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Isolation and Characterization of α-amylase by using Conventional Koji Tray Culture

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α-amylase family enzymes have significant importance in wide area with potential application. α-amylase was isolated from air, cultured under optimum condition and subjected for characterization. Each colony is observed under microscope using lacto phenol cotton blue and three different types of *Aspergillus* species was found i.e *A.niger, A.flavus, A. Oryzae*. The optimum pH for the activity of the enzyme *in A.flavus* and *A.niger* was 4.5 and in *A. oryzae* optimum pH was 5.0. The optimum temperature for enzyme production was 30°C for *A. flavus* and *A. oryzae* and for *A. niger* it is 40ºC.

Key words: *A. flavus,· A. oryzae, A. niger,* α-amylase,· Koji tray culture.

α-amylase is an exoenzyme having hydrolytic property, that hydrolyses starch into disaccharide and monosaccharides, these enzymes are called as α-1,4-glucan 4-glucanohydrolases [1,2]. This enzyme is extensively used in starch liquefaction, paper industries, food, pharmaceutical and sugar industries [3]. Amylases are starch degrading enzymes have great deal of attention because of their perceived technological significance and economic benefits, hence are used for commercial production of glucose. The new potential of using microorganism as biotechnological sources of industrially relevant enzymes has renewed interest in the exploration of extra cellular enzymatic activity in several microorganisms[4]. These enzymes are found in animals, plants and molds [5,6].

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Amylases, their sources and properties are reported by Adebiyi and Akinyaanju [7] and Akpan *et al.* [1]. 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 amylase family of enzymes has great significance due to its wide area of potential application in pharmaceutical and clinical sector also requires high purity amylases. Thus, it is significant to develop economic processes for their purification to obtain pure enzymes with maximum specific activity [8]. The amylases of fungal origin are most stable than the bacterial origin [9], enzymes are stable under extreme condition of pH (7.0) and temperature (70ºC). The selection of suitable production media is essential for growth of microorganisms and production of enzymes. The ability to degrade starch is used as criteria for the determination of amylase production by microbe.

Studies on fungal amylase have concentrated mainly on *Rhizopus sp.*, and *Aspergillus spp.*, probably because of the ubiquitous nature and non fastidious nutritional

requirements of these organisms [10]. It has been reported that while a strain of *Aspergillus niger* has produced 19 types of enzymes, á-amylase was being produced by as many as 28 microbial cultures [8]. *A.oryzae* and *A.flavus* were traditionally distinguished based on morphological, physiological and culture based characteristics [11]. Thus, the selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions. Optimization of growth conditions is important for best growth of fungi. The growth requirements for fungi may vary from strain to strain, although cultures of the same species and genera tend to grow best on similar media. Similarly growth responses of fungi also vary from strain to strain though they are grown on same conditions [12]. Most common fungi grow well over the range pH 3 to 7, although some can grow at pH 2 and below e.g., *Moniliella acetoabutans, Aspergillus niger, Penicillium funicilosum*[12].

Aspergilli are known to produce different groups of enzymes but the selection of a particular strain remains a tedious task especially when commercially competent enzyme yields are to be achieved. Therefore, the present study was undertaken to identify the indigenous efficient alpha-amylase producer strains of *Aspergillus niger, Aspergillus oryzae* and *Aspergillus flavus* among the fungal species.

MATERIAL AND METHODS

Isolation of *Aspergillus species*

Aspergillus culture was isolated using Martins Rose Bengal Agar media. Composition of Martins Rose Bengal Agar medium was Agar: 15.0g, Glucose: 10.0g, Peptone: 1.25g, KH_2PO_4: 1.0g, $MgSO_4.7H_2O$: 0.5g, Rose Bengal: 0.05g, Streptomycin: 0.1g, Water: 1000ml. Then expose the petri plate containing media to air b/w 5-6PM for 1min, incubate at room temp for 48 hrs and developed colonies were observed.

Identification of *Aspergillus* species

Colonies were identified by observing under microscope using Lacto phenol cotton blue. It stains the fungal cytoplasm and provides a light blue background against which the walls of hippie can readily be seen. It contains four constituents: phenol, lactic acid, cotton blue, glycerine. Sub culturing of colonies were done by transferring the colonies to freshly prepared Martins Rose Bengal Agar media and pure culture were obtained.

Confirmation test for amylase production

Prepare Starch agar media and then transfer each pure culture on to the starch agar media. Pour the iodine solution on the culture media. Starch, in the presence of iodine produces a dark blue color in the medium and yellow color zone around a colony, indicates the presence of amylase.

Induction of enzyme

In order to increase the enzyme production *Aspergillus spp* were seeded on to Rice agar media. Its composition includes Rice flour: 5g, Glucose: 2g, Agar: 2-2.5g, Water: 100ml. Koji tray culture is similar to solid state fermentation (SSF). SSF is the cultivation of microorganisms under controlled condition in the absence of free water. Koji media composition consists of wheat flour: 50g, Gram seed: 50g.

Preparation of koji media

Weigh 50g of wheat distribute into six petri plates equally. Keep at 150°C for 12 hrs in hot air oven. 50g of Gram seed were soaked in water for overnight, autoclaved at 121°C for 15 min. Mix the Gram seed with wheat flour in petri plate aseptically.

Preparation of suspension of *Aspergillus* culture

Take 2-3 forceps of culture grown on Rice glucose agar media. Transfer it into 5ml of sterile distilled water. Transfer 5ml of suspension of *Aspergillus* culture on to the koji media uniformely. Incubate at room temp for 48hrs.

Extraction of crude α-amylase

1gm of culture from koji media is mixed with 10ml of water. The mixture was then centrifuged at 8000 rpm for 15 min. Filter the content of centrifuge tube using whatmann filter paper.

Activation of starch solution

Add 2.5ml of 0.1N HCl to 175ml water in beaker and boil it. Add 5g of dry starch that is creamed with 20ml of water with constant stirring and boil for 5min. Add 12.5ml of buffer solution of pH 6.0 and 25ml of 0.1N NaOH and make the volume to 500ml with water.

Activation of enzyme on starch

5ml of extract was added to 50ml of

activated starch, keep it in water bath for 10min at 30^0C. 1ml of fresh activated enzyme added to 4ml of diluted iodine solution. OD of sample was measured at 640nm. Again 1ml of activated enzyme was added to another tube at 5min of interval until the end point was approached. Reaction time was noted and enzyme activity was observed for every 24hrs for 4 days.

Characterization of enzyme : Substrate concentration, Temperature and pH

Effect of substrate concentration on enzyme activity was measured at different concentrations of starch in the reaction mixture (1, 2, 3, 4, 5mg). Effect of pH on amylase activity was determined by incubating the reaction mixture at pH values ranging from 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8. Optimum temperature for enzyme activity was determined by conducting the assay at different temperatures ranging from 5, 10, 20, 30, 40, 50, 60ºC.

RESULTS

Many colonies are formed on the Martin Rose Bengal Agar media after exposed to air. Each colony is observed under microscope using lacto phenol cotton blue. Three different types of *Aspergillus* species found i.e *A.niger, A.flavus, A. oryzae*. Brown coloured colonies are reported as *Aspergillus oryzae*. *Aspergillus niger* colonies appear black in colour. Green coloured colonies are reported as *Aspergillus flavus.* Each colony is subcultured until pure culture is obtained. Colonies of *A.niger, A.flavus, A.oryzae* obtained on Rose Bengal Agar medium was shown in Fig. 1, 2, 3.

Clear zones of round colonies, confirms the amylase production by *Aspergillus* species. The *Aspergillus* culture production is increased on the Rice Glucose Agar media. The increase in *Aspergillus flavus, Aspergillus oryzae and Aspergillus niger* culture production was clearly

Fig. 1-3. *Aspergillus niger, A. oryzae* and *A. flavus* on Martin Rose Bengal Agar media

Fig 4-6. *Aspergillus flavus, A. oryzae* and *A. niger* cultures on Rice Glucose Agar media

photographed in Fig. 4, 5, 6.

The activity levels of the enzyme was determined with different substrate concentrations from 1mg to 9mg. Finally it was found that the activity was maximum at 6 to 7mg substrate concentration which was represented in Fig. 7. Assay of enzyme production was carried out at various temperature ranges from 10°C to 60°C. It was found that *Aspergillus spp* have considerable growth at 20°C, but there was no enzyme production. However, the optimum temperature for enzyme production was 30°C for *A.flavus* and *A.oryzae* and it is 40°C for *A.niger*. Enzyme activity on different temperature was graphically represnted in Fig 8. The production of á-amylase was found to be best at the pH level 4 in *A.niger*, 4.5 in *A.oryzae* and 4.2 in *A.flavus*. The production of α-amylase was found to be the best at pH 5.5. Enzyme production started at pH 3.0 and cease at pH 8.0 was represented in Fig 9. Maximum enzyme production of enzyme occurred at pH 4 to 5, very little growth was observed without enzyme production in medium at initial pH 3 to 4. The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is very essential for the production of α-amylase.

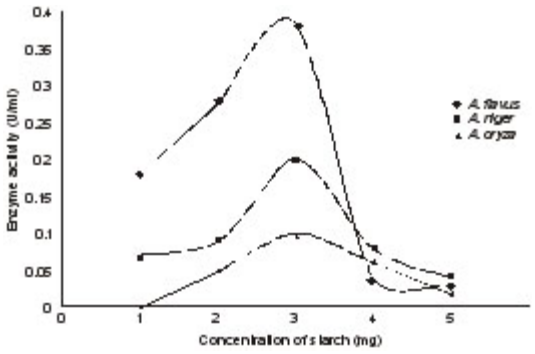

Fig. 7. Effect of substrate concentration on enzyme activity

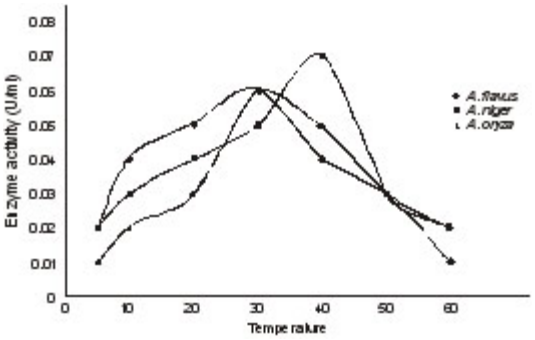

Fig. 8. Effect of temperature on enzyme activity

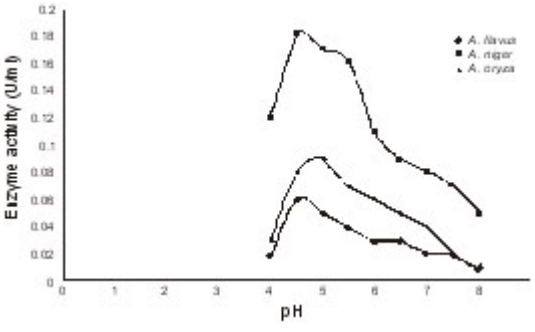

Fig. 9. Effect of pH concentration on enzyme activity

DISCUSSION

Enzyme preparation of *Aspergillus* species consists of atleast two enzymes glucoamylase and α-amylase reported by Bergmann et al. [13]. α-amylase differ in their pH optimum, temperature optimum, temperature stability and in several other physiochemical depending on the species origin. Hence different enzymes have found specific applicability in different industries. The influences of various environmental conditions like effect of pH value and temperature optimum and incubation period on the production of α-amylase by *Apergillus* species was reported [14,15,16,17]. Increase in the incubation period resulted decrease in the production of α-amylase by culture of *Aspergillus niger*. It may be due to the fact that maximum production of α-amylase enzyme, the production of other product and depletion of the nutrients. These byproducts inhibited the growth of fungi and hence enzyme formation was estimated by the method of Duochaun et al. [9]. The enzyme is very sensitive to pH. Therefore selection of optimum pH is very essential for the production of α-amylase [18].

The result of this conceptual study clearly reflect that pH value of medium has the ability to

affect the growth rate and consequently on proliferation of these fungi. The relative intensity of this effect however varies with the species involved. A wide body of evidence indicates that optimum fungal growth occurs in acidic media; however, the range of pH that will permit growth varies with the species and the composition of the culture medium [19]. It is evident from the present study that the extent of zones of starch digestion was significantly enhanced by lowering the pH to 4.5. In similar investigations Kim *et al.* [20] have reported the pH range 3-6 for *Sphaeropsis pyriputrescens* and optimum pH between 3-4 which is comparable to other such studies on fungi. It is enumerated from the spectrum of microbial cultures employed for enzyme production in solid state fermentation that four isolates among the tested species gave the highest detectable quantities of starch hydrolysis. These findings are in line with the work reported by Omemu *et al.* [6] where the selection of potent species was made by plate method. However, zonation cannot in any way be correlated quantitatively with the amount of enzyme produced. Therefore, the isolation of enzyme producers using starch plates can only be partially selected. So, the selection of more efficient amylolytic strains was made on biochemical basis [21].

Another important feature of this study was the significant differences of amylolytic activities associated with different fungal isolates. These findings are parallel with the work carried out by Alazard & Raimbault [22] in which they compared the amylolytic enzyme production potential of *Aspergillus niger* in liquid and solid state cultivation. In another study by Mikiashvili *et al.* [23] significant difference among enzyme activities showed that extensive differences in enzyme production may exist among fungal species and even among strains of same species was demonstrated with species of genera *Ganoderma, Omphalotus* and *Pleurotus* etc.

Koji culture is representing solid state fermentation to go for the extraction of the á-amylase enzymes from micro organisms like fungus (*Aspergillus*). This is the cultivation of micro organisms under controlled conditions. For the production of á-amylase both organic, inorganic sources are essential. The enzyme is very sensitive in different pH, so selection of optimum pH is essential for production of á-amylase [18]. The production of á-amylase was found to be best at pH level 4 in *A. niger*, 4.5 in *A. oryzae* and 4.2 in *A. flavus*. The production of á-amylase was found to be best at pH 5.5. The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is very essential for the production of á-amylase [18]. Enzyme production started at pH 3.0 and cease at pH 8.0 maximum enzyme production of enzyme occurred at pH 4 to 5. Assay of enzyme production was carried out at various temperature ranges from 10°C to 60ºC. It was found that *Aspergillus* spp have considerable growth at 20ºC, but there was no enzyme production. However, the optimum temperature for enzyme production was 30°C for *A. flavus* and *A. oryzae* and for *A. niger* it is 40ºC.

CONCLUSION

The present study shows that *Aspergillus* species is a good producer of á-amylase. All three different species of *Aspergillus* are showing comparatively better response in substrate concentration (starch), different pH level and temperature level. Both organic and inorganic nutrients are required for the optimum growth of *Aspergillus* species that leads to the rate production of á-amylase. This enzyme is used in the industries like starch degradation, paper industry, food industry, change in the food properties also in the textiles. Present work shows that like solid state fermentation the koji tray culture is also an important method to extract á-amylase from the *Aspergillus* species of *A.oryzae, A. flavus* and *A.niger*. Though *A.niger* is pathogenic, it is showing better extraction of amylase in the variation of pH level and temperature. Starch substrate is showing very good extraction of amylase in *A.oryzae*. By providing different temperature and pH level it is possible to get higher production of amylase in these three *Aspergillus* species (*Aspergillus niger, Aspergillus oryzae, Aspergillus flavus).* Koji tray culture is the simplest way to go for the extraction of á-amylase.

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