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OPTIMIZATION OF SOME FERMENTATION CONDITIONS FOR THE PRODUCTION OF EXTRACELLULAR AMYLASES BY USING ASPERGILLUS SPECIES

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ABSTRACT:
Amylolytic fungal isolates were obtained by starch-agar plate method from soil sample collected from santra market of Nagpur. Various amylolytic fungi were isolated and out of which three *Aspergillus species*, named as *Aspergillus niger, Aspergillus flavus* and *Aspergillus oryzae* were selected based on clear zone ratio. Amylase production was optimized using basal media. The maximum level of amylase production was achieved from *Aspergillus niger, flavus* and *oryzae* after 72 h of cultivation. The optimal temperature for amylase production was in the range of 40 to 45 ° **C** and pH of the media in the range 7 to 7.5.Under the optimized fermentation conditions Aspergillus species produced almost the similar amount of amylase with organic agro-wastes compared to the basal media. Results reported herein support the notion that all *Aspergillus species* can be used to produce industrially important amylases by utilizing agro-wastes.

Keywords :- Aspergillus niger, Aspergillus flavus, Aspergillus oryzae, Amylase production and agro-wastes.

INTRODUCTION :

In recent era, the applications of microorganisms has great importance in food, textile and detergent industries and has gained great interest into the exploration of enzyme activity in microorganisms (Sivaramakrishnan *et al.*, 2006). Amylases are industrially important enzymes which hydrolyze starch a homopolysaccharide to give various products like dextrin and smaller polymers of glucose unit (Gupta *et al.*, 2009). Amylases are extracellular enzymes which are most important for biotechnology with great significance and contribute approximately 25% of the world enzyme market. Amylases can be obtained from several sources, such as plants, animals and microorganisms. However, a large number of microbial amylases are available commercially and completely replaced chemical hydrolysis of starch,a substrate for amylase in starch processing industry. Major benefit of using microorganisms for the production of amylases is the economical bulk production capacity and they are easily manipulated to obtain enzymes of desired characteristics (Karnwal and Nigam, 2013). Amylase can be obtained from various sources like fungi, yeast, bacteria and actinomycetes; however, especially fungi, have gained highest attention because of the availability and high productivity of fungi, which are also amenable to genetic manipulation. Many fungi had been found to be good sources of amylolytic enzymes. Many studies indicated that amylases of fungal origin are more stable than those of bacterial origin (Sanghvi *et al.,* 2011). Starch is the best substrate for production of yeast cells in a large scale due to its low price and easily available raw material in most regions of the world. Because most of yeasts from environments are safe (GRAS) compared to bacteria, interest in amylolytic yeasts has increased in recent years

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as their potential value for conversion of starchy biomass to single-cell protein and ethanol has been recognized.

Amylases can be grouped into two classes, endoamylases (α-amylase) and exoamylases (glucoamylase). α-amylase catalyze hydrolysis of α-1,4-glucosidic linkages in the interior of starch molecule in a random manner producing branched and linear oligosaccharides (dextrin, maltose, maltotriose, glucose) of different chain length while glucoamylases catalyze hydrolysis of α- 1,4- and α-1,6-glucosidic linkages in starch molecule (amylase and amylopectin) from its nonreducing end yielding glucose (Khan and Priya, 2011).

Genetically modified microorganisms can be used for the production of various types of enzymes having different characteristics of interest. Microbial technology plays an vital role for the production of industrially important enzymes and now a days they are commercially available.

Since Amylases are industrially important therefore, in this study, we aimed to isolate and screened amylase producing fungi from soil to determine the amylase activity.

MATERIALS AND METHODS :

Isolation of amylase producing fungi

Soil samples were collected from different areas of Santra Market Nagpur. Serial dilution was made and plated on potato dextrose agar and starch mineral agar medium by spreading 0.1ml of the diluted sample. Then the plates were kept for incubation at 37°C for 4-5 days. The pure cultures were identified by their morphology and colony characteristics and sub-cultured. The isolates were maintained on PDA medium. All the fungal strains were subjected to lactophenol cotton blue staining for studying the morphology. All the fungal cultures were confirmed as *Aspergillus species* by studying the morphology and the spore color.

Lactophenol Cotton Blue Staining

A rectangular slab of PDA was prepared and was placed on a clean glass slide. The culture was inoculated and another glass slide was placed on top of it to form a sandwich. This was kept for incubation for 3 days at room temperature. A loop of fungal cultures were placed on a clean glass slide, a drop of lactophenol cotton blue stain was then mixed with the culture. A clean coverslip was placed over the culture and viewed under the microscope (45 X) and the morphology of *Aspergillus species* were observed.

Determination of Amylase activity

All the *Aspergillus species* isolates were tested for amylase production by starch hydrolysis. When starch agar medium (Peptone – 0.5g, Beef Extract – 0.15g, Yeast extract – 0.15g, NaCl – 0.5g, Starch – 1g, Agar – 2g, Distilled water – 100ml) was inoculated with the organism and subsequently flooded with iodine solution (Iodine – 0.2%, Potassium Iodide – 0.4%, Distilled water – 100ml), the zone of clearance around the microbial growth indicated the production of amylase. On the basis of the area of clearance, three fungal isolates was selected for further studies on amylase production.

Amylase Production using Solid State Fermentation

The *Aspergillus species* were subjected to solid state fermentation in different agro-wastes like rice bran, wheat bran, coconut oil cake, groundnut oil cake, and gingely oil cake which was used as solid substrates for SSF.5g of each bran was weighed and hydrated with 5ml of basal salt solution $(NH_2)_2SO_4$ – 2g/l, KH_2 PO $_4$ – 1g/l, MgSO $_4$. $7H_2O$ – 0.5g/l, $ZnSO_4$ – 0.1g/l and adjusted with moisture content from 43-81%. 1% of inoculum was inoculated after sterilization and incubated at room temperature for four days.

Amylase Enzyme Extraction:

22ml of 0.1M phosphate buffer saline (pH 7) was added to each of the inoculated substrate

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beds and was vigorously shaken in rotary shaker for 15 minutes at 120rpm. The mixture was filtered through cheese cloth and centrifuged at 8000rpm at 4^{0}C for 15min. The supernatant was filtered through cheesecloth and the filtrate was used as the crude enzyme preparation. Enzyme amylase was assayed by Dinitrosalicylic acid method.

Specific activity : One unit of amylase activity is defined as the amount of enzyme, which released 1 µM of glucose per minute per milligram protein (U/mg).

Effect of Incubation Time

The effect of incubation period on amylase production was investigated by checking the amylase activity on2nd 4th, 6th, 8th and 10th days of incubation at pH 7 and at room temperature.

Effect of pH

Solid State Fermentation investigated the effect of different pH on amylase production by adjusting the pH of basal salt solutions to 6, 6.5, 7.0, 7.5, 8 and 8.5. The flask were then incubated for 5 days at room temperature.

Effect of Temperature

The effect of temperature on enzyme production was investigated by SSF in different substrates and incubated at 30^{0}C, 35^{0}C, 40^{0}C, 45^{0}C, 50^{0}and 55^{0}C at pH 7 for 5 days.

RESULTS AND DISCUSSION :

Strain Selection

Fungal cultures were isolated from soil sample by serial dilution on Potato Dextrose Agar medium. The cultures were observed after Lacto phenol cotton Blue staining and the isolates were confirmed as *Aspergillus niger*, *Aspergillus flavus and oryzae*.All the three fungal isolates were tested for amylase production by starch hydrolysis. When starch agar medium was inoculated with the organism and subsequently flooded with iodine solution, the zone of clearance around the microbial growth indicated the production of amylase. On the basis of the area of clearance, all three *Aspergillus species* were selected for further studies of amylase production.

Solid State Fermentation

Different agro-wastes like rice bran, wheat bran, black gram bran, coconut oil cake, and gingely oil cake were used as solid substrates for SSF. After inoculation and incubation for six days at room temperature with pH 7, the enzyme amylase was extracted using phosphate buffer and was estimated for the protein content and the amylase activity. A lower yield of extracellular amylase production under SSF by *A. oryzae* using sugarcane bagasse (Tunga and Tunga, 2003) has been reported.

Effect of Time of Incubation

The incubation period varies with enzyme production. Short incubation period offers potential for inexpensive production of enzyme (Somjoy *et al.,* 1995). In the present study, six days of incubation gives promising results for the production of amylase by *Aspergillus species*. Similar results were reported earlier that the mycelial growth on starch reached a maximum after five days and maximum amylase activity was produced after two days of cultivation (Ely *et al.,* 2002). The decreased activity in the later phase of growth was probably due to catabolite repression by glucose released from starch hydrolysis.All three *Aspegillus species* gave highest activity on 6th day.

Effect of pH

Aspergillus species were inoculated into different substrates and incubated at room temperature for four days. The enzyme was extracted and amylase produced at different pH were recorded.

The maximum yield of amylase was at pH 7.0 for Aspergillus niger and at pH 7.5 for Aspergillus oryzae and Aspergillus flavus. In our study the effect of pH on the enzyme activity indicates that the amylase is active in the pH range 7 – 8, both neutral and alkaline.

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Effect of Temperature

Aspergillus species were inoculated at different temperature 30^{0}C, 35^{0}C, 40^{0}C, 45^{0}C, 50^{0}and 55^{0}C and showed maximum yield of amylase in the temperature range of 40^{0}C to 45^{0}C. It is reported that best enzyme production in *A. niger* at room temperature both in SmF and SSF (Varalakshmi *et al.,* 2009)and reported 30^{0}C to be the best for enzyme production by *Penicillium fellutanum* (Kathiresan and Manivannan, 2006).

CONCLUSION :

Isolation of fungi from soil sample and the rapid screening by plating on starch agar plates led to the finding of three *Aspergillus species* capable of producing amylase. These fungal strains were confirmed as *Aspergillus niger,Aspergillus flavus* and *Aspergillus oryzae*. The best amylase producer was identified as *Aspergillus niger*. Thus all the three *Aspergillus species* were found to be the best producer of amylase. The strain is maintained in our laboratory for further explorations regarding industrial applications.

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Figure1: Amlyase activity of selected *Aspergillus species* at different period incubation

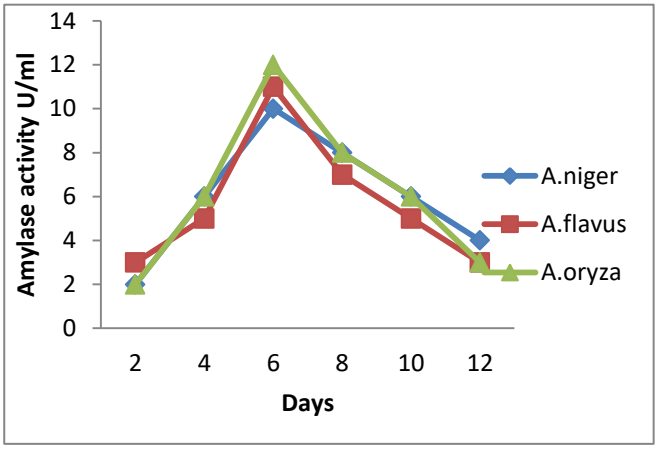


Figure2: Amlyase activity of selected *Aspergillus species* at different pH

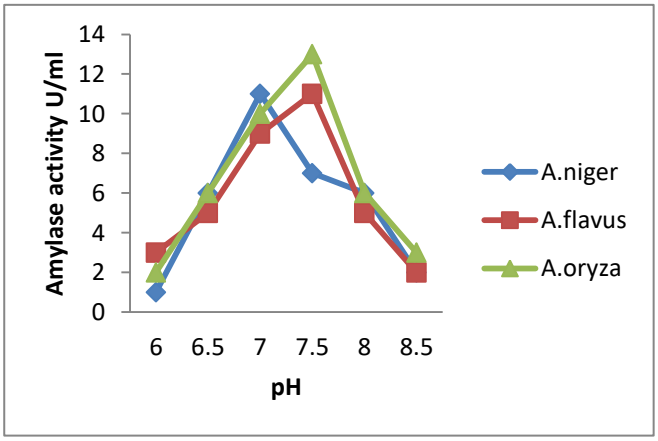
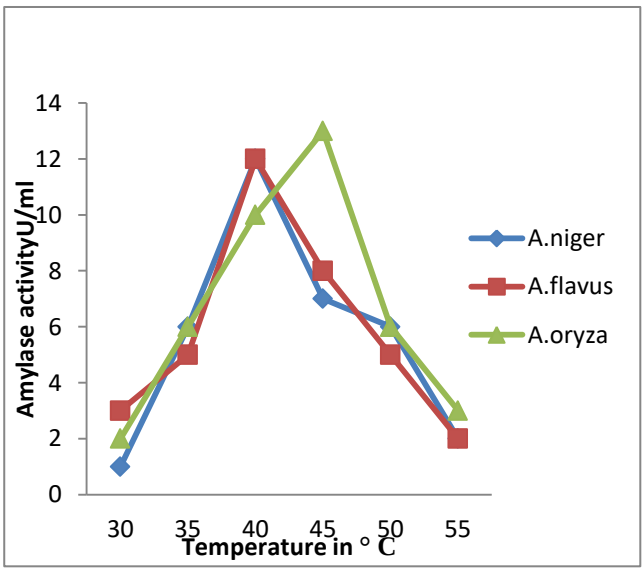


Figure3: Amlyase activity of selected *Aspergillus species* at different temperature



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