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Production of α-amylase by *Acremonium sporosulcatum*

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Production and partial purification of alpha amylase by mesophilic mold *Acremonium sporosulcatum*. Using substrate starch solution pH 4.5 with 0.2 M sodium acetate buffer. The production maximum in maize starch, it has optimum growth temperature at 30 °C, the pH 7.0 was found to be the optimum for the best synthesis of the enzyme. The influence of incubation temperature was studied by growing different temperature 20°, 30 °, 35 °, and 37 °C. Fermentation carried out using potato, sweet potato, maize, maize cob powder, rice, and tapioca starch in different concentrations of sugar present in the basal medium. 7.5% of starch is for maximum yield, for nitrogen source among inorganic sodium acetate and organic yeast extract is found best production. In effect of aeration 50 ml is carried in 250 ml flask, partial purification of enzyme was active in wide range of values in pH 5-8 optimum activity exhibited at pH 7.0 and temperature 70 °C after the enzyme inactive slowly in 15 minutes for incubation at 100 °C it completely loss activity

Key words: Isolation, submerged fermentation, alpha amylase activity, *Acremonium sporosulcatum*.

Critical survey of the available literature indicates that no species of *Acremonium* has been investigated for the production of α-amylase. The only publication dealing with biochemical activities of *Acremonium* are those which deals with the production of exo-polysaccharides (Stasinopoulos and Seviour, 1989) and β-glucanases (Pitson *et al.,* 1991,1997). The factors influencing the synthesis of the enzyme are presented. Amylases are a group of enzymes that break down starch or glycogen. They are produced by a variety of living organisms including fungi, bacteria (Fodel, 2000; Yabuki *et al.,* 1977; Young *et al.,* 2001) and also higher plants. Microorganisms synthesize and release amylases extracellularly. Amylases are classified on the break down pattern of starch molecule.

α-amylases (endol-1-4-α-D-glucan glucohydrolase, EC 3.2.1.1) reduce the viscosity of starch by breaking down 1-4-α-D-glucosidic bonds at random in the linear amylase chain and thus producing varied sized chains of glucose. These are endo enzymes that split the substrate in the interior of the molecules and are classified according in their action and properties. The families of amylase enzymes are of grate significance due to its areas of potential applications in higher frucotose corn syrup preparations, additives to detergents to remove strains, saccharification of starch for alcohol production and brewing etc. Interestingly amylase from fungal source was the first to be produced and as pharmaceutical aid for the treatment of digestive disorders as early as 1894 (Crueger and Crueger, 1989). Amylases find potential application in a number of industrial processes like food, textiles, and paper industries. Microbial

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amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. They would be potentially useful in pharmaceutical and fine chemical industries if enzyme with suitable properties could be prepared (Fogarty, 1980 and Kelley *et al.,* 1997). Emergence of biotechnology offered new avenues of amylase application in many other fields like clinical, medical and analytical chemistry. Recently Witczak (1999) presented a review of thio-sugars as potential new therapeutics, which are gaining substantial attention. Enzyme controlled mechanisms involving amylases and lipases have significantly contributed to the understanding of the biological processes. There are several processes in medical areas that involve the application of amylases (Sutton *et al.,* 1999; Giri *et al.,* 1990; Ch *et al.,* 1997; Stanberg *et al.,* 1999). a-Amylase is useful as thermistor for the biochemical analysis of cyclodextrins (Kolb *et al.,* 1996).

In the recent years genetic engineering has been used extensively for cloning of amylase producing strains mainly a-amylase and glucoamylase with the view to achieve desirable production levels in the cloned host in addition to co-expression of two enzymes by the same organism. A grate deal of work has been done on cloning a-amylase producing strains more commonly employing *Saccharomyces cerevisiae* or *E. coli* (Liebl *et al.,* 1997; Birol *et al.,* 1998).

MATERIAL AND METHODS

Seed development and fermentation conditions

Like any other fermentation process dealing with secondary metabolites, amylase production by *A. sporosulcatum* (IMI 393096, Kew (UK), NCIM 1319, Pune (India) was carried out as biphasic system i.e. development of seed followed by fermentation process. These steps are essential because environmental factors required for optimum growth and reproduction of the strain might differ from those required for the production of the enzyme. These parameters include carbon and nitrogen source, pH of the medium, temperature of incubation, aeration and inoculum concentration.

A well grown, heavily sporulating culture slant was selected and 5 ml sterile distilled water was aseptically added. With the help of a sterile inoculation needle the spores were carefully scraped off and the whole suspension was added to 250 ml Erlenmayer flask containing 75 ml sterile distilled water. 0.5 ml of this, was used to inoculate 50 ml sterile seed medium present in 250 ml Erlenmayer flask

2.0 ml of fresh, healthy and actively growing seed was used to inoculate 250 ml Erlenmayer flasks containg 50 ml fermentation medium. The composition of the fermentation medium is same as the seed medium except it contained microelement solution. Triplicates were used for each experiment. The culture was grown as before under submerged conditions on reciprocating shaker for 5-6 days. At the end of every 24 hrs. samples were collected to determine pH, growth pattern and purity of the culture and enzyme activity. At the end of 144 hrs. of incubation, the contents of all the flasks were pooled, pH determined using a pH meter and mycelial mat was separated by filetration through Whattman number 1 filter paper fixed in a buchener funnel. The filtrate was collected for enzyme estimation.

Analytical procedures

α-Amylase activity was assayed by the method of McMahon et al (1977) with slight modification of optimum temperature and pH. One unit of amylase activity (mmol/min) was defined as the activity of 1 ml of enzyme solution that produced 1mmol of reducing sugar per minute from starch at pH 4.5 and 50^{o} C. Reducing sugar was determined by the 3,5-dinitrosalicylic acid (DNS) method (Samarntarn and Tanticharoen 1999) using glucose as a standard. The fermentation broth contained some suspended particles, which interfered with the determination of cell weight by filtration and drying. Thus protein content was used in stead of dry mass for evaluation of cell growth. Protein contents were determined by the standard Kjeldahl method (Kjeldahl,1983). All the fermentation experiments and enzyme assay were carried out in triplicate with analytical grade reagents and the mean values were reported.

Properties of Partial Purification Enzyme

In order to examine the properties of the enzyme, the amylase form the culture broth was partially purified. A precipitation test was

conducted using ammonium sulphate. After salting out with ammonium sulfate ranging from 30 to 80 % saturation, recovery of a-Amylase activity was below 50 % under all test conditions. On the basis of these results, salting out with 60 % ammonium sulphate was the most effective from the perspective of recovery and specific activity of α-Amylase.

Partially purified enzyme was found active is wide range of pH 5 – 8. Optimum activity was exhibited at ph 7.0 and temperature 70°C. The enzyme became inactive at 100°C in 15 min incubation.

Hydrolysis of Raw starch

The cornstarch separated from the kernel by the wet milling process is generally 99% pure and contains 0.25-0.35% protein, 0.5-0.6% lipid and less than 0.1% minerals. 35% of the industrially prepared cornstarch is utilized by the food industry; the remainder of the starch is further refined or modified for use in the paper and construction industries. A significant proportion of the cornstarch derived from the wet-milled process used for food goes into the fermentation of beer. To be useful to the breweries, it first has to be converted into dextrose or d-glucose.

RESULTS

Effect of hydrogen ion concentration

Hydrogen ion concentration of the medium exerts tremendous influence on carbon nitrogen metabolism, growth, sporulation biosynthesis and release of metabolites by microorganisms. Generally each organism has a range of pH over which it will be able to grow and synthesize the metabolic products. However synthesis of metabolic products occurs in a narrow range of pH than the range in which growth occur. But this range varies from culture to culture or even strain to strain, and also the biosynthetic product it synthesis. For example in *Aspergillus ochraceus* maximum growth occurred at pH 6.0 while the enzyme production occurred at pH 5.0 (Ely and Waldemarin, 2002). *Aspergillus sp.* NH_4 was reported to produce a-amylase in the pH range of 5.0-8.8 (Warin, 2004). Therefore the effect of hydrogen ion concentration on the synthesis of a-amylase by *A. sporosulcatum* was investigated by inoculating buffered nutrient medium having different pH values. 2 % spore inoculum was used to inoculate 50 ml of sterile medium in 250 ml Erlenmeyer flasks. Culture was allowed to grow for 144 hrs. under aerated and agitated conditions on a rotary shaker as before. At the end of the growth cycle final pH of each was noted. Mycelial mats were separated by filtering through previously weighed Whattman filter paper dried to constant weight in a hotair oven. Mycelial mats were microscopically examined to note any abnormalities if present.

At acidic pH 3 culture growth was poor and enzyme activity was negligible. Increase in the initial pH of the growth medium upto pH 6.0 gradually increased the biomass and also enzyme synthesis. But best growth and enzyme formation occurred when the initial pH of the fermentation medium was adjusted to pH 7.0) Enzyme synthesis reached maximum, when the final pH of the fermented broth tend to become alkaline (pH 8.7) but when the initial pH was 8.0 and above decrease in enzyme activity was noted because it effected the growth of the mold. Therefore in all the experiments the initial pH of fermentation medium was adjusted to neutrality. Generally the fermentations were terminated when the pH reached 8.7.

Effect of temperature

Biological activities of all living organisms proceed in a series of intricately coordinated and controlled reactions catalyzed by specific biological catalysts. The rate of these reactions is dependent upon the temperature of the environment in which microorganisms are allowed to grow, and it constitutes one of the essential parameters for the metabolic process to proceed effectively. A small amount of thermal energy needed to activate the molecules participating in the enzyme reaction is met by the organism itself while major part of the heat is obtained from the environment. In any given media the range of temperature over which growth and metabolic activity is maximum is considered as the optimum temperature for that organism. The optimum range of temperature varies with the strain, chemical composition of the medium and also physical condition of the environment.

Therefore influence of incubation temperature was studied by growing the culture

at different temperature like 20 °C, 30 °C, 35 °C and 37 °C. At 20 °C the mold growth was relatively poor and enzyme activity was also low. The mycelium under microscope appeared relatively thin and vacuolated. The pH of the culture broth at the end of fermentation remained near neutrality with increase in temperature to 30 °C. There has been gradually increase of the mycelial growth and synthesis of the enzyme. At 144 hrs. of the fermentation, the pH becomes alkaline and the enzyme synthesis reached maximum. This has synchronized with excellent development of biomass. The mycelium appeared healthy with out much vaculation. The color of the broth was also developed relatively deep blue black. Further increase in incubation temperature did not show any more improvement in enzyme activity. Therefore 30 °C incubation temperature is taken as optimum for maximum growth as well as synthesis of alpha-amylase under present experimental condition.

Effect of carbon source

The production medium should contain cheap and easily available carbon source, and a nitrogen source and trace elements like minerals for the synthesis of enzyme. These substances should be supplied in optimum concentration so that maximum levels of product formation can be achieved.

Therefore in the present investigation in order to determine the best source of carbon which promote growth and synthesis of the enzyme is investigated incorporating substances like potato starch, sweet potato starch, maize starch, maize cob powder, rice starch and tapioca starch at the same concentration as sugar in the basal medium. Fermentation was carried out as before. Of these maize starch was founded the best source of carbon as the enzyme yield was found, highest and the biomass turn over was also good. Microscopically mycelium appeared healthy with no vacuoles. Potato starch was found the next best

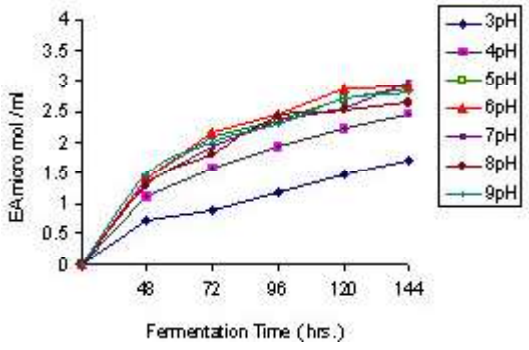

Fig. 1. Effect of pH on the synthesis of α-amylase by *Acremonium sporosulcatum*

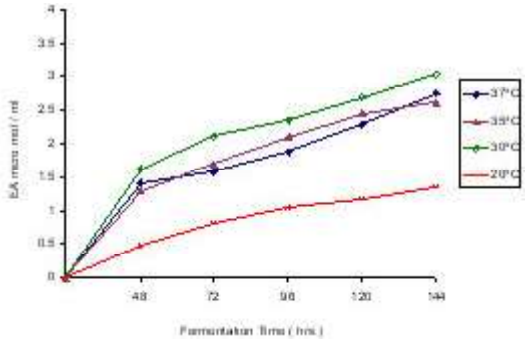

Fig. 2. Effect of Temperature on the synthesis of α-amylase by *Acremonium sporosulcatum*

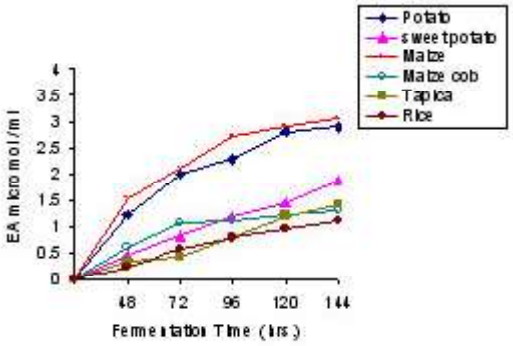

Fig. 3. Effect of different carbon substances on the synthesis of α- amylase by *Acremonium sporosulcatum*

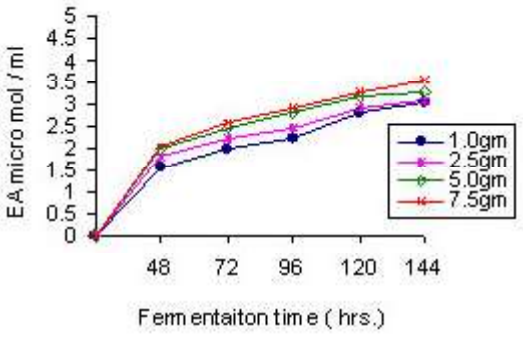

Fig. 4. Effect of different concentrations of Maize starch on the synthesis of α-amylase by *A. sporosulcatum*

source of carbon. Maize cob powder sweet potato powder, tapioca starch and rice starch are less preferred sources of carbon and stand in descending order of preference for the synthesis of the enzyme. Therefore maize starch was selected as the best carbon source and used.

In order to determine the optimum concentration of maize starch for maximum synthesis of a-amylase it was incorporated in different concentrations in the fermentation medium and the culture was allowed to grow as before. Samples were taken at 24 hrs. interval to test sterility, enzyme activity and pH. Of the five concentrations studied at 7.5% maize starch concentration was found the best for the optimum synthesis of the enzyme. Decrease in maize starch concentration progressively decreased the enzyme synthesis. Therefore 7.5% maize starch concentration was taken as optimum and used in all experiments. Addition of small quantities of glucose in the initial stages of fermentation did not show any stimulatory effect as claimed for *Aspergillus ochraceus* producing α-Amylase (Ely and Waldemarin, 2002).

Effect of nitrogen source

Generally in addition to an energy source and trace elements a suitable nitrogen source in adequate quantities should be essential for the rapid growth of the mold. Many fungi are reported to use inorganic nitrogen either in the form of nitrate or ammonia. But the presence of an organic source of nitrogen in the growth medium exerts a stimulatory effect on the growth and metabolism of fungi.

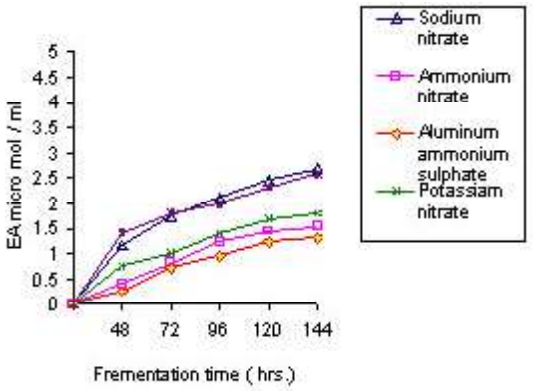

Fig. 5. Effect of different inorganic compounds on the synthesis of a- amylase by *Acremonium sporosulcatum*

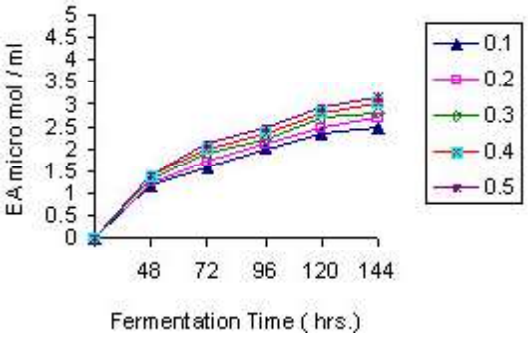

Fig. 6. Effect of different concentrations of sodium nitrate on the synthesis of α-amylase by *Acremonium sporosulcatum*

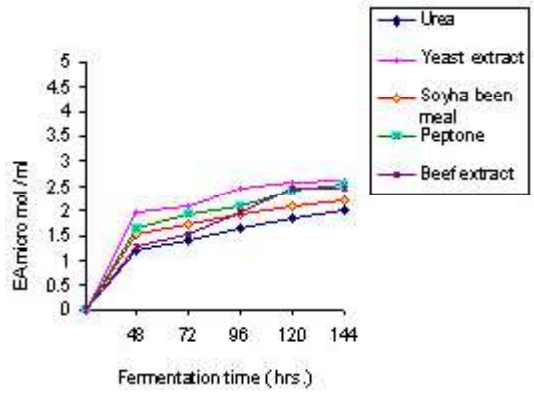

Fig. 7. Effect of different organic sources of nitrogen on the synthesis of α-amylase by *Acremonium sporosulcatum*

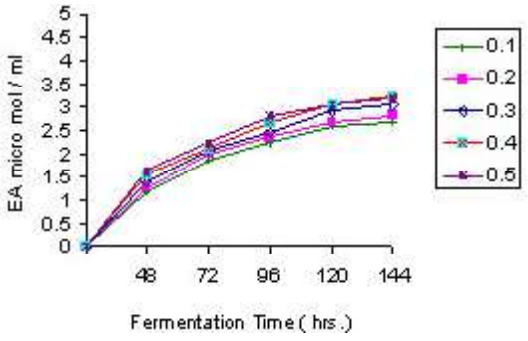

Fig. 8. Effect at different concentrations of yeast extract on the synthesis of α-amylase by *Acremonium sporosulcatum*

Therefore a variety of inorganic and complex organic nitrogen substances have been incorporated in the basal medium and their effect on the synthesis of the enzyme have been studied. Of all the inorganic nitrogen sources sodium nitrate was found to be best inorganic source of nitrogen for the mold to synthesize the enzyme. Increase in sodium nitrate concentration from 0.1-0.5 % progressively increased the enzyme activity. Further increase in sodium nitrate concentration did not help to increase the enzyme synthesis. Ammonium sulphate was found next best source of nitrogen but inferior to sodium nitrate.

Low activities of the enzyme were observed in the presence of potassium nitrate, aluminum ammonium sulphate. In the presence of aluminum ammonium sulphate the mycelium poorly developed with large vacuoles.

Effect of inoculum concentration

Inoculum concentration and its quality, purity and age are some of the important factors that affect the final synthesis of the fermentation product. The strain must retain its product forming capacity even after repeated transfers. The strain purity is of utmost importance in the formation biosynthetic products.

The seed was grown as before for 72 hrs. and at the end of which sample was aseptically drawn to determine packed cell volume pH, sterility both by microscopic examination and also by inoculation of a loop-full of the seed in a sterile nutrient solution and incubation at 37°C temperature.

When the seed was found pure, and in required state of growth it was used to inoculate 50 ml sterile fermentation medium present 250 ml Erlenmayer flasks. The inoculum was used in 0.5 ml to 2.5 ml quantities with 0.5 ml instruments. Inoculum concentration from 0.5 ml to 1.5 ml gradually increased the production of biomass and also enzyme activity. At 2.0 ml concentration of the inoculum and above there was gradually decrease in the enzyme activity. Probably because the feed back repression. Therefore 1.5 ml inoculum was found optimum for the maximum yield of the enzyme.

Effect of aeration

In all aerobic fermentations the quantum of available oxygen has tremendous influence on the growth of the microorganisms and also on the product formation. In order to determine quantamum of air for optimum synthesis of the enzyme, different quantities of fermentation medium were used. via 25 ml to 125 ml in 25 ml increments present in 250 ml Erlenmeyer flasks and the fermentations were carried out as before at the end of which the final pH, sterility and

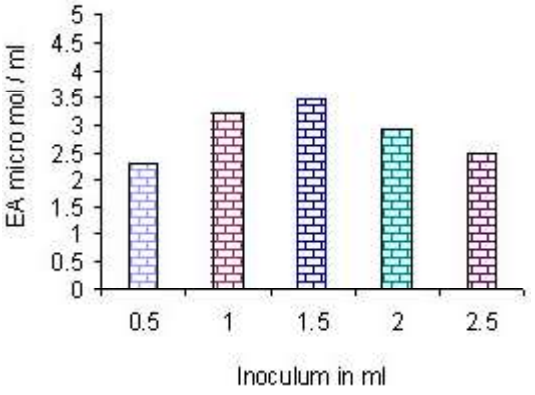

Fig. 9. Effect of inoculum concentration on the synthesis of α-amylase by *Acremonium sporosulcatum*

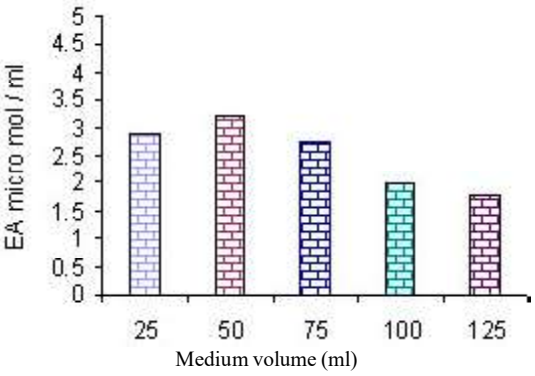

Fig. 10. Effect of aeration on the synthesis of α-amylase by *Acremonium sporosulcatum*

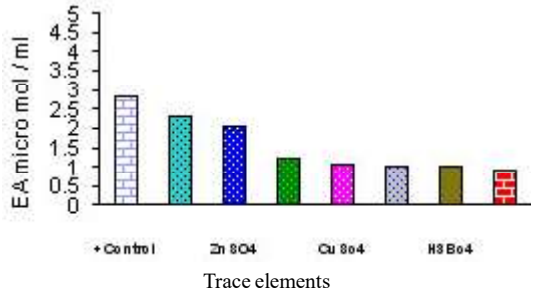

Fig. 11. Effect of trace element solution on the synthesis of α-amylase by *Acremonium sporosulcatum*

enzyme activity were estimated.

Increase in medium volumes from 25 ml to 50 ml increased the enzyme activity. Further increase in medium volume from 75ml to 125 ml as the aeration was reduced, affected the enzyme synthesis. Poorest yields were seen when 125 ml medium in 250 Erlenmeyer flasks was used. Therefore 50 ml volume in 250 ml Erlenmeyer flask was found to be the best production.

Effect of trace elements

In order to study the effect of trace elements, 0.1 ml of trace elements solution was added to each flask of containing 50 ml sterile fermentation medium in 250 ml Erlenmeyer flasks. Simultaneously the individual components of the trace elements solution were separately added to each flask containg 50 ml fermentation medium. Triplicates were used for each element. Fermentation was carried out as before at the end of which enzyme activities and final pH of each test were noted. Addition of complete trace element solution has contributory role in enhancing enzyme activity. In comparison with other Fe $(NH_3)_2$ SO_3 is found essential for the enzyme synthesis.

DISCUSSION

Composition of the culture medium, initial concentration of polysaccharides, cultivation conditions, genetic composition of the strain, all these factors are found to influence and regulate the enzyme synthesis (Schmidell *et al.,* 1988). Induction of amylase requires a substrate having a-1,4 glucoside bond including natural substance like starch (Domingnes and peralta, 1993). However it is observed that type of starch and its concentration greatly influence the enzyme activities. Thus maize starch at 7.5 % concentration was found the best carbon source for the maximum synthesis to the enzyme, Presence of in organic nitrogen like yeast extract is found essential for the maximum synthesis of amylase since nutritional control of á- amylaseis known (kunder *et al.,* 1973; Gogoi *et al.,* 1987). But evidence has also been presented to slow that stationary phase gene control of a-amylase production (Hiller *et al.,* 1997). Biphasic process of fermentation produced better results in short time as building up of biomass is much rapid when seed is grown in a defined medium and transferred to fermentation vassel leading to better yields of enzyme (Kelley *et al.,* 1997). In general, fermentation processes handling secondary products of metabolism get completed by 120 hrs. but in the present process of amylase synthesis was extended 144 hrs. This is contrast with amylase process by *Aspergillus ochraceus*, which reached maximum after 48hrs. of incubation (Adms, 1985; Chapman *et al.,* 1975).

CONCLUSION

It has wide range of temperature tolerance form 4° C to 37° C with an optimum temperature of 30° C for growth, reproduction and elaboration of α- amylase. Similarly it grows and produces the enzyme form pH 3 – 9 although pH 7.0 appears optimum.

Fermentations were carried out as biphasic system – ie the seed stage and fermentation stage. Several of the naturally occurring starchy substances like potato starch, maize starch, tapioca starch etc are studied out of which maize starch at a concentration of 7.5 % was founded optimum for the synthesis of the enzyme. The fungus needs both inorganic and organic nitrogen source like sodium nitrate and an organic nitrogen source like yeast extract are found essential for the synthesis of á- amylase. Aeration, inoculum concentrations, effect of micronutrients are also investigated.

The enzyme is partially purified by ammonium sulphate precipitation method and it is found to be stable upto 70°C. The culture is deposited at different international culture collection centers.

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