Full Length Research Paper

Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture

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***Aspergillus* species isolated from various seeds were screened for their ability to produce amylase. A selected strain, *Aspergillus* sp. JGI 12, showed the highest amylase activity in solid state fermentation. Different substrates were screened for enzyme production. Coconut oil cake, groundnut oil cake and rice bran were found to be very good substrates for enzyme production. Different combinations; wheat bran : groundnut oil cake : rice bran (1:2:2) was used which resulted in higher enzyme titre. This combination of substrates was used for further studies on amylase production and characterization. The enzyme amylase was found to be thermostable and active at wide range of pH.**

Key words: fungal amylase, *Aspergillus sp.* JGI 12, solid state fermentation, solid substrate, enzyme production.

INTRODUCTION

In recent years the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan et al., 1999; Pandey et al., 2000; Abu et al., 2005). Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents (Akpan et al., 1999; Mitchell and Lonsane, 1990). Although amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand (Pandey et al., 2000). Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. Besides their use in starch saccaharification, they also find potential application in a number of industrial processes such as in food, baking, brewing, detergent, textile and paper industries. 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 (Pandey et al., 2000).

During the last decade, an increased attention was paid to the use of various agro industrial wastes for value addition using solid-state fermentation (SSF) by filamenttous fungi (Pandey et al., 1999, 2000a, 2000b, 2001). It has been reported that SSF is the most appropriate process in developing countries due to the advantages it offers (Carrizales and Jaffe, 1986). The hyphal mode of growth and good tolerance to low water activity (a_w) and high osmotic pressure conditions, make fungi most efficient for bioconversion of solid substrates (Raimbault, 1998). The objective of this study were selection of a suitable strain for the production of glucoamylase, screening of different agricultural byproducts as substrates for maximum enzyme production, application of different combinations of these substrates for enzyme production, and optimization of cultural conditions for the production of glucoamylase.

MATERIALS AND METHODS

Isolation of Microorganism

Several species of *Aspergillus* were isolated from different seeds obtained from various locations by Standard

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Blotter Method (Abdul Baki and Anderson, 1973). They were identified on the basis of morphological characteristics.

Isolation and screening of isolates

Primary screening was done by starch agar plate method. The isolate *Aspergillus* sp. JGI 12 showed a maximum hydrolysis halo on this medium and was selected for further investigation.

Substrates

Wheat bran (WB), black gram bran (BGB) and rice bran (RB) were obtained from mills in Mangalore, India. Coconut oil cake (COC), groundnut oil cake (GnOC) and gingelly oil cake (GiOC) and corn cob (CC) were procured from Bangalore, India. The substrates were ground into coarse powder with a blender.

Solid state fermentation

For production of enzymes in SSF, the fungi were grown at 25ºC in 250 ml Erlenmeyer flasks containing 5 g of the coarsely ground substrate. Distilled water was used to adjust the moisture content from 43 to 81%.

Optimization of culture conditions

During comparison of substrates wheat bran and the other substrate were taken in a ratio of 1:1. Two other combinations of substrates were also used at various ratios based on the studies of comparison. To ascertain the effect of culture conditions the present study was carried out at different incubation periods (4, 5, 6, 7, 8 and 9 days), temperature (25, 30, 40, 50 and 60ºC) and pH (3.6, 4.2, 5, 5.8, 6.5, 7.0, 7.5, 8 and 9). Different carbon sources such as glucose, maltose, lactose, sucrose and starch were used to determine their effect on amylase production.

Enzyme extraction

22 ml of 0.1 M phosphate buffer (pH 6.5) was added to the cultures, the mixtures were shaken for 30 min at 19ºC and 140 rpm on a rotary shaker. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4ºC for 15 min. The supernatant was filtered through Whatmann number 1 filter paper and the filtrate was used as the crude enzyme preparation.

Assay of enzyme activity

Amylase activity was estimated by analysis of reducing sugar released during hydrolysis of 1% (w/v) starch in 0.1 M phosphate buffer, pH 6.5, at 25ºC for 20 min by the Dinitrosalicylic acid method (Miller, 1959). One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as glucose per min under the assay conditions. Enzyme activity is expressed as specific activity, which is represented as U/mg of protein.

Assay of protein concentration

The protein concentration was determined by the Lowry's method (Lowry et al., 1951) using bovine serum albumin as the standard.

Enzyme substrate reaction time

To ascertain the effect of incubation time on enzyme activity the enzyme substrate reaction mixture was incubated for different incubation periods of 5, 10, 20, 30, 40 and 50 min and enzyme activity was studied.

Substrate concentration

Effect of substrate concentration on enzyme activity was measured at different concentrations of starch in the reaction mixture (0.25, 0.5, 0.75, 1.0, 1.25, 1.5 and 1.75 %).

Temperature and pH

Effect of pH on amylase activity was determined by incubating the reaction mixture at pH values ranging from 3.6 to 9.0. Optimum temperature for enzyme activity was determined by conducting the assay at different temperatures ranging from 25 to 70ºC.

Thermo stability of amylase

The thermal stability of the enzyme was determined by incubating the enzyme fraction at various temperatures between 25 to 60ºC, without the substrate, for 1 h. At 10 min intervals, aliquots of 0.5 ml of the incubated enzyme were assayed for activity.

Statistical analysis

All experiments were carried out in triplicates, and repeated three times. The samples collected from each replicate were tested for amylase production and activity. Means of amylase activity and production were calculated and significant differences were calculated by determining standard error.

RESULTS AND DISCUSSION

Screening of isolates

Primary screening was carried out by starch hydrolysis method. Among the isolates the fungal isolate *Aspergillus*

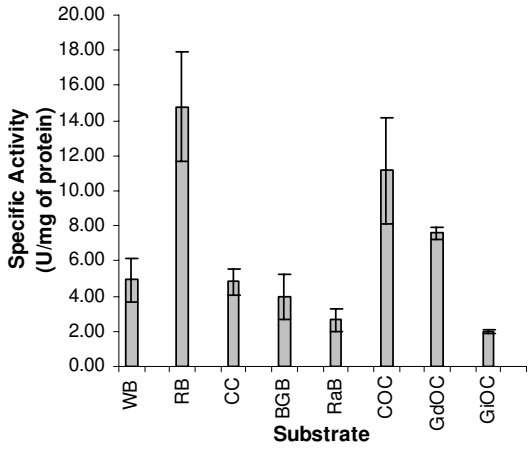

Figure 1. Comparison of *Aspergillus* sp. JGI 12 amylase activity of different substrates.

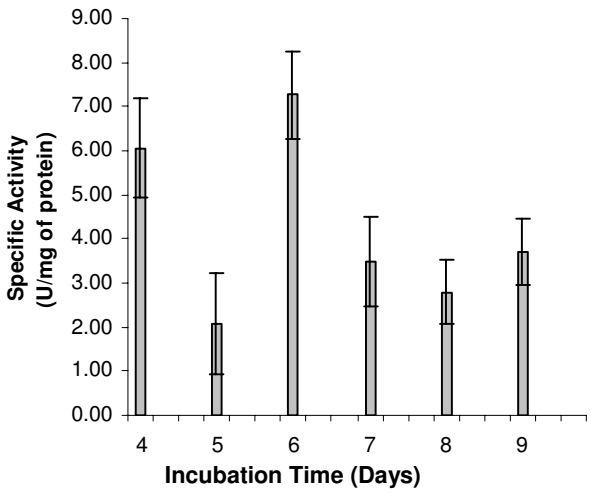

Figure 2. Effect of combination of substrates (RB, COC and GOC in the ratio of 2:1.5:1.5) on the production of *Aspergillus* sp. JGI 12 amylase.

sp. JGI 12 exhibited higher amylolytic activity in starch agar medium and was selected for further studies.

Comparison of substrates

Mixed substrate fermentation was carried out with 2.5 g of any one of the substrates with 2.5 g of wheat bran for the production of amylase at 25ºC using *Aspergillus* sp. JGI 12 for 6 days. Rice Bran and Gingelly Oil Cake gave the highest and lowest specific activity of glucoamylase - 16.42 and 2.03 U/mg, respectively (Figure 1).

Optimization of culture conditions

In the comparative studies, mixed substrate fermentation

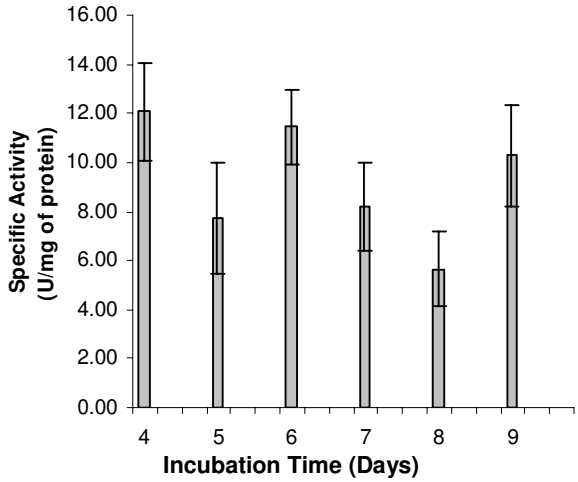

Figure 3. Effect of combination of substrates (WB, RB and GOC in the ratio of 1:2:2) on the production of *Aspergillus* sp. JGI 12 amylase.

was carried out using three substrates. Combination 1consisted of RB, COC and GOC in the ratio of 2:1.5:1.5 (Figure 2). Combination 2 consisted of WB, RB and GOC in the ratio of 1:2:2. Combination 2 showed significant increase in enzyme yield compared to combination 1 (Figure 3).

Effect of temperature, pH and supplementation with carbon source

Combination 2 was considered for further studies as it showed higher amylase activity. Maximum enzyme yield was observed at 30℃ (Figure 4) and pH 5.8 (Figure 5). Maximum production of amylase was achieved when glucose was the carbon supplement (Figure 6) Addition of glucose, sucrose or starch gives higher amylase yields than the unsupplemented substrate, thus highlighting the importance of supplementation for higher enzyme

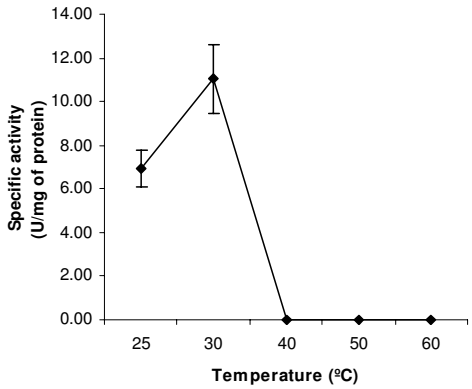

Figure 4. Effect of temperature on production of *Aspergillus* sp. JGI 12 amylase.

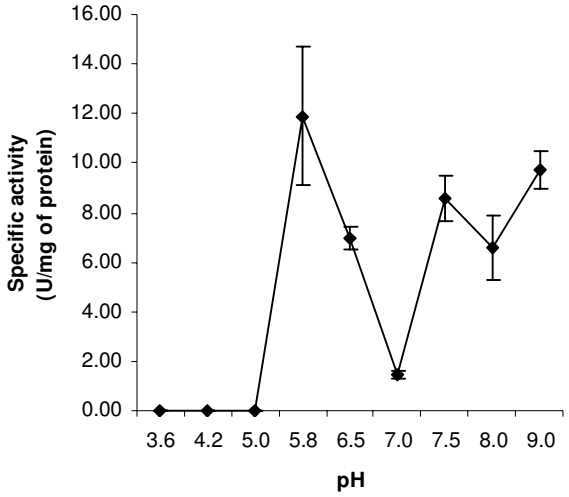

Figure 5. Effect of pH on production of *Aspergillus* sp. JGI 12 amylase

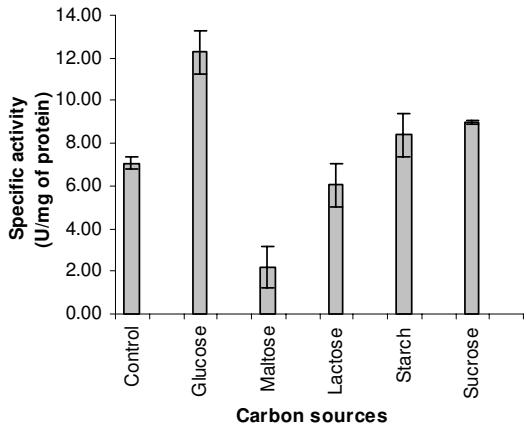

Figure 6. Effect of supplementation of carbon sources on *Aspergillus* sp. JGI 12 amylase production

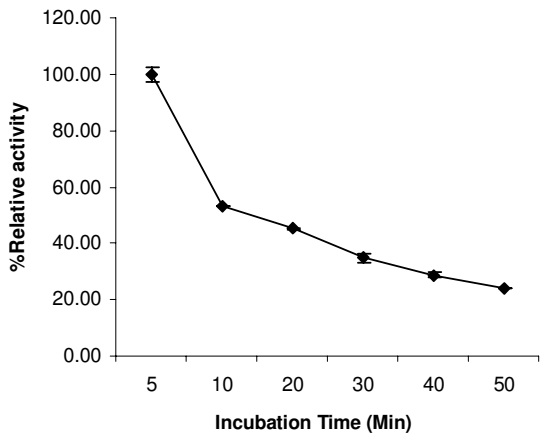

Figure 7. Effect of incubation time on *Aspergillus* sp. JGI 12 amylase activity.

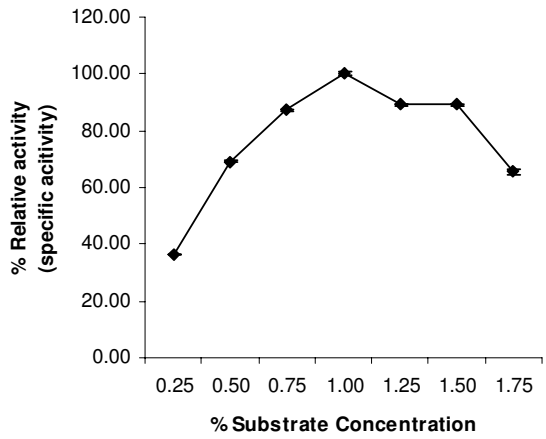

Figure 8. Effect of substrate concentration on *Aspergillus* sp. JGI 12 amylase activity.

production. Maltose and lactose marginally inhibited the enzyme production (Figure 6).

Enzyme-substrate reaction time

The culture filtrates of the fungal isolate exhibited highest amylase activity when the enzyme substrate reaction mixtures were incubated for 5 min. As time of incubation increased there was a gradual decrease in the enzyme activity (Figure 7).

Substrate concentration

The crude enzyme extract was allowed to react with different substrate concentrations (0.25 to 1.75%) and maximum activity was found with 1% starch as the substrate (Figure 8).

Temperature and pH

Temperature and pH are the most important factors, which markedly influence enzyme activity. Maximum amylase activity was recorded at 30ºC. Further increase in temperature resulted in decrease in the activity of amylase (Figure 9). The effect of pH on the enzyme activity indicates that the amylase is active in the pH range 5.8 - 9.0 with two peaks, one acidic (pH 5.8) and one basic (pH 9.0) (Figure 10). This suggests that the enzyme would be useful in processes that require wide range of pH change from slightly acidic to alkaline range and vice-versa. The multiple pH optima observed suggests the presence of at least two amylolytic activities in the crude amylase preparation; an alpha amylase and a glucoamylase (Yamasaki et al., 1977; Useda et al., 1981; Bergmann et al., 1988; Hayoshida et al., 1988; Abu et al., 2005). Some of these enzymes act synergistically in starch degradation (Useda et al., 1981).

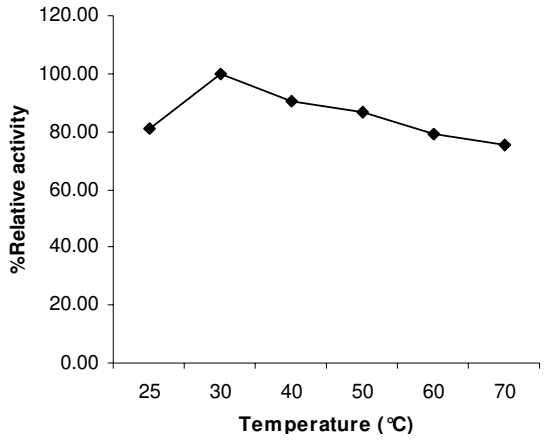

Figure 9. Effect of temperature on *Aspergillus* sp. JGI 12 amylase activity.

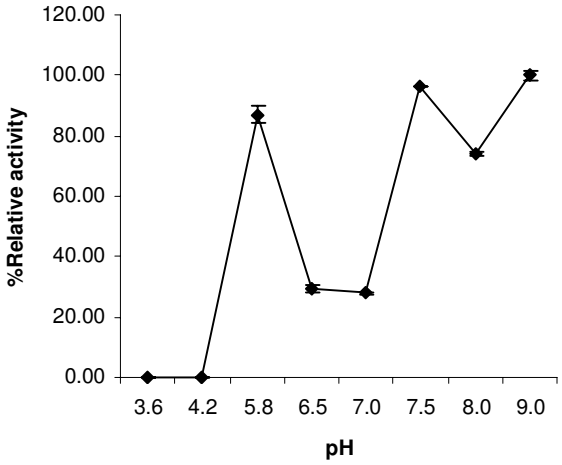

Figure 10. Effect of pH on *Aspergillus* sp. JGI 12 amylase activity.

Temperature stability

The temperature stability studies indicate a general decrease in the stability of the enzyme with time (0 - 50 min) at all temperatures (25 - 60ºC) as shown by decreased enzyme activity. The enzyme retained 60% of its activity even at 60ºC for 50 min, indicating its thermostability. It showed the highest stability at 25ºC and least stability at 60ºC (Figure 11). The high temperature inactivation may be due to incorrect conformation due to hydrolysis of the peptide chain, destruction of amino acid, or aggregation (Schokker and van Boekel, 1999).

Conclusion

The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is essential for the production of alpha and glucoamylases (McMahon et al., 1999). In this

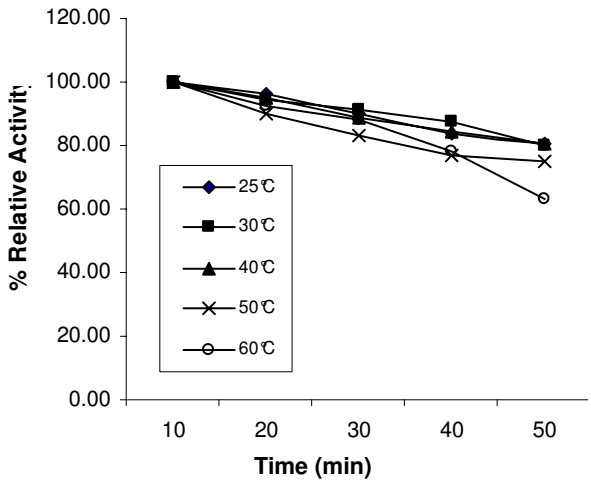

Figure 11. Effect of temperature on enzyme stability.

study, the effect of initial pH of solid substrate was studied (Figure 5). The solid substrates were maintained at different pH (3.6 - 9.0). Production of glucoamylase was the best at pH 5.8. Further increase in pH resulted in decrease of amylase production by the fungus. However, another peak in enzyme production was observed at pH 9.0. Two pH optima observed indicate the synthesis of at least two amylases, an alpha amylase and a glucoamylase (Yamasaki et al., 1977; Useda et al., 1981; 2005). This property makes the enzyme suitable for industrial production of paper and detergents.

It can be concluded that *Aspergillus* sp. JGI 12, can be industrially exploited for the synthesis of α-amylase and glucoamylase, and strain improvement studies can be carried out to enhance enzyme production.

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