the evolutionary history of the taxa analyzed [17]. The evolutionary distances were computed using the Kimura2-parameter method [18] and evolutionary analyses were conducted in MEGA5 [19].

## 2.5 Spore Harvesting and Storage

After observing the amylase activity the fungal isolate was further used by harvesting the spores. The spores were harvested after the culture had been incubated for 14days. During this stage the culture was fully sporulated. The

petridish was flooded with a mixture of 0.5ml sterile distilled water and 5.0ml of 0.05% Tween-80, then rubbed the culture gently with a sterile bent glass rod. The spores were released. Then the spore suspension was roughly filtered through sterilized glass wool positioned in the funnel into a 50ml Erlenmeyer flask. Spore suspensions were transferred directly to previously sterilized 50ml screw cap tubes and centrifuged for 5mins at 1000rpm at 4°C using REMI centrifuge. After centrifugation, supernatant was removed and the pellet was resuspended in 10ml sterile distilled water containing 0.1% (v/v) Tween-80, using vortex shaker for 30sec to suspend it completely. Then it was centrifuged once again to wash the spores free of debris. This process was repeated thrice and finally resuspended in 5.0ml sterile distilled water containing 0.1% (v/v) Tween-80 and shaken for 30sec before storage. The spore suspension was stored at 4°C in darkness until its requirement.

## 2.6 Amylase Production

The isolate (15F) was subjected to fermentation medium containing (KH<sub>2</sub>PO<sub>4</sub>-0.14g, NH<sub>4</sub>NO<sub>3</sub>-1g, KCl-0.5g, MgSO<sub>4</sub>·7H<sub>2</sub>O-0.01g, FeSO<sub>4</sub>·7H<sub>2</sub>O-0.001g, soluble starch-2g, Distilled water-100ml at pH6.5). Erlenmeyer flasks were sterilized by autoclaving at 121°C for 15mins. Spore suspension (0.5ml) was added and incubated in a shaking incubator for 48hrs, at 200rpm and 28°C temperature.

### 2.6.1 Extraction of Amylase from Fungal Isolates

The fermented broth was centrifuged at 7000rpm for 30mins. The cell free supernatant was used for the estimation of amylase.

### 2.6.2 Demonstration of Enzyme Activity/Enzyme

#### Assay

One milliliter of culture extract (enzyme) was pipetted into test tube, and 1.0 ml of 1% soluble starch in citrate phosphate buffer pH 6.5 was added and incubated in water bath at 40°C for 30mins. Then treated with DNS(Dinitro salicylic acid) and the reaction was stopped by boiling for 5mins, and cooled to room temperature and 20ml of distilled water was added and color intensity was measured at 540nm. One unit of amylase activity was defined as the amount of enzyme that releases 1 $\mu$ mol of maltose per minute under the assay conditions. Activity of the enzyme is expressed in units per mg protein.

## 2.7 Optimization of Cultural Conditions

### 2.7.1 Effect of Different Cereal Flours on Enzyme Production

The effect of different cereal flours such as sorghum, sammai, finger millet, pearl millet and horse gram on enzyme production was carried out at pH 5.6 at 30°C for 48hrs.

### 2.7.2 Effect of Temperature

The effect of temperature on enzyme production was investigated by incubating the fermentation medium at 25<sup>0</sup>C, 30<sup>0</sup>C, 35<sup>0</sup>C, 40<sup>0</sup>C and 45<sup>0</sup>C at pH 5.6 for 90hrs.

### 2.7.3 Effect of Incubation Period

The effect of incubation period on enzyme production was investigated by incubating the fermentation medium at regular intervals of 18, 36, 54, 72 and 90 hrs at pH 5.6 and at 35<sup>0</sup>C.

### 2.7.4 Effect of Different Nitrogen Sources

The effect of different Nitrogen sources on enzyme production was investigated by adding ammonium nitrate, ammonium chloride, ammonium sulphate, and sodium nitrate separately to the fermentation medium and incubating them at pH 5.6 for 90hrs at room temperature.

### 2.7.5 Effect of Different Concentrations of Phosphate

The effect of phosphate concentration in the enzyme production was investigated by adding 0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1.4% of phosphate in the medium at pH 5.6 for 90hrs of incubation.

\*Statistical analysis: All the experiments were repeated thrice and standard deviation was calibrated.

## 3. RESULTS

### 3.1 Soil Sample Analysis

Fungal isolate from soil have beneficial role in industrial area [20, 21] and their saprophytic ability mainly depends on the chemistry, texture, salinity, and water holding capacity [22, 23]. Some of the physico chemical characteristics of the soil from which the microorganisms isolated are shown in table 1.1. Soil pH is one of the most indicative measurements of the soil because it is an important factor for the survival of microorganisms [24]. In the present study the pH of the soil was determined to be 7.0.

**Table 1.1:** Physico chemical characteristics of the soil samples

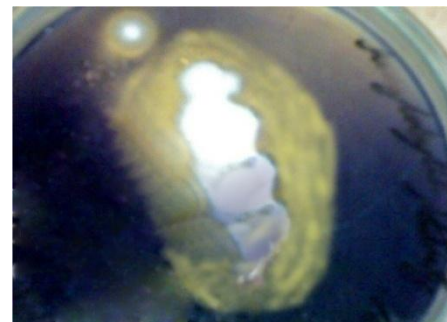
Characteristics	Soil sample
Color	Brown
Odour	Normal
pH	7.0
Organic carbon(kg/*A)	65
Phosphorous(kg/*A)	0.60
Potassium(kg/*A)	0.56

\*A=Acre

### 3.2 Isolation and Identification of Fungal Isolates

#### 3.2.1 Morphological Characterization of Isolates

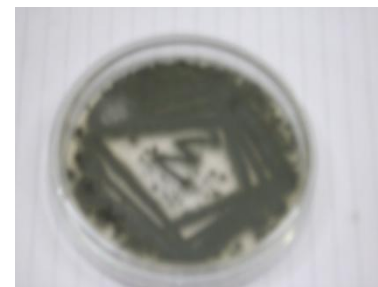
There are various methods for isolating the fungi [25, 26] but the simplest one is dilution plate method and the lifting of conidia from sporulating conidiophores. According to the starch hydrolyzing capacity, five fungal isolates were isolated from soil samples. Among these only one isolate (15F) showed the maximum zone of clearance on starch agar media as shown in figure 1.1 (a). The isolate (15F) was characterized morphologically by lactophenol cotton blue staining and scanning electron microscopic analysis the details are presented in table 1.2. The macro and microscopic images of isolate (15F) was shown in figure 1.2.



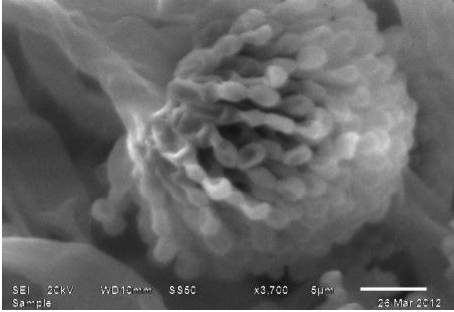
**Fig 1.1 (a)** Starch hydrolyzing activity of isolate (15F) on starch agar media

**Table 1.2:** Macroscopic and microscopic characteristics of fungal isolate (15F).

Parameter	Isolate
Colony color	Green powdery.
Colony diameter (cm)	3.0- 6.0
Hyphae	Septate branched.
Spore arrangement	Bunch of spores with single chains.
Spore shape	Oval



**(a)** Isolate-15F macroscopic image



(b) SEM image of Isolate-15F

Fig 1.2: Macro and Microscopic images of fungal isolate (15F).

### 3.2.2 Molecular Characterization of Isolate (15F)

Molecular characterization of the isolate (15F) was performed by ITS/5.8S rRNA and β-tubulin gene sequence analysis and the isolate was confirmed as *Aspergillus fumigatus* (MTCC Acc No 11399). ITS/5.8S rRNA gene sequence, β-tubulin gene sequence data and phylogenetic Evolutionary relationship of taxa based on ITS Sequence, β-tubulin gene Sequence data of *Aspergillus fumigatus* were shown in figures 2(a), 2(b), 2(c), 2(d).

ATGCTTAAGTTCAGCGGTATCCCTACTGATCCGAGG  
 TCAACCTTAGAAAAATAAAGTTGGGTGTCGGCTGGC  
 GCCGGCCGGGCTACAGAGCAGGTGACAAAGCCCCA  
 TACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCT  
 TTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGGGCC  
 CAACACACAAGCCGTGCTTGAGGGCAGCAATGACGC  
 TCGGACAGGCATGCCCCCGGAATACCAGGGGGCGC  
 AATGTGCGTTCAAAGACTCGATGATTCACCTGAATTCT  
 GCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTC  
 ATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGT  
 TTTAACTGATTACGATAATCAACTCAGACTGCATACT  
 TTTCAGAACAGCGTTCATGTTGGGGTCTTCGGCGGGC  
 GCGGGCCCGGGGGCGCAAGGCCTCCCGCGGCCGTC  
 GAAACGGCGGGCCCGCCGAAGCAACAAGGTACGAT  
 AGACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCA  
 CTCGGTAATGATCCTTCCGCAGGTTACCTACGGAAA  
 CCTTGTTACGAT

Fig 2(a) *Aspergillus fumigatus*, Strain “(15F)”, ITS/5.8S rRNA gene sequence data

GTGCTGCTTTCTGGTATGTCTTGACCTCAAAGCTTGG  
 ATGACGGGTGATTGGGATCTCTCATCTTAGCGGGNTA  
 CCTCCATGGGTTTCAGCCTCACTGTCATGGGTATCAGC  
 TAACAAATCTACAGGCAGACCATCTCTGGTGAGCAT  
 GGCCTTGACGGCTCTGGCCAGTAAGTTCGACCTATAT  
 CCTCCCAATTGAGAAAGCGGCGGAAACACGGAAAAC  
 AAGGAAGAAGCGGACGCGTGTCTGATGGGAAATAAT  
 AGCTACAATGGCTCCTCCGATCTCCAGCTGGAGCGTA  
 TGAACGTCTATTTCAACGAGGTGTGTGGATGAAACTC  
 TTGATTTATACTATTTTCGGCCAACATCTCACGATCTG

ACTCGCTACTAGGCCAACGGTGACAAATATGTTTCCTC  
 GTGCCGTTCTGGTCGATCTCGAGCCTGGTACCATGGA  
 CGCTGTCCGTGCCGGTCCCTTCGGCGACTATTCGCTC  
 CCGACAACCTTCGTCTTCGGCCAGTCCGGTGCTGGTAA  
 CAACTGGGCCAAGGGT

Fig 2(b) *Aspergillus fumigatus*, Strain “(15F)”, β-tubulin gene sequence data

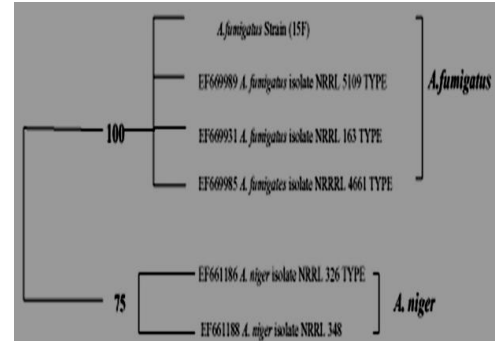


Fig 2(c) Evolutionary relationship of taxa based on ITS Sequence data.

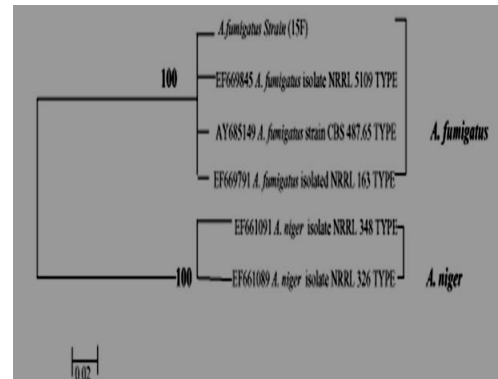
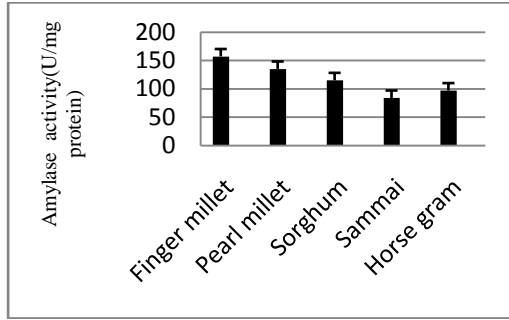


Fig 2(d) Evolutionary relationship of taxa based on β-tubulin gene Sequence data.

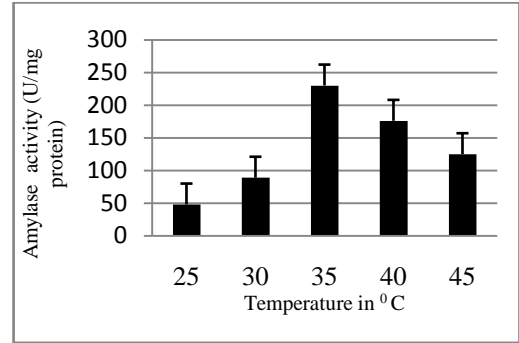
### 3.3 Effect of Different Cereal Flours on Enzyme Production

Addition of different cereal flours like finger millet, pearl millet, sorghum, sammai and horse gram to the fermentation medium, maximum enzyme activity was observed with finger millet (157±0.5U/mg protein) and minimum with sammai (84±0.5U/mg protein) by *Aspergillus fumigatus* at 30°C temperature as shown in figure3(a).



\*Different cereal flours on amylase production by *Aspergillus fumigatus* at 30°C for 48 hrs of incubation

**Fig 3(a)** Effect of different cereal flours on amylase production

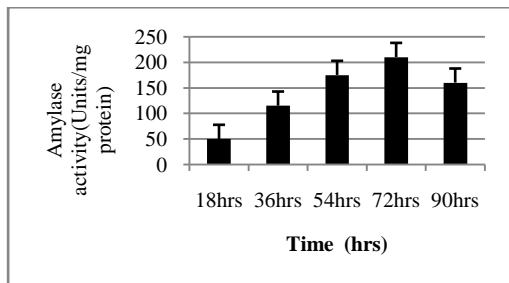


\*Different temperatures (25-45°C) on amylase production by *Aspergillus fumigatus* with finger millet at 72hrs of incubation

**Fig 3(c)** Effect of different temperatures on amylase production

### 3.4. Effect of Incubation Period

Incubation period varies with the type of enzyme production. Short incubation period offers potential for inexpensive production of enzyme [27]. As shown in figure 3(b), incubation of fermentation medium at different time intervals 18, 36, 54, 72, and 90 hrs, maximum (210±0.6U/mg protein) and minimum (50±0.63U/mg protein) enzyme activity was observed within 72hrs and 18 hrs respectively at 30°C temperature.



\*Different incubation periods (18-90hrs) on amylase production by *Aspergillus fumigatus*

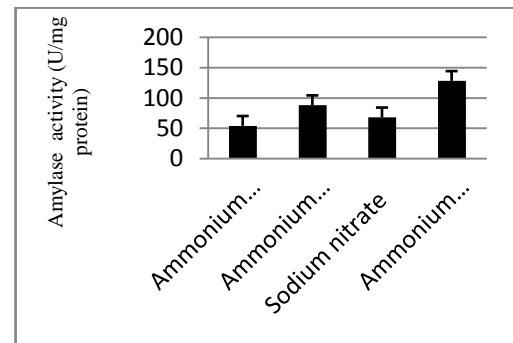
**Fig 3(b)** Effect of incubation period on amylase production

### 3.5 Effect of Temperature

Incubation of fermentation medium at different temperatures (25°C, 30°C, 35°C, 40°C, and 45°C) was carried out. Maximum (230±0.7U/mg protein) and minimum (48±0.57U/mg protein) amylase activity was observed at temperature 35°C and 25°C respectively by *Aspergillus fumigatus* at 72hrs of incubation period as shown in figure 3(c).

### 3.6 Effect of Different Nitrogen Sources on Enzyme Production

Ammonium nitrate, ammonium chloride, ammonium sulphate, and sodium nitrate were used as nitrogen sources separately along with fermentation media. Maximum (128±0.43U/mg protein) and minimum (54±0.5U/mg protein) amylase activity was observed in Ammonium nitrate and ammonium chloride respectively with *Aspergillus fumigatus* at 35°C for 72hrs of incubation as shown in figure 3 (d).

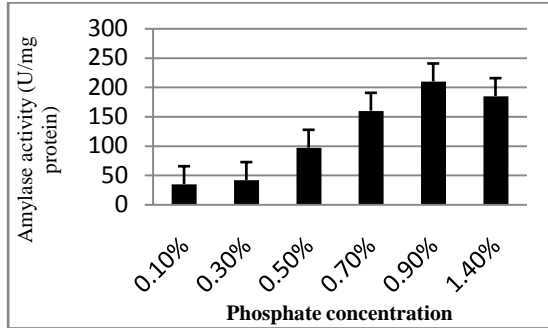


\*Different nitrogen sources on amylase production by *Aspergillus fumigatus* at 35°C for 72

**Fig 3(d)** Effect of different nitrogen sources on amylase production

### 3.7 Effect of Different Concentrations of Phosphate

Incubation of fermentation medium with different concentrations of Phosphate (0.1%, 0.3%, 0.5%, 0.7%, 0.9%, and 1.4%) the maximum (210±0.65U/mg protein) and minimum (35±0.7U/mg protein) amylase activity was observed in 0.9% and 0.1% phosphate respectively with *Aspergillus fumigatus* at 35°C for 72hrs of incubation as shown in figure 3(e).



\*Different percentages of phosphate concentrations on amylase production by *Aspergillus fumigatus* at 72hrs of incubation.

**Fig3 (e)** Effect of different phosphate concentrations on amylase production

#### 4. DISCUSSION

Isolation of fungi from soil samples and the rapid screening by plating on starch agar plates led to finding of five fungal isolates capable of producing amylase. Morphological and molecular characterization confirmed the isolate (15F) as *Aspergillus fumigatus*. Different cereal flours used in submerged fermentation, maximum amylase activity ( $157 \pm 0.5$  U/mg protein) was observed in *Aspergillus fumigatus* with finger millet at  $30^\circ\text{C}$  for 48hrs of incubation. Gomes [28] reported that amylase production was maximum (140U/mg protein) with sorghum flour with *Aspergillus niger* at 72hrs of incubation period. Incubation of fermentation medium at different time intervals the maximum amylase activity ( $210 \pm 0.6$  U/mg protein) was observed at 72hrs in *Aspergillus .fumigatus*. Dhurba Sharma and Shukla [29] reported the maximum production of amylase (185U/ml) with *Aspergillus fumigatus* for 6 days of incubation at  $30^\circ\text{C}$ . Incubation at different temperatures the maximum amylase activity ( $230 \pm 0.7$  U/mg protein) was observed at  $35^\circ\text{C}$  by *Aspergillus fumigatus*. Got [30] reported maximum production of amylase (193U/ml) and glucoamylase (200U/ml) by *Aspergillus fumigatus* at  $37^\circ\text{C}$ . By the effect of different nitrogen sources maximum activity of amylase ( $128 \pm 0.43$  U/mg protein) was observed in *Aspergillus fumigatus* with ammonium nitrate. It was reported that peptone, sodium nitrate and casein hydrolysate were good nitrogen supplements for amylase production in *Aspergillus fumigatus* by Got [30], in *Aspergillus niger* by Pandey [31] and in *Aspergillus oryzae* by Pederson and Nielson [32]. With different phosphate concentrations, maximum amylase activity ( $210 \pm 0.65$  U/mg protein) was observed in 0.9% of phosphate concentration by *Aspergillus fumigatus* at  $35^\circ\text{C}$  for 72hrs of incubation period. Nguyen [33] reported that *Thermomyces lanuginosus* showed the maximum amylase activity (190U/ml) with 0.7% of phosphate concentration at  $30^\circ\text{C}$  of incubation. Peixoto [34] elicited same rate of amylase activity with *Rhizopus microsporus*, a thermotolerant fungi when compared with *Aspergillus fumigatus* at 0.5% of phosphate concentration.

#### 5. CONCLUSIONS

The fungal isolates were collected from soil, analysis of soil was performed. Morphological characterization of isolate (15F) was carried out through scanning electron microscope which reports that the conidiophores are in septate, oval shape. The molecular characterization of the isolate (15F) was carried out with the help of ITS/5.8 S rRNA and  $\beta$ -tubulin gene sequence analysis and the isolate was confirmed to be *Aspergillus fumigatus* (MTCC Acc No 11399), which is closely related to *Aspergillus niger*. Optimization of cultural conditions was done for maximal production of amylase using different cereal flours, incubation periods, temperatures, nitrogen sources and with different phosphate concentrations. The maximum amylase activity ( $230 \pm 0.7$  U/mg protein) was observed on production media containing finger millet as the carbon source, and ammonium nitrate as the nitrogen source at  $35^\circ\text{C}$  for 72hrs incubation period by *Aspergillus fumigatus*. The carbon source was cheaply available throughout the year and easy for storage of seeds for further usage. Amylases have wide range of applications in lactic acid production, antibiotics, food, bio fuel, and detergent industries. Amylolytic activity of filamentous fungi helps in the production of lactic acid. Lactic acid mainly helps in the formation of biodegradable plastics. Applications of amylase in food industries extensively employed in baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups. Ethanol is the most utilized liquid bio fuel. The bioconversion of starch into ethanol involves liquefaction and saccharification. Amylases are used in textile industry for desizing process.

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