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Research Paper

ISOLATION, CHARACTERIZATION AND OPTIMIZATION OF CULTURAL CONDITIONS FOR AMYLASE PRODUCTION FROM FUNGI

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Abstract

The soil samples were collected and 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 isolates exhibiting the maximum zone of clearance on starch agar media by iodine were identified and were subcultured on potato dextrose agar (PDA). The isolates were morphologically characterized by performing cotton blue staining and were identified as *Aspergillus niger, Penicillium chrysogenum, Microsporium sp and Fusarium sp*. Isolate *Penicillium chrysogenum* was the most potent producer of alkaline amylase was identified showing highest activity in the optimized medium at pH 8.0, temperature 45ºC, with 1% wheat bran and peptone incubated for 7 days.

Key words: Amylase, *Aspergillus fumigatus, Penicillium chrysogenum,* Agrobased waste.

INTRODUCTION

In modern times most important products of biological origin are enzymes, because they have numerous applications in various industrial processes. Enzymes are produced by various micro-organisms including bacteria, fungi and yeast and are considered as important products obtained for human needs through microbial sources.

The advantage of using micro-organisms for the production of enzymes is that bulk production is economical and microbes are easy to manipulate to obtain enzymes with desired characteristics. Fungal enzymes are preferred over other microbial sources owing to their widely accepted Generally Regarded As Safe (GRAS) status [1].

Amylases are a group of hydrolases which can specifically cleave glycosidic bonds in starch. α- amylases (endo-1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that can randomly cleave 1,4-α-D-glucosidic linkages between adjacent glucose units inside the linear amylose chain [2, 3, 4].

The microbial amylases could be potentially useful in various pharmaceutical, fine-chemical industries etc., with the event of new frontiers in biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. Amylases are also used in baking, brewing, textile, detergent, paper and distilling industries. These uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes [5].

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 [6]. Hence, there is an increasing worldwide interest in the screening of new microorganisms producing amylases suitable for industrial applications. [7,8].

When these amylase producers are isolated, there is need to explore their production efficiency through studying of their best conditions for a higher productivity. These stimulated our interest in this research to optimize the cultural conditions of some isolated α-amylase producing fungi to enhance their potentials for more effective industrial applications.

MATERIALS AND METHODS

Collection of Soil Samples

The soil samples were collected under sterile conditions to avoid contamination from different depths viz. (10, 15, 20cms).

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 [9]. 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 280C for 2-3 days.

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 pH 6) were inoculated with the isolates and incubated for 48 hrs at 28^0C. 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.

Microscopic Identification of Fungal Isolates

Identification of fungal species was done as per the manuals of Domsch [10] and Barnett and Hunter [11]. 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 under required magnifications to observe morphology of mycelium and spore structures.

Amylase Production

The isolates were subjected to fermentation medium containing (KH_2PO_4-0.14g, NH_4NO_3-1g, KCl-0.5g, $MgSO_4.7H_2O$-0.01g, $FeSO_4.7H_2O$-0.001g, soluble starch-2g, Distilled water-100ml at pH6.5). Erlenmeyer flasks were sterilized by autoclaving at 121^0C for 15mins. Spore suspension (0.5ml) was added and incubated in a shaking incubator for 48hrs, at 200rpm and 28^0C temperature.

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.

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 40^0C 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 1µmol of maltose per minute under the assay conditions. Activity of the enzyme is expressed in units per mg protein.

Optimization of Cultural Conditions

Effect of Different Carbon sources on Enzyme Production

The effect of different Carbon sources such as Glucose, Sucrose, Starch, Lactose and Fructose on enzyme production was carried out at pH 5.6 at 30^0C for 48hrs.

Effect of Temperature on Enzyme Production

The effect of temperature on enzyme production was investigated by incubating the fermentation medium at 35^{0}C, 40^{0}C, 45^{0}C, 50^{0}C, 55^{0}C and 60^{0}C at pH 5.6 for 48hrs.

Effect of Different Nitrogen Sources on Enzyme Production

The effect of different Nitrogen sources on enzyme production was investigated by adding Ammonium sulphate, Urea, Peptone and yeast extract separately to the fermentation medium and incubating them at pH 5.6 for 48hrs at room temperature.

Effect of pH on Enzyme Production

The effect of pH was studied by varying the pH of the medium at 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. After the inoculation of the fungus, the medium was incubated at 30ºC for 48hrs. The optimum initial pH of the solid substrate achieved by this step was fixed for subsequent experiments.

Effect of different Agrobased waste material

The effect of different Agrobased waste material on enzyme production was investigated by adding groundnut cake, coconut cake, Soya cake and wheat bran separately to the fermentation medium and incubating them at pH 5.6 for 48hrs at room temperature.

RESULTS AND DISCUSSION

Isolation of Fungal Isolates from Soil Samples

Fungal isolate from soil have beneficial role in industrial area [12, 13] and their saprophytic ability mainly depends on the chemistry, texture, salinity, and water holding capacity [14, 15]. Different Fungal colonies were isolated form cow dung samples enriched for amylase producing microorganisms by serial dilution process where in PDA (potato dextrose agar) media was prepared, autoclaved and poured in sterile Petri plates. 50 µl of serially diluted samples diluted up to 10^{-5} dilutions were spread on respective solidified PDA plates. The inoculated petriplates were incubated at 28°C for 48 hours. All the strains were screened for the Amylase activity by plate assay method. Four fungal isolates were selected and differentiated as *Aspergillus, Penicillium, Fusarium and Microsporium* Strains on the basis of physical characteristics [16]. The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 28°C for 48 hours in order to obtain pure fungal plates.

The production of fungal enzymes in nature has a role in their pathogenicity or degradative capacity [17]. Priest (1984) [18]showed that there are several possible regulatory mechanisms in enzyme production, including enzyme induction. Optimization of process parameters is very important for overproduction of enzymes to meet industrial demand [19].

Optimization of Cultural Conditions

Effect of Different Carbon sources on Enzyme Production

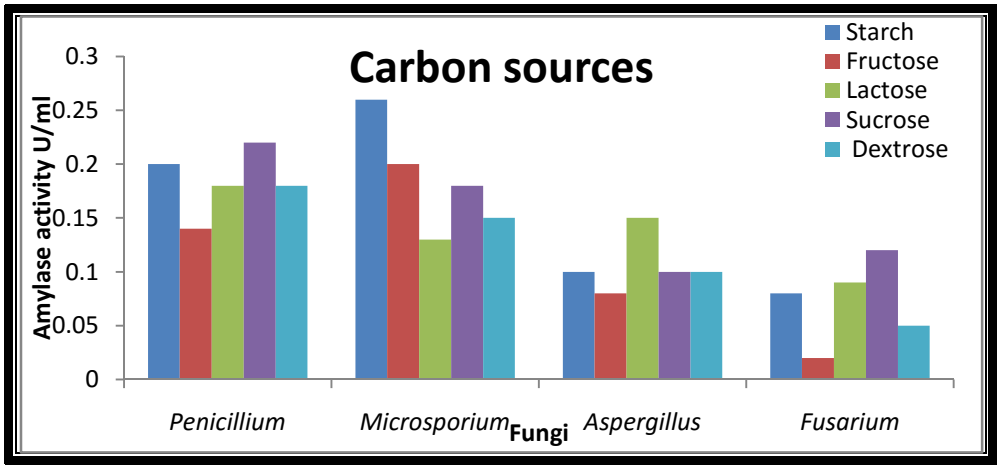

Fig 1: Effect of different carbon sources on Fungi

Addition of different carbon sources like finger Dextrose, Lactose, Sucrose, Fructose and Starch to the fermentation medium, maximum enzyme activity was observed with Starch (0.26U/ml) with *Microsporium* and minimum with Dextrose (0.05U/ml) by *Fusarium* at 30^{0}C temperature as shown in Figure 1. This finding is in line with the report that the best carbon substrate for amylase production is sweet potato starch [20].

The result of this investigation showed that the fungus had the ability to utilize the various carbon and nitrogen sources as good substrates for growth as well as for the concomitant production of amylase in submerged cultivation. Fungi being heterotrophs obtain their required nutrients from the organic matter in the environment through the presence of efficient and extensive systems of powerful enzymes. Thus, they are able to utilize complex carbon sources as their energy source [21, 22]. The dominance of polysaccharides over disaccharides and monosaccharides in supporting the growth of fungi had earlier being reported by [23] and Akinyosoye et al. [24], who reported that starch supported the maximum biomass yield of *Geotrichum candidum* and *Phoma sorghina* better than disaccharides (maltose and lactose), monosaccharides (glucose, fructose, and galactose).

Effect of Temperature on Enzyme Production

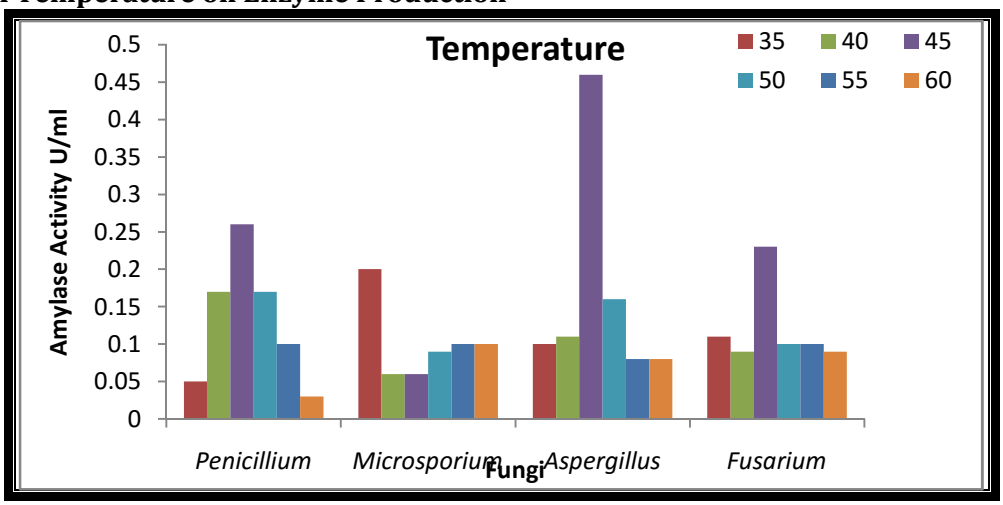

Fig 2: Effect of different Temperatures on Fungi

Incubation of fermentation medium at different temperatures (35^{0}C, 40^{0}C, 45^{0}C, 50^{0}C, 55^0 C and 60^{0}C) was carried out. Maximum (0.46 U/ml) and minimum (0.03U/ml) amylase activity was observed at temperature 45^{0}C by *Aspergillus fumigatus* and 60^{0}C by *Penicillium* sp respectively at 72hrs of incubation period as shown in Figure 2.

Temperature is a critical factor which markedly influences enzyme activities. In the present study, maximum α-amylase activity of the fungal isolates was obtained at 45 ^{o}C. This observation agrees with the earlier report that amylases differ in their temperature optimum, pH optimum and several other physiochemical properties depending on their origin. Hence, different enzymes have found specific application in different industries [25].

Effect of Different Nitrogen Sources on Enzyme Production

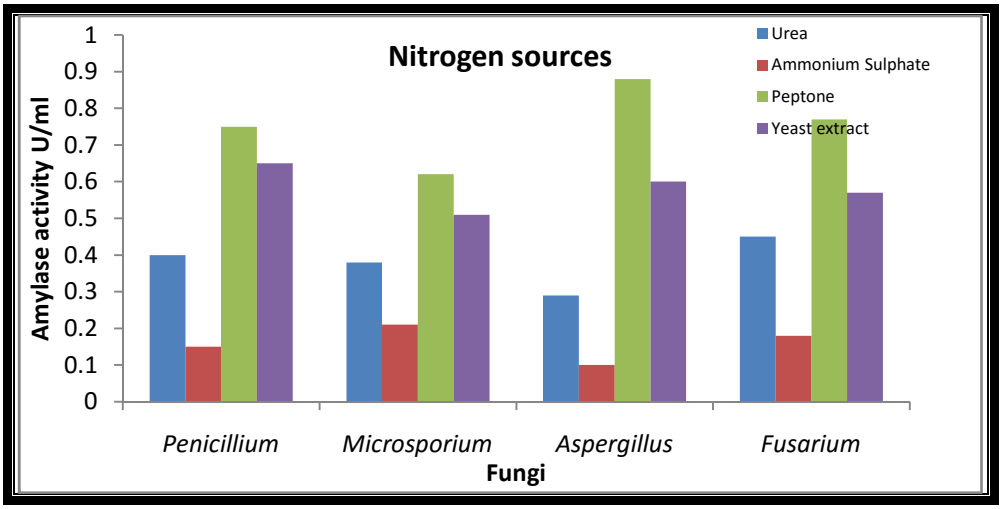

Fig 3: Effect of different nitrogen sources on Fungi

Urea, Ammonium sulphate, Peptone and Yeast extract were used as nitrogen sources separately along with fermentation media. Maximum (0.88 U/ml) with Yeast extract by *Aspergillus* and minimum (0.1U/ml) amylase activity was observed in Ammonium sulphate respectively with *Aspergillus* at 35^{0}C for 72hrs of incubation as shown in Figure 3.

The production of primary metabolites by organisms is highly influenced by their growth which depends on the nutrients provided. Similar type of observation found by Muhammad *et al*., (2012) [26] when using *A. niger* ML- 17 and *R. oligosporus* ML- 10. Many workers Oshoma *et al., (*2010) [27]; Valaparla, (2010)[28]; Anto *et al., (*2006)[29]; Pederson and Neilson (2000)[30], reported that Peptone and yeast extract as an organic nitrogen source produces maximum amylase production.

Vahidi et al. [31] reported that good growth and antifungal activities were observed when complex nitrogen sources—yeast extract, peptone—were used compared to inorganic nitrogen source (NH_4Cl and $NaN0_3$). Akhilesh et al. [32] equally reported best polygalacturonase production with *Mucor circinelloides* ITCC 6025 when casein hydrolysate and yeast extract were used together, while Sasi et al. [33] reported that organic nitrogen induced the highest amylase activity in estuarine strain of *Aspergillus* spp. This preponderance of organic nitrogen sources on inorganic sources might be due to the fact that the organic nitrogen sources were better good growth stimulators. During growth and enzyme production, the fungus strain probably hydrolyzed the organic nitrogen releasing their mineral component and other growth factors in them into constituents that can be easily incorporated into cellular metabolism.

Effect of pH on Enzyme Production

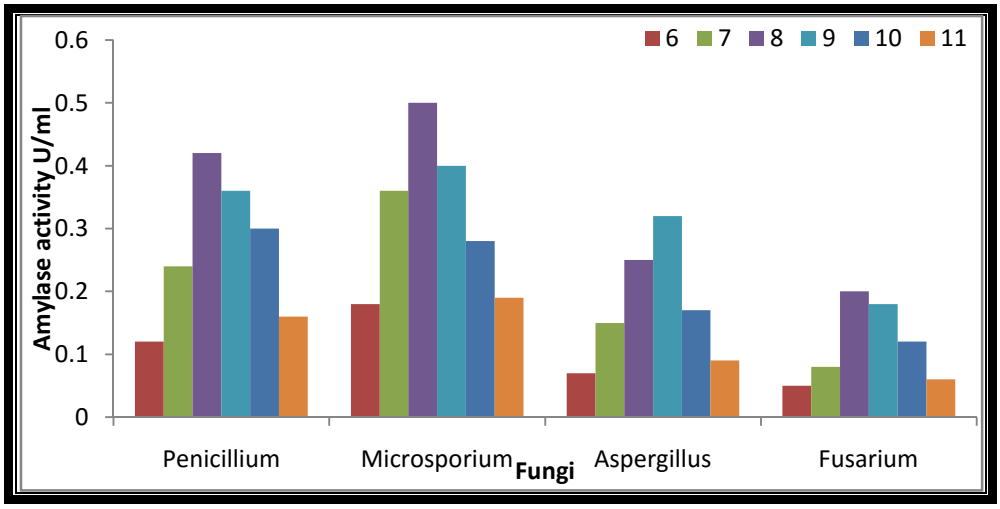

Fig 4: Effect of different pH on Fungi

The effect of pH on amylase activity of different fungal sp was studied by varying the pH from 6.0 to 11.0. The results depicted in Figure 4 indicate that with increase in pH value from 6 to 8, the activities of amylase enzyme reached to the maximum followed by a gradual decrease thereafter. It is clear that pH of 8.0 was found to be best for amylase activity and maximum activity recorded was 0.5 U amylase from *Microsporium* and 0.42 with *Penicillium* sp.

In the course of the investigation, it was observed that the pH of the culture media varied over a wide range of values within the acidic region on the pH scale. Fungi generally alter the pH of the medium in which they grow, due to uptake of the anions or cations in the medium [34]. Therefore, the varied changes witnessed in the pH values of the culture media may be as a result of the utilization of some compounds in the culture media.

Effect of Agrobased waste on Enzyme Production

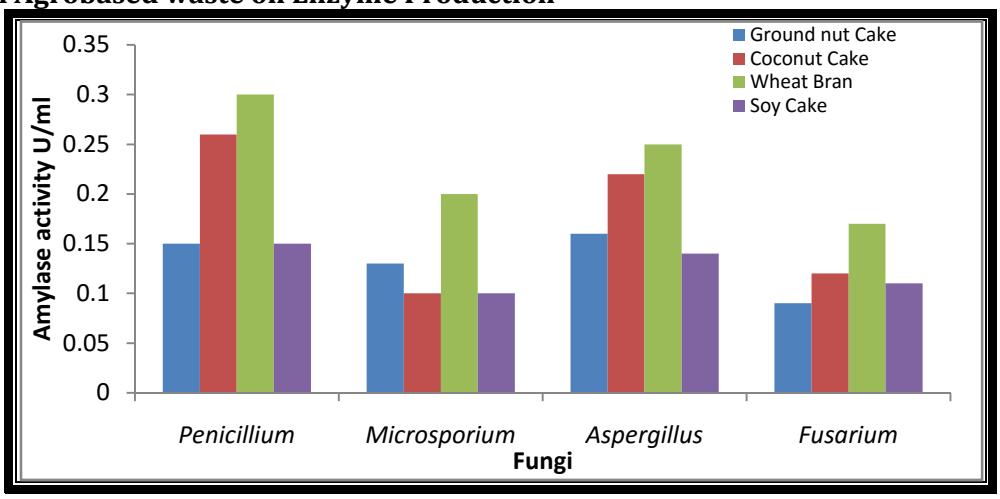

Fig 5: Effect of different Agrobased waste on Fungi

Four different agricultural residues viz. Groundnut cake, wheat bran, Soycake and Coconut cake were fermented in the solid state by different fungal strains using mineral medium (pH 5) at 28 ± 2°C for five days. The enzymes were partially purified by isopropanol using the mold filtrate. The precipitates obtained were dissolved in citrate buffer (pH 5) and were used further for the estimation of their respective enzyme activities. The results presented in Figure 5 show that out of four agricultural residues, wheat bran as substrate yielded maximum enzyme activities of alpha amylase (0.3U) by *Penicillium* and *Aspergillus* sp. Similar finding on SSF based amylase production was also reported by Sindhu *et al*., 2009[35].

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