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Isolation, Optimization, and Partial Purification of Amylase from *Chrysosporium asperatum* by Submerged Fermentation

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A potent fungus for amylase production, *Chrysosporium asperatum*, was isolated from among 30 different cultures obtained from wood samples collected in the Junagadh forest, India. All of the isolated cultures were screened for their ability to produce amylase by submerged fermentation. Among the selected cultures, *C. asperatum* (Class Euascomycetes; Onygenales; Onygenaceae) gave maximum amylase production. In all of the different media tested, potato starch was found to be a good substrate for production of amylase enzyme at 30°C and pH 5.0. Production of enzyme reached the maximum when a combination of starch and 2% xylose, and organic nitrogen (1% yeast extract) and ammonium sulfate were used as carbon and nitrogen sources, respectively. There was no significant effect of metal ions on enzyme activity. The enzyme was relatively stable at 50°C for 20 min, and no inhibitory effect of Ca^{+2} ions on amylase production was observed.

Keywords: Amylase, *Chrysosporium asperatum,* submerged fermentation, optimization

Amylases are hydrolytic enzymes that are widespread in nature, being found in animals, microorganisms, and plants. They are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents [5]. Although amylases can be obtained from several sources including plants and animals, those from microbial sources generally meet industrial demand [36]. These enzymes have found numerous applications in commercial processes, including thinning and liquefaction of starch in the alcohol, brewing, and sugar industries. In addition to starch saccharification, amylases are also useful in many industrial processes in the food, baking, brewing, detergent, textile, and paper industries. Their application has been expanded into many other fields including medicinal and analytical chemistry [36]. Various starch hydrolysates have been used as sweeteners in the food industry [24]. Amylases have also been utilized to convert cereal starch into fermentable sugars for yeast strains in the brewing industry [24]. Both detergent and paper industries use both amylase and cellulase enzymes [9, 10, 30, 37, 38].

Recently, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several strains. Although they are also produced by plants and animals, amylases from different microbial strains have successfully replaced chemical hydrolysis and met the industrial demand [36]. The most important reason for using amylases of microbial origin is their high productivity and maximum thermostability in industrial applications [11]. Currently, a large number of microbial amylases have been commercialized in the starch processing industries [36]. Starch hydrolytic enzymes comprise 30% of the world's enzyme consumption [43].

Evidence of amylases in yeast, bacteria, and molds have been reported and documented [5, 4, 12]. Among the microorganisms, many fungi had been found to be good sources of amylolytic enzymes. A perusal of the literature indicates that amylases of fungal origin are more stable than those of bacterial origin [15]. In-depth studies on fungal amylases have been concentrated mainly on *Rhizopus* sp. and *A. niger*, probably because of the ubiquitous nature and nonfastidious nutritional requirements of these organisms [1]. Other such species may exist in nature but there is not much information on those that produce amylase. However, a large number of researchers have isolated *Chrysosporium* species from many different habitats around the world. Until recently, interest was restricted only to occurrence; however, as research into this fungus has gained momentum, it has been evaluated for its ability to produce commercially important enzymes such as cellulase, xylanase, and lignolytic

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enzymes. Most of the work has made use of *Phanerochaete chrysosporium* for production of enzymes and biodegradation of xenobiotic compounds, whereas less attention has been paid to other species fungal. The present work is therefore focused on the screening and isolation of potential species for amylase production, characterization of an amylase-producing strain, and optimization of culture conditions to maximize enzyme activity and production in this strain. On the basis of amylase activity, the most promising strain, *Chrysosporium asperatum*, was investigated in detail by the submerged fermentation method.

Materials and Methods

Isolation and Screening of Fungi for Amylolytic Enzyme

Isolation of fungal cultures was carried out using 30 different samples collected from Gir forest, Junagadh, Gujarat, India. Isolation and establishment of pure cultures of these strains was carried out by the routine method. Initially, these samples were plated on malt agar (MA) plates and purified cultures were maintained at 4°C in the refrigerator on MA medium containing 1% starch. Based on the zone of clearance shown by the cultures on starch agar plates and their individual amylase activity, strain KSR-1 was selected for further study. This strain was identified by morphotaxonomic features/characters at the Agharkar Research Institute, Pune, India and was found to be *Chrysosporium asperatum* (Class Euascomycetes; Onygenales; Onygenaceae).

Innoculum Preparation

For the preparation of inoculum, a 72-h-old culture grown on MA + 1% starch agar medium was used and a loopful culture of *C. asperatum* was suspended in Tween 80 (0.1%). Innoculum with spores having 10^6 spores/ml was used to inoculate the production medium.

Enzyme Production and Extraction

Extraction and production of amylase was carried out by the submerged fermentation technique. The medium contained 10 g malt extract, 3 g peptone, 1 g K_2HPO_4, 0.04 g KH_2PO_4, and 30 g/l potato starch. Into a 250 ml conical flask, 100 ml of medium was dispensed, autoclaved at 121°C at 15 psi for 20 min, and cooled at room temperature. The inoculated medium was shaken in an incubator at 150 rpm for different time periods. Every 24 h over a period of 5 days, the culture in the flasks was harvested to measure enzyme activity. Medium containing crude enzyme was centrifuged at 10,000 rpm at 4°C for 15 min in a cooling centrifuge and pellets were dissolved in acetate buffer (pH 5.0). The filtrate was then subjected to ammonium sulfate precipitation and purification.

Optimization of Process Parameters and Effect of Additional Nutrients

A spore suspension was prepared in 0.1% Tween 80 from a 72-h-old culture of *C. asperatum.* The inoculum size was varied from 5×10^5 to 5×10^6 and 5×10^7 spores/ml of mineral solution. The effect of pH on the production of amylase was determined at values ranging from 2 to 9. The carbon sources glucose, maltose, lactose, sucrose, fructose, and xylose (2%); 1% of the organic nitrogen sources yeast extract, peptone, beef extract, and malt extract; and the inorganic nitrogen sources ammonium sulfate, sodium nitrate, ammonium molybate, and aspargine were supplemented as individual components in the production medium to determine their effects on enzyme production.

Effects of Metal Ions

Four different metal ions, Cu^{+2}, Zn^{+2}, Fe^{+3}, and Mg^{+2}, in the form of their salts, were supplemented in the medium to determine their effects on the production of enzyme. Each metal ion was added to a reaction mixture at a concentration of 1 mM and their effects were monitored at 540 nm, with the exception of Ca^{+2}, which was evaluated at two different concentrations (5 mM and 10 mM).

Parameters for Amylase Enzyme Assay

Amylase enzyme assay. Amylase activity was determined by incubating a mixture of 0.1 ml of enzyme and 1% starch dissolved in 0.1 M sodium acetate buffer, pH 5, at 30°C. The reducing sugar released after 25 min was measured as described by Miller [33]. One unit (U) of amylase activity is defined as the amount of enzyme that releases 1 μmol of reducing sugar as glucose per minute under the defined assay conditions. All of the sets of reactions were performed in triplicate and the standard errors were reported.

Optimization of enzyme activity. Six different concentrations of potato starch, 0.5%, 1%, 1.5%, 2%, 2.5%, and 3%, were used to determine the effect of substrate concentration on enzyme activity. The effect of temperature on enzyme activity was determined within a range of 10–60°C. The effect of pH on enzyme activity was evaluated by incubating the enzyme for 25 min with potato starch in 0.1 M acetate buffer at 37°C and at pH 2 to 6.

Purification of enzyme and determination of molecular weight. Crude filtrate was subjected to ammonium sulfate precipitation, and 20%, 40%, 60%, and 80% fractions were collected. Maximum activity was observed in the 60% fraction; thus, it was further subjected to affinity precipitation. Purification of amylase was carried out by affinity precipitation with alginate as described by Teotia *et al.* [42]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 12% gel following the procedure of Lammeli [29] using a Medox electrophoresis unit (Medox India Pvt. Ltd., Ahmedabad, India).

Native PAGE was performed as described in Sambrook and Russell [40] using 1% starch as the substrate. Once electrophoresis was completed, gels were stained with 0.1% iodine solution, and then destained with 1 M NaCl until clearance bands of amylase activity were obtained.

Results and Discussion

Selection of Strains for Amylolytic Activity

Out of the 30 fungal cultures isolated and purified from the wood samples, one culture giving promising results was selected. To enable the production of significant levels of amylase activity, this culture was studied further for its ability to grow in liquid medium containing starch as the sole carbon source at 30°C. It was identified by morphotaxonomic features as *Chrysosporium asperatum* (Class Euascomycetes; Onygenales; Onygenaceae). Its maximum growth at 30°C indicates that the fungus is mesophilic in nature. The organism

is considered to be mesophilic with an optimal temperature for growth between 25°C and 35°C. It is aerobic in nature and can grow over a wide range of hydrogen ion concentrations [35]. Among the Euascomycetes, *Chrysosporium* sp. was explored earlier by many scientists [19, 26] owing to its ability to degrade xenobiotic compounds and produce commercially important enzymes. However, detailed studies on the purification of α-amylases from fungi have been conducted on only a few species [3]; thus, there is no such information available on the species investigated in this study.

Optimization of Amylase Enzyme During Submerged Fermentation

Effects of inoculum size and incubation time on enzyme production. Inoculum size plays an important role in the production of amylase. However, little significant differences were observed when inoculum size was varied relative to that of optimum inoculum size. It was found that high inoculum levels led to a decrease in the level of enzyme production, which in turn was inhibitory in nature (data not shown). As reported earlier by Hema *et al.* [22], no effect due to inoculum size was observed on enzyme production in the present study.

The incubation time for achieving the maximal enzyme level is governed by the characteristics of the culture and is based on the growth rate and enzyme production [27]. Production of amylase increased with increasing incubation time, and it was found to be maximal after 72 h following inoculation. A further increase in the incubation period resulted in a decrease in the production of amylase. This result might be due to the production of byproducts and depletion of nutrients after 72 h. Duochuan [15] reported that such byproducts inhibit growth of fungi and hence enzyme production.

Effects of different substrates and substrate concentrations on production of enzyme. The ideal substrate for improving fungal amylase production should combine a biomass-increasing property with enzyme synthesis induction. Substrate concentrations ranging from 1–6% were evaluated for optimization of amylase activity. The maximal activity was observed in medium containing 3% potato starch after 72 h of incubation, as shown in Fig. 1. Initially, as the concentration of the substrate was increased, there was a significant increase in enzyme activity, but as soon as all of the substrate binding sites were filled, there was a decline in enzyme activity. The effect of six different substrates on enzyme activity was determined, and the maximal production of enzyme (96.85 IU/ml/min) was obtained using potato starch after 72 h of incubation. After 24 h of incubation, corn starch gave the maximal activity (76.45 IU/ml/min), but after 72 h of incubation, potato starch gave the maximal activity. Amylase activity was highest on potato starch. The amylose and amylopectin contents and quantity of lipids in

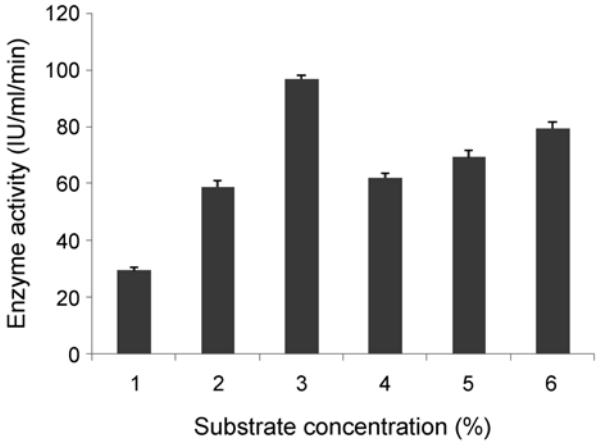

Fig. 1. Effect of substrate (potato starch) concentration on production of enzyme.

starch often varies, depending on its origin [2, 23]. According to Aberle *et al.* [2] and Hoover [23], the amylose, amylopectin, and lipid contents are approximately 28%, 72%, and 6.0% in cereal starch and 20%, 80%, and 0.1% in tuber starch, respectively. Therefore, it is inferred that the composition and variation in molecular weight of starch could affect enzyme action [14]. It follows that a change in the composition and molecular weight might be responsible for changes in the production of enzyme. A lower affinity for low-molecular-weight substrates than for highly polymerized glucan is consistent with the properties of α-amylase and glucoamylase. When the amount of starch was increased, the production of enzyme was reduced. This result may be due to an increase in the amount of the carbon source above the optimal level, leading to a reduction in enzyme formation [8].

Effect of carbon source on production of enzyme. On supplementation of the production medium with different carbon sources, slightly better amylase production was

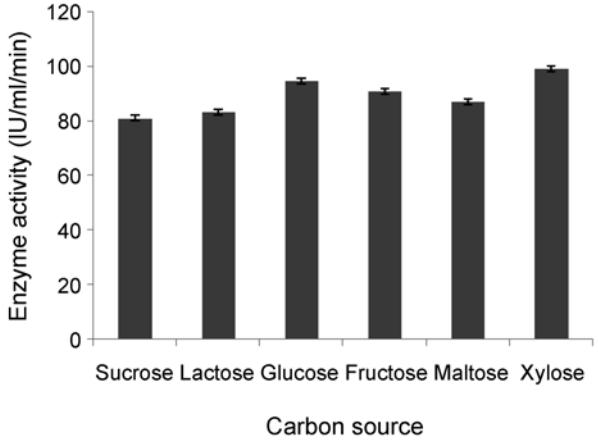

Fig. 2. Effect of carbon source on production of enzyme.

observed when a combination of 2% xylose and 3% potato starch was used (Fig. 2). After 72 h of incubation, maximal activity (99.19 IU/ml/min) was found in medium supplemented with xylose, whereas minimal activity (81.09 IU/ml/min) was found in medium containing sucrose. Addition of supplements in solid-state fermentation (SSF) for amylase production has been studied previously; the results indicated that glucose and sucrose are important supplements for amylase production by *A. niger*, which belongs to the class Ascomycetes, as does *C. asperatum* [7]. Earlier workers dealing with SSF have suggested that soluble starch is the best source for production of amylase enzyme with *A. fumigatus* and *A. niger* [20], but our results suggest that a combination of two carbon sources increases the production of amylase by submerged fermentation.

Effect of nitrogen source on production of enzyme. Both organic and inorganic nitrogen sources were added as supplements in the medium. After 72 h of incubation, maximal enzyme production using yeast extract as the nitrogen source was observed, as shown in Fig. 3. Similar findings were also reported using SSF, where yeast extract was added as a supplement with coarse waste of rice bran [22]. Inorganic nitrogen sources also had an inducing effect on the production of amylase. Among those evaluated, ammonium sulfate was found to be the best in this regard (data not shown). Lineback *et al.* [31] have reported the regulation of amylase formation by nitrogen source only; an easily metabolizable nitrogen source such as ammonium sulfate was better than other nitrogen sources. Herein, ammonium sulfate showed maximum enzyme production after 48 h of incubation.

It has been reported that during the production of amylase by SSF using wheat bran, urea is the best nitrogen source for induction of amylase activity [18]. However, according to Haq *et al.* [21], urea releases ammonium ions slowly and was thus found not to be a good source of nitrogen. This observation was attributed to the low urease activity of the organism.

Effects of pH and temperature on production of enzyme. Since enzymes are very sensitive to pH, determination of the optimal pH is essential to the production of amylase [32]. In the present study, the effect of pH on the production of enzyme was thus studied by carrying out fermentation over a range of pH (2.0–9.0). The production of amylase was found to be highest at pH 5. There are reports that fungal cultures give optimum enzyme production at pH 5 using various substrates [18, 34]. It is evident from the data that amylase production by *C. asperatum* and enzyme activity showed a broad range of pH preference, thus allowing the use of the enzyme under both acidic and alkaline conditions. Maximal activity (96.15 IU/ml/min) was observed at a temperature of 30°C. The optimal temperature for amylase activity required by various fungi has been previously studied; they have a wide range of temperature preferences depending upon the nature of their adaptation [17, 27, 35]. Amylase activity increased progressively with increasing temperature from 20°C, reaching a maximum at 60°C, in *Aspergillus niger* [35], whereas the optimum was found to be 35°C for *Aspergillus falvus* var. *columnaris* [17]. In thermophilic species such as *Thermomyces lanuginosus*, the optimal temperature was reported to be 50°C [27]. In the present study, *C. asperatum*, being a mesophilic species, showed an optimal temperature of 30°C for maximal enzyme activity.

Effects of temperature and pH on enzyme activity. Temperature and pH are the environmental factors that most markedly influence enzyme activity. For *C. asperatum*, the optimal pH was observed to be 5.0, as indicated in Fig. 4. Above pH 5.0, there was a continuous decline in the activity of amylase enzyme. Enzyme activity increased with increasing temperature from 10°C and reached the maximum at 30°C (Fig. 5). However, the enzyme retained 80% of its activity at 50°C after 20 min, indicating that it is thermostable. The enzyme showed the highest activity at 30°C and the lowest activity at 10°C. The lowest activity was also observed at 60°C. Inactivation at high temperature may be due to incorrect conformation caused by hydrolysis

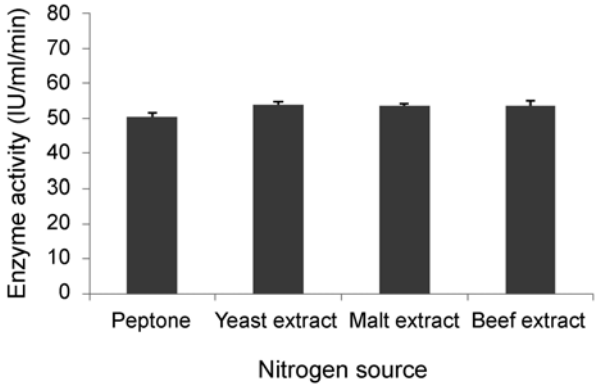

Fig. 3. Effect of nitrogen source on production of enzyme.

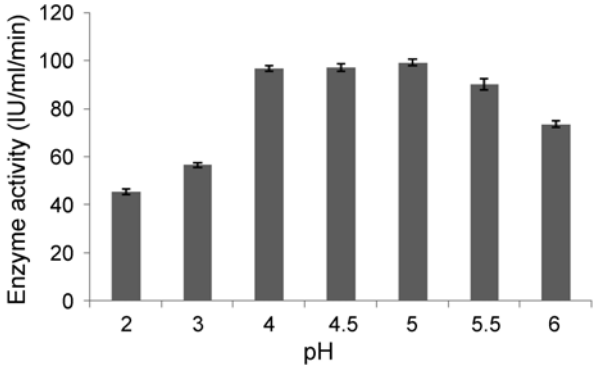

Fig. 4. Effect of pH on enzyme activity.

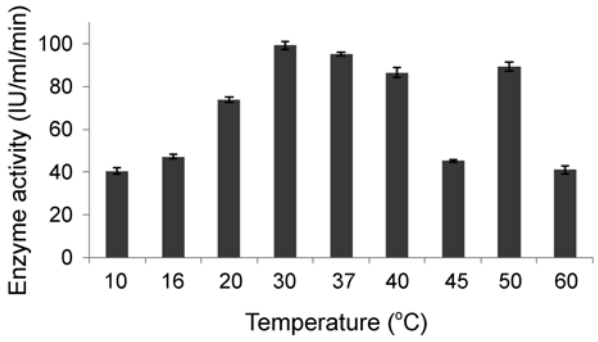

Fig. 5. Effect of temperature on enzyme activity.

of the peptide chain, destruction of amino acids, or aggregation [41].

Effect of substrate concentration and incubation time on activity. The effects of different concentrations of the substrate potato starch from 0.5–2.5% were determined. Maximal activity (96.35 IU/ml/min) was found after 20 min of incubation with 1% substrate (data not shown). When the amount of starch was increased in the enzyme–substrate mixture, there was an increase in amylase activity. Thereafter, when the substrate concentration exceeded 1%, a decline in enzyme activity was noticed. This reduced enzyme activity may have been associated with the saturation of the catalytic sites of the enzyme by the substrate.

As for incubation time, the maximal activity (99.19 IU/ml/min) was observed at 20 min, after which it began to decline, as shown in Table 1. The lowest activity (23.75 IU/ml/min) was observed at an incubation time of 5 min.

Effects of metal ions on production of enzyme. Four different metal ions, Cu^{+2}, Zn^{+2}, Fe^{+3}, Mg^{+2} in the form of their salts at a concentration of 1 mM, were supplemented into the medium to investigate their effects on the production of enzyme. The results are shown in Table 2. The addition of metal ions caused no significant increase or decrease in the production and activity of the enzyme. The effect of metal ions on the activity of amylase was also studied by earlier workers, who found that the enzyme did not require any specific ion for catalytic activity [39]. The effect of Ca^{+2} at two different concentrations (5 mM and 10 mM) was also investigated and no inhibitory effect was found

Table 1. Effect of incubation time on enzyme activity.

Incubation time (min)	Enzyme activity (U/ml/min)
5	23.75±1.54
10	40±1.33
15	65.65±0.98
20	99.19±0.90
25	98.25±1.25
30	99±1.10
40	72±1.45

Table 2. Effects of metal ions on enzyme activity.

Metal ion	Enzyme activity (U/ml/min)
$CuCl_2$	97.15±0.95
$ZnCl_2$	96.25±1.12
$FeCl_3$	98.32±1.25
$MgSO_4$	98±0.65

on enzyme activity. The inhibition of amylase activity in the presence of Ca^{+2} has been reported in the case of metalloenzymes containing a metallic ion for catalytic activity [28, 39]. On the contrary, other reports also indicate no effect of Ca^{+2} on amylase activity [6, 16, 39].

Ammonium sulfate precipitation and purification of enzyme. Crude filtrate was subjected to ammonium sulfate precipitation. Maximal activity was observed in 60% fractions, which were subjected to further purification by affinity precipitation using sodium alginate. SDS–PAGE of the purified enzyme showed the homogeneous nature of the band pattern (MW, 55,000 Da), which was further characterized by native gel electrophoresis (Fig. 6). Khoo *et al.* [25] purified the α-amylase enzyme produced by *Aspergillus flavus* using ammonium sulfate precipitation and ion-exchange chromatography. Their study reported that the enzyme is homogeneous on SDS–PAGE. Abou Zeid [3] also purified extracellular α-amylase from the same species by a starch adsorption method. Similarly, Chakraborty *et al.* [13] purified a thermostable α-amylase enzyme by ammonium sulfate fractionation and ion-exchange column chromatography on DEAE-cellulose to obtain a homogeneous product.

In conclusion, screening of various fungal strains for production of amylase showed a good producer that was identified as *C. asperatum* by morphotaxonomic characteristics. Overall, results of this study indicate that *C. asperatum* species is a potent producer of amylase in the family Onygenaceae, *P. chrysosporium*. After affinity precipitation, purified amylase showed maximal activity at pH 5.0 and a temperature of 30°C. The enzyme showed no inhibitory

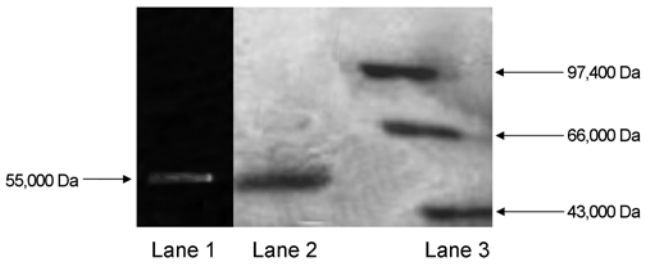

Fig. 6. SDS–PAGE and Native-PAGE of purified enzyme.
Lane 1: Native PAGE of amylase. Lane 2: Purified enzyme. Lane 3: Ladder Medium Range (Bangalore Gene Pvt .Ltd.; Cat. No. RPMW-M 106005).

effect in the presence of Ca^{+2} ion. The purified enzyme was homogeneous in nature with a single band of molecular mass 55,000 Da in denaturing gel electrophoresis, and the enzyme was further characterized using native gel electrophoresis.

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