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ORIGINAL ARTICLE **OPEN ACCESS**

Production and Optimization of α-amylase from *Aspergillus ochraceus*isolated from Marakkanam Saltpans, Tamil Nadu, India

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ABSTRACT
Alpha-amylases are an important class of industrial enzymes, finding wide scale applications in various industries. The use of halophilic α-amylase in bioprocesses presents the advantage to obtain optimal activities at high salt concentrations. The present study was concerned with the production and optimization of alpha amylase from halophilic fungi isolated from Marakkanam Saltpan, Tamil Nadu, India. Twenty-one fungal strains were isolated from sediment sample and these isolates were screened for the production of alpha amylase enzymes. Based on the clear zone, the maximum zone producing strain of Aspergillus ochraceus was selected for optimization study. The maximum microbial amylase production could be achieved using an optimized medium of the following incubated at shaking condition (agitation speed 150 rpm), pH 8, temperature 40°C, the best starch, Sodium nitrate and 4% of NaCl concentration that provided the highest enzyme production from A.ochraceus. For purification, the specific activity of the purified amylase from A. ochraceuswas found as 3670.51 (units/mg prot/ml). Approximately 5.66 folds of purification were found by purification with 81.45 % yield. According to this finding, the halophilic fungi of A. ochraceusshowed promising amylolytic activities for biotechnological applications.
Keywords: *Aspergillus ochraceus, saltpan, α-amylase, optimization*

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INTRODUCTION

Extremozymes are the enzymes derived from extremophilic microorganisms, are an attractive option to regulating a given biocatalyst for a specific industrial application, since they can catalyze reactions in non-aqueous environments, water/solvent mixtures, at extremely high pressures, acidic and alkaline pH and also at very high temperatures. [1]. Extremophilic microorganisms can flourish in extreme environments such as unusual levels of salt, pH, pressure, temperature, etc., and those which are adapted to live in hypersaline habitats are considered halophiles [2]. The halophilic microorganism able to tolerate in the presence as well as in the absence of salt is categorized as halotolerant [3]. Halophiles represent valuable sources of a range of biomolecules which can offer potential applications for biocatalysis and biotransformation [1]. Most of the studies involving halophilic and halotolerant microorganisms have focused on halophilic bacteria and archaea, whereas there have been a few reports on halophilic and halotolerant fungi communities [4].

The ability of extremophiles to produce hydrolytic extremozymes has been much studied for its possible applications in industries [5,6]. Mostly halophilic hydrolases such as amylases, cellulases, lipases, xylanases, and proteases have been reported from halophilic bacteria [7]. Except for few preliminary studies there have not been many investigations on the extremozymes from halophilic fungi, particularly obligate halophilic fungi [1, 8].Black yeasts were the first documented fungi that form an active population in hypersaline seawater [9]. Studies on the fungi in hypersaline environments have showed the abundant and consistent occurrence of some specialized fungal species (10). One of the major reasons for the special interest in the extremophiles is to provide the opportunity for harsh industrial processes performed by their robust enzymes (11,12).

α-amylases are an important class of industrial enzymes, finding wide scale applications in food, textile, paper, detergent, analytical chemistry, beverage and pharmaceutical industry. Demand for α-amylase is projected to increase further in the coming years due to its use in diverse industrial sectors [13]. Yet search continues for novel α-amylase to increase the realm of processes where it can be used. In this context, isolation and screening of extremophilic organisms for α-amylase of desired trait is a contemporary research area. The use of halophilic α-amylase in bioprocesses presents the advantage to obtain optimal activities at high salt concentrations. Halophilic α-amylases also might be particularly resistant to organic solvents because they function under conditions where water activity is low. With this background, the present investigation was made on production and optimization of alpha amylase from halophilic fungi.

MATERIAL AND METHODS

Collection of Samples

Sediment sample was collected from saltpan of Marakkanam, Tamil Nadu, India in a sterile plastic covers and brought to the lab, stored in the refrigerator at 4 °C until it was used.

Isolation of fungi

One gram of collected soil sample was diluted in 99 ml blank and from the dilution 1 ml was serially diluted to the test tubes containing 9 ml of sterile distilled water and dilutions were made up to 10^{-6}. From the each dilution, 1 ml of dilution suspension was pipetted and plated on a sterile PDA. Bacterial contamination was inhibited by adding 0.05% of chloramphenicol in PDA. All plates were incubated at 25ºC for 5-7 days.

Screening for amylase production

Twenty one fungal strains were isolated in Czapeck Dox Broth (CDB) containing 1% soluble starch for the screening of amylase production. Sterile50 ml of CDB medium with 1% starch inoculated with two mycelial discs (8 mm) of a fungal strain and incubated 37^0C in rotary shaker for 6 days. The culture filtrate was separated and centrifuged at 8000 rpm for 15 minutes. Mycelium free culture filtrate obtained was used for the qualitative assay of amylase and activity.

Water agar medium (14) (1.8g agar in 100ml of 50% Seawater) supplemented with 1% soluble starch and 1% casein were poured in to petriplates and after solidification wells were made using a 8 mm diameter cork borer. 100µl of culture filtrate was poured in one well and same volume of uninoculated medium was added in a well as control. After 24 h, for amylase screening, the plates were flooded with Grams Iodine solution [0.3% (w/v) and I 3% (w/v) KI in 100ml distilled water] which produces a zone of clearance in starch utilized area. The zone of starch lysis indicated the production of amylase enzyme by the fungal strain. The diameter of zone of starch lysis was determined for all the amylase positive isolates. Based on the clear zone, the strain *Aspergillus ochraceus* was selected for further study. The strain was maintained on potato dextrose agar and used for further to study on amylase production.

Optimization for amylase production

Factors affecting cell growth and α-amylase production were investigated using one factor at a time method. The optimized parameters viz., static and shaken conditions (50 to 250 rpm), carbon source (0.5% of starch, wheat bran, glucose, skim milk and sucrose), nitrogen source (0.5% of beef extract, peptone, gelatin, sodium nitrate and ammonium nitrate), temperature (20°C to 60°C), pH (2.0 to 10.0), salt concentrations (1 to 5 %) were tested

Purification of enzyme

Ammonium sulphate precipitation

Purification steps were carried out at 4ºC. In the initial purification step, the supernatant containing the extra cellular amylase was treated with different saturation levels of solid ammonium sulphate (up to 80% saturation level) as described by Wang *et al*., 2006, with continuous overnight stirring. The precipitated proteinaceous material was collected by centrifugation (10,000 rpm for 15 min) and dissolved in 0.1M phosphate citrate buffer (pH 5.0). The enzyme solution was dialyzed using dialysis membrane No-150 (Himedia) against the same buffer for 48 h with several intermittent buffer changes. The dialyzed protein fraction was lyophilized to a powder.The enzyme activity was assayed in each and every step following the procedure stated previously.

RESULTS AND DISCUSSION

Isolation and screening of amylolytic fungi

All the isolated 21 fungal strains belonging to nine genus were qualitatively screened by well diffusion assay method using water agar medium supplemented with 1% starch. Based on the diameter of zone of clearance they were classified into three categories *viz*.,high enzyme activity (+++), medium enzyme activity (++) and low enzyme activity (+). The strains that failed to produce clear zones were denoted as

non producers (-). Among the 21 strains, only 4 namely, *A. flavus, A. fumigatus, A. niger*and *A. ochraceus* were belonged to the +++ category, 13 strains of ++, 2 strains of + category and another 2 strains did not exhibit amylase activity. Among the +++ category, *A. ochraceus*exhibited largest zone of starch lysis (8.3 mm). Hence it was selected for further studies on production and optimization of amylase enzyme.

Optimization of α-amylase production conditions

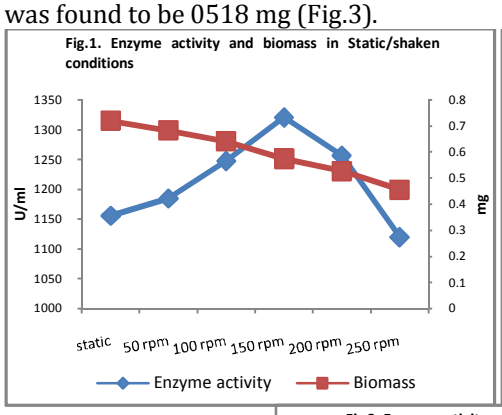
Selected strain *A. ochraceus*was subjected to various culture conditions to investigate the optimum culture conditions for α-amylase production.

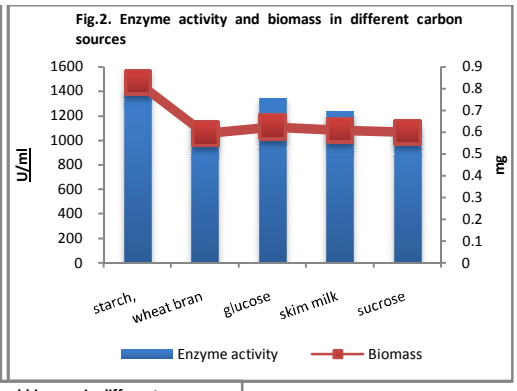
Effect of static and shaking conditions on α-amylase production

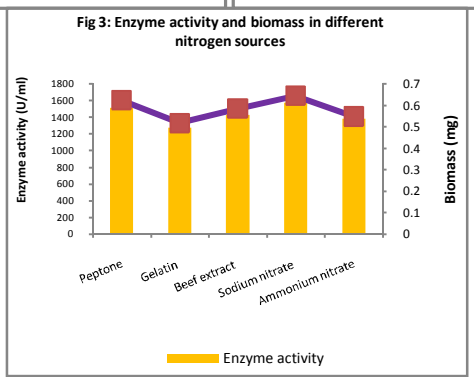
The results obtained from the static and shaking conditions in submerged culture cleared that the α-amylase productivity was gradually raised with the increment in the shaking condition. Shaking condition of150 rpm was the best for α-amylase productivity (1321 U/ml of enzyme).Minimum was observed in static as well as at 300 rpm. However the biomass during static conditions was found to be high (0.721 mg) compared to other shaking conditions (Fig 1).

Effect of different carbon and nitrogen sources on α-amylase production

The broth amended with 05 various carbon sources at the concentration of 0.5 % was inoculated with *A. ochraceus*and kept in a shaker at 150 rpm for five days. The highest enzyme activity was obtained in starch amended medium (1415 U/ml) where the biomass produced was about 0.621mg (Fig 2). Sodium nitrate gave the maximum enzyme activity of about 1575 U/ml and the biomass obtained was 0.645 mg. Likewise the minimum enzyme activity (1276 U/mg) was found in gelatin amended medium where the biomass was found to be 0518 mg (Fig.3).

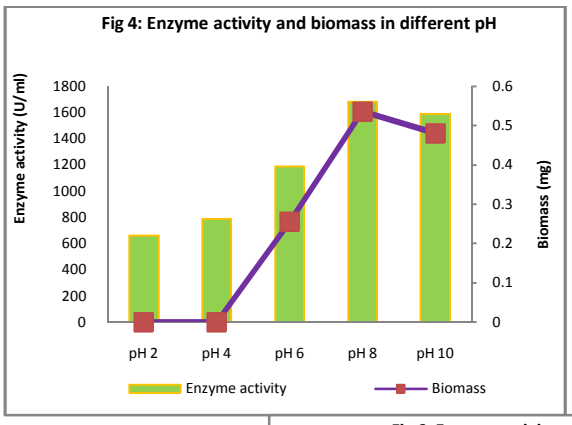


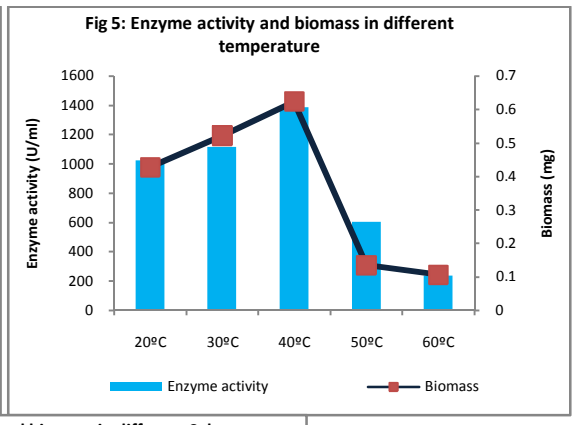


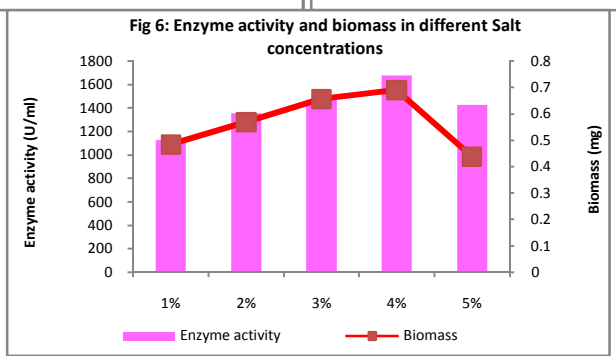


Effect of different pH, temperature and salt concentrations on α-amylase production

The initial medium pH ranging from 2-10 was studied to detect the effect on amylase production by *A. ochraceus* in MSM broth with previously optimized parameters. pH 8 supported maximum amylase production (*i.e.*)1685 U/ml with the biomass of 0.536 mg. The lowest pH 3 and 4 did not produce amylase enzyme even though minimum growth (0.264 mg) was observed in pH 6 (Fig.4). Optimum temperature for amylase production was standardized for which different temperatures ranging from 20°C to 60°C were examined. The result showed the maximum amylase activity at 40ºC (1386 U/ml) while the minimum was observed at 60°C (238 U/ml). The biomass also found to be maximum at 40°C (0.624 mg) and minimum (0.107) at 50°C (Fig.5). As the fungus *A. ochraceus* was isolated from a salt environment, the effect of varying percentage of NaCl concentration on amylase activity was studied. At 4% of salt concentration, it gave maximum amylase activity (1677 U/ml) and minimum enzyme activity was obtained at 1% of salt concentration (1126 U/ml (Fig.6).







Purification of enzyme

As shown in Table (1), the specific activity of the purified amylase from *A. ochraceus*was found as 3670.51 (units/mg prot/ml). Approximately 5.66 folds of purification were found by purification with 81.45 % yield

Table 1:Purification of amylase enzyme by ammonium sulfate precipitation

Purification step	Total enzyme Activity (U)	Total protein (mg)	Specific activity U/mg protein)	Purification (fold)	Recovery (%)
Culture supernatant	2025	7.6	280.5	1	100
Ammonium sulphate precipitation	1742	2.8	1674.0	5.96	81.45

Discussion

Many attempts have been made to find suitable fungus strains for the production of amylases with desirable properties [15]. Mesophilic fungi are reported to be the principal amylase producers and especially members of the *Aspergillus* and *Penicillium* genera that appear to be the dominant producing species [16]. Fungal amylases are preferred for use in various industries, including the food and pharmaceutical industries, due to their nontoxic characteristics [17,18]. Consequentially, *Aspergillus* species, such as *Aspergillus niger* and *Aspergillus oryzae*, are frequently used in the industrial production of amylases [19], but there are few reports on the purification and detailed characterization of α-amylases from halophilic fungi [17].

The results obtained from the static and shaking conditions in submerged culture cleared that the α-amylase productivity was gradually raised with the increment in the shaking condition. Similarly, Valaparla [20] found maximum amylase activity in Maize starch substrate of 13.59 U/ml and lowest activity in Tapioca starch substrate of 4.66 U/ml at 30°C in shaking condition from *Acremonium sporosulcatum*

Some of the most known substrates as a carbon source for microorganisms to produce alpha-amylase include maltose, glucose, and sucrose. In the present study, the highest enzyme activity was obtained in starch amended medium (1415 U/ml) where the biomass produced was about 0.621mg. Like that, a study on *Aspergillus oryzae* S2 showed that the proper concentration of starch (10%) are the best carbon source to produce alpha-amylase [21].As nitrogen contents in culture medium have a significant role in the growth of microorganisms. Different nitrogen sources have been widely studied for the optimization of

alpha-amylase production, including the organics such as yeast extract, soybean, and peptone, which are the most applicable nitrogen sources in culture medium; other sources are the inorganics, such as ammonium hydrogen phosphate, ammonium sulfate, and ammonium chloride [22]. In this study, Sodium nitrate gave the maximum enzyme activity of about 1575 U/ml and the biomass obtained was 0.645 mg.
The pH characterization study shows the typical behavior of halophilic fungi adaptation to its habitat. The hypersaline habitats are mostly found to have neutral to alkaline range of pH (4). In this study, pH 8 supported maximum amylase production (*i.e.*) 1685 U/ml with the biomass of 0.536 mg. The pH values of 9 and 10 are mostly considered enough to designate the enzyme as alkalophilic [23, 24]. Alkalophilic amylases are mostly applied in the detergent industries [25].Most of the enzymes are unable to perform the catalytic activity at high temperature values of 50-60ºC [26]. The α-amylase in this study has been found to have optimum activity at 50ºC and retain more than 85% of its activity at high temperatures of 40-50ºC, which are considered as thermophilic range for enzymes [27, 28]. This character shows that, it has the capability to withstand high temperature processes that are mostly carried out in starch industries [26].This extreme characteristic requires modification in the working capabilities of the metabolites in the halophiles. In this study, at 4% of salt concentration, it gave maximum amylase activity (1677 U/ml) and minimum enzyme activity was obtained at 1% of salt concentration (1126 U/ml). The catalytic ability of α-amylase in this study at high salt concentrations makes this enzyme novel. This capability is much greater than the previously reported bacterial and Archeal amylases [25, 29]. Ability of this amylase to work at extreme salt concentrations makes them the best available candidate to work for bioremediation processes, particularly, saline waste management [30, 31].
40 % ammonium sulphate saturation was found suitable to precipitate protein with the highest enzyme activity. Lawal et al. (32) reported 60 % ammonium sulphate saturation for α-amylase from *Aspergillus niger*. Sidkey et al. (33) and Varalakshmi et al. (34) reported that 40-60 % saturation gave the highest enzyme activity of α-amylase isolated from *A. flavus* and *A. oryzae*, respectively. These results are in line with that obtained from the present study. Generally, proteins become more soluble in aqueous solution when a salt such as ammonium sulphate is added. Low salt concentration stabilizes the various charged groups on a protein molecule, thus enhancing the solubility of proteins in aqueous solution (35).

CONCLUSION

Results obtained from this study have shown that that the amylolytic enzyme yield from *A. ochraceus*can be increased by the optimized condition. The pH and temperature optimum of 9.0 and 40-50 °C together with the evidence of a wide range of pH and temperature stability shows feasibility for application of this enzyme in detergent industry, textile desizing and paper industry. Further studies to purify, characterize and various application ofthe amylase enzyme produced by this strain will be investigated.

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