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PARTIAL PURIFICATION AND CHARACTERIZATION OF AMYLASE FROM *ASPERGILLUS FLAVUS* USING POD BIOMASS OF *PITHECELLOBIUM DULCE* AS SUBSTRATE

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ABSTRACT

Nature represents a formidable pool of bioactive compounds and is more than ever a strategic source for new and successful commercial products. The potential of using microbes as biotechnological sources of industrially important enzymes has triggered renewed cynosure in the exploitation of microbial communities as bioreactors. Among these bioreactors, amylases have received great deal of attention. In these endeavours this study is conducted to search for new amylase with waste pod biomass of *Pithecellobium dulce* as substrate. A partial purification and characterization of the amylase from *Aspergillus flavus* was carried out in this study. The extracellular amylase extract was concentrated using ammonium sulfate precipitation and dialysis. The optimum conditions for the partially purified amylase were evaluated. The optimum pH and temperature were observed 6 and 60°C respectively. Furthermore, the analysis of kinetic showed that the enzyme has K_m of 1.43 mg/ml and V_{max} of 250 μmol/min/mg. The results indicate that the enzyme reflects their potentiality towards industrial applications.

INTRODUCTION

In recent, industrially important enzymes has triggered renewed cynosure in the exploitation of extracellular enzymatic activity in several microorganisms because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation (Chakrabortya *et al*., 2009) .The use of enzymes created opportunities for developing a green, sustainable and modern industrial chemistry due to excellent specificity, being economic, energy-saving process and simplicity .Commercial enzyme production has grown during the past century in volume and number of products, in response to expanding markets and increasing demand for novel biocatalysts. Out of which amylases constitute approximately 25% of enzyme market (Kumar *et al*., 2012).

The spectrum of amylase applications has expanded in several fields such as food, paper, textile clinical, medicinal and analytical chemistry (Kammoun *et al*., 2008). It is an important enzyme of starch processing industry to form simpler sugar constitutes by hydrolysis of polysaccharides by cleaving (1→ 4, 1→6) linkage of starch (Vardhini *et al*., 2013). Although amylases can be derived from several sources, such as plants, animals and microorganisms, the enzymes from microbial sources are preferred in industrial sector and a large number of them are available commercially (Naga Raju et el., 2013). Moreover, due to their diversity, fungi have been recognized as a source of new enzymes with useful and novel characteristics (Bakri *et al*, 2009). Species of *Aspergillus like Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus* are the major fungal species that are capable of producing industrially functional enzymes (Sahni and Goel 2015). In recent years the cost of carbon source for microbial enzymes production has necessitated a drive towards cheaper and sustainable sources (Ibrahim, 2012).

Thus the commercial success of amylases is linked to utilization of starchy biomass as an industrial raw material.The successful implementation of enzymes as industrial biocatalysts requires the availability of suitable enzymes with high activity, specificity and stability.Starch is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato (Agrawal *et al*., 2005). But these major energy-providing material in human diets (Kolawolea., 2011). Therefore, the use of waste pod biomass of *pithecellobium dulce* as substrate not only provide as an alternative but also viable option to value addition to the agriculture commodities which are produced in huge quantities annually and have no value in the market.

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MATERIALS AND METHODS

Source and Identification of Fungi

The fungus was isolated from soil samples by serial dilution method. The soil samples were collected from different sites of Haryana. The qualitative and quantitative screening were conducted to know the best amylolytic fungal strain. The primary identification of potent amylase producing fungal isolate was done on the basis of morphological characteristics.

Substrate

The pod biomass of *Pithecellobium dulce* was collected from Rohtak city which is located at a latitude of 30°1`N and longitude of 75°17`E and its nearby places. The pods biomass washed with tap water and then oven-dried at 70°C till constant weight. Then the biomass grinded and sieved to obtain mean particle size (0.2 to 1.0 mm) .It stored in sealed plastic bags at room temperature for carrying out further experiments of amylase production in solid state fermentation

Culture Conditions and Preparation of Inoculum

The isolate was cultured and maintained on Potato Dextrose agar plates. The fungus Sterilized normal saline (20-40 mL) containing Tween-80 (0.1% v/v) was added to five days old slants of fungal culture grown on PDA medium. The spores were scratched by sterile wire loop and shaken vigorously for preparing a homogenous spore suspension. A standard spore count was done using a hemocytometer. One milliliter of spore suspension containing $1x10^7$ spores was used as inoculum for solid state fermentation.

Enzyme Extraction

Erlenmeyer flasks of 500 ml containing Mendel and Reese media (Mandel & Reese, 1957) with 10g pod biomass of *Pithecellobium dulce* (1:1 v/w) were autoclaved at 121°C for 20 minutes and, after cooling, inoculated with 1.0 ml of spore suspension containing $5x10^7$ spores. All the contents of flasks were mixed well and incubated at 30°C under static conditions. After 72 hours of incubation, the contents of the flasks were extracted using 1:20 ratio of substrate to phosphate buffer (0.05M, pH 6.5). The flasks were kept in an incubator shaker for one hour at 120 rpm and 30°C, after which the entire slurry was squeezed through muslin cloth. The enzyme extract was centrifuged at 10,000 rpm for 15 minutes at 4°C to remove spores and debris. The clear supernatant was used as crude amylase.

Partial purification by Ammonium sulphate fractionation

The crude broth obtained after fermentation was centrifuged at 5000 RPM for 15 min to remove the cell biomass. The crude amylase supernatant was treated with ammonium sulphate (analytical grade) at different saturation ratio (20 to 80 %) to reach the optimum ratio of ammonium sulphate by adding gradually the amount to each 20 ml of the crude amylase enzyme in ice bath. Then stirred for 60 min and precipitation was allowed at 4°C for overnight. Solution was centrifugation at 10,000 rpm for 15 minutes at 4°C and amylase pellets collected. Chilled 0.05 M phosphate buffer (pH 6.5), was added to dissolve pre- chilled amylase pellet at a ratio of 1:0.5 followed by centrifugation at 10,000 rpm for 15 minutes at 4°C. The supernatant was recovered and pellets were then resuspended in buffer followed by centrifugation under same conditions. These steps were continued 2 - 3 times to obtain amylase as supernatant.

The ammonium sulphate was used in enzyme precipitation because it high soluble and cheap compared with the other salts, unaffected in pH and enzyme stability. The concentration by ammonium sulphate depending on equilibrate the charges found in protein surface and disrupt of the water layer surrounding it, that leads to precipitate it.

Dialysis against buffer

The obtained ammonium sulphate precipitate (in solution) was introduced into dialysing membrane against phosphate buffer (pH 6.5, 0.05 M) with frequent changes of buffer till no more ammonium sulphate could be detected. The undissolved dialysate was removed by centrifugation (10,000 rpm, 4 °C, 20 min).The supernatant was collected and assayed for amylase activity for further characterization.

Amylase assay

The dialysed sampleswas assayed for amylase activity. The enzyme sample was incubated with soluble starch at desired temperature and pH. After 10 mintues, the liberated reducing sugars was estimated by using 3,5- dinitrosalicyclic acid (DNS) method (Miller, 1959). The color developed was read by measuring its optical density using a spectrophotometer at 540 nm. One unit of enzymatic activity (IU) was defined as the amount of enzyme releasing 1 µmol of sugar in 1 minute under standard assay conditions.

Protein Content Estimation

The protein content was estimation by standard Lowry method (Lowry, 1951). Different dilutions from 0.2 mg/ml to 0.8 mg/ml of Bovine Serum Albumin (BSA) solutions were prepared by mixing of stock BSA solution (1 mg/ ml) and distilled water in test tubes to final volume of 5ml. 0.2 ml protein solutions from each were taken in fresh test tubes and 2 ml of alkaline copper sulphate reagent was added to each. The solutions were mixed well and incubated at room temperature for 10 min. 0.2 ml of Folin Ciocalteau solution was then added to each tube and incubated for 30 min in dark place. The absorbance of each mixture was then measured using spectrophotometer at 600 nm. The absorbance was plotted against protein concentration to get a standard calibration curve. The concentration of the unknown samples was determined from the plotted standard curve.

Characterization of Partially Purified amylase

Determination of optimum pH for amylase activity

The optimal pH of partially purified amylase was determined by conducting the amylase assays at different pH using citrate buffer (0.05 M) in the pH range of 3.5-5.5 and phosphate buffer (0.05 M) in the pH range from 5.5-8. Optimum pH was defined as the pH at which maximal enzyme activity was obtained in the assay.

Determination of optimum temperature for amylase activity

The optimum reaction temperatures of partially amylase was

determined at the optimum pH using standard assay conditions at different temperatures ranging from 20 °C to 100°C. Optimal temperature was defined as the temperature at which maximum enzyme activity was obtained.

Determination of temperature stability of amylase

The partial purified amylase was incubated in tubes in different temperatures (30, 40, 50, 60, 70) °C and were taken from each test tube after every 20 minutes interval of times (20, 40, 60, 80,100, 120 minutes) to check amylase stability. Amylase assay were conducted to calculate the amylase activity.

Zymogram analysis

Native polyacrylamide gel electrophoresis (PAGE) was performed on a 10% polyacrylamide gel by the method of Laemmli (1970). 0.5 % Starch was incorporated into 10% polyacrylamide gel prior to polymerization as substrate for the preparation of zymogram. After electrophoresis, the gel containing 0.5 % starch, incubated at 50^{o}C for 30 minutes in 0.05 M citrate buffer (pH 4.8). Then, the gel containing starch, stained with iodine solution (0.2%) for 30 minutes and the excess stain was removed by placing the gel in 1.0 M NaCl solution.

RESULTS AND DISCUSSION

Table 1 Summary of Purification

Purification Step	Amylase activity (Units)	Total protein (mg)	Specific Activity (U/ml)	Fold Purification	Yield (%)
Crude Extract	1680.34	3.38	497.14	1	100
Ammonium Sulphate precipitation	1370.54	0.61	2245.90	4.52	81.56
Dialysis	1189.23	0.34	3497.05	7.034	70.77

Detection of Amylase activity by zymogram staining

The zymogram of amylase was prepared by incorporating 0.5% starch into 10% native PAGE as shown in Figure 1. The zymogram staining of partially purified amylase revealed one clear and distinct band named as G1, indicating the presence of single forms of amylase. The light zone against the dark background indicated the amylase activity.

Fig 1 Zymogram staining on native PAGE of partially purified amylase

Determination of Vmax and Kmax

The partially purified amylase enzyme was incubated with various concentrations (0.1–1.0mg/ml) of pod biomass starch to determine the kinetic parameters (Roy *et al*., 2014). The Michaelis-Menten constant (Km) and maximum velocity (Vmax) values were determined from Lineweaver-Burk plots. The Km and Vmax values were calculated from the kinetic data. The enzyme has Km of 1.43mg/ml and Vmaxof 250 μmol/min/mg for starch (Figure 3).

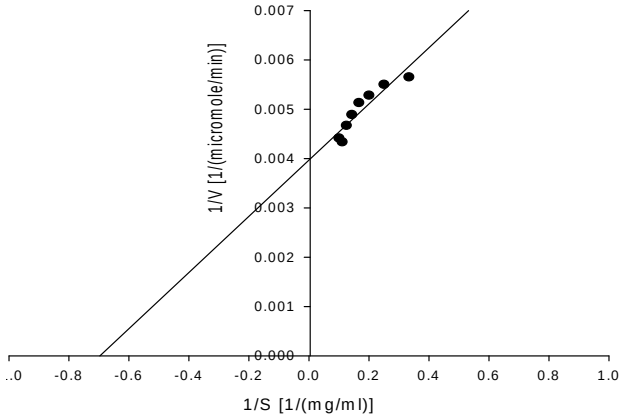

Fig 2 Lineweaver-Burk plot for amylase enzyme

The results are coincide the fact that, Km of fungal and yeast amylases have been reported to be between 0.13 - 5 mg/ml (Pandey *et al*., 2000, Gupta *et al*., 2003). The Km value calculated in the present study is lower than that reported for the GA from *A. flavus* (2.85 mg/ml) (El-Abyad *et al*., 1994) as well as for *Aspergillus awamori*, Km was calculated as 9.79 mg/ml (Negi and Banerjee, 2009). Since lower Km values allow for faster and easier industrial processes, so it can be concluded that its industrial potential is high.

Determination of optimum pH for amylase activity

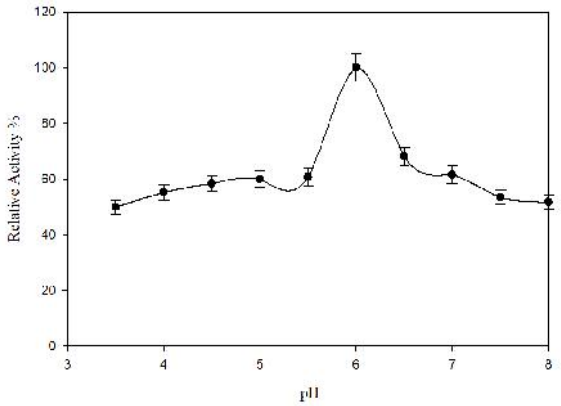

Fig 3 Characterization of partially purified amylase for optimtm pH

The maximum amylase activity was measured at pH 6.0 (Figure 3). 60% of maximum activity was determined between pH 3.5 - 5.5 and at pH values ranging between 6.5 and 7.5. Amylase activity started to decrease at pH values over pH 6.0. This data shows that the enzyme is active in a slightly acidic range of pH values 6. Similarly, Norouzian *et al*., (2006) also reported that high activity is associated with acidic pH values. While, Mohammed *et al*. (2014) concluded that the purified α-amylase showed the optimum pH for glucoamylase (B)

stability ranging between (6 - 6.5) and the stability was decreased in extreme acidic and alkaline pH. Oyewale, (2012) also concluded that the purified α-amylase showed maximum activity was observed at 7.0. Gomes *et al*., (2005) reported that *Aspergillus flavus* glucoamylase are stable at pH values between 4.0 and 9.0. Whereas, the optimal pH values for other *Aspergillus* GAs (Glucoamylase) were found to be between 4.5 and 7.0. Moreover, α -Amylase and glucoamylase enzymes are used in starch liquefaction and saccharification and the process is carried out at pH 4.2 - 6.2 (Moreira *et al*., 2004). Hence obtained amylase can be used for the same for industrial applications.

Determination of temperature for amylase activity

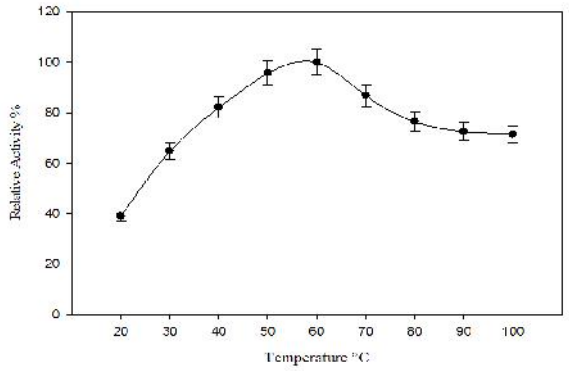

Fig 4 Characterization of partially purified amylase for optimum temperature

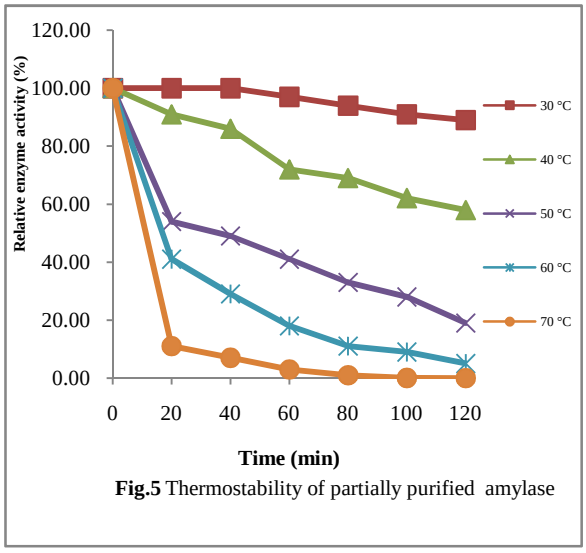

Fig.5 Thermostability of partially purified amylase

Amylase Activity increased from 20 - 60°C and reached a maximum at 60°C (Figure.4) Almost 95% of maximum activity was retained at 50°C, suggesting a high thermal stability. The GA activity decreased rapidly above 60°C. Amylase shows stability from 30 - 60°C till 20-60 minutes (Figure. 5. Most raw starch-digesting glucoamylases are known to exhibit optimum temperatures between 50 and 70°C and are remarkably stable at high temperatures (Norouzian *et al*., 2006). Similarly, Chakraborty *et al*, (2000) found that the maximum activity of a thermostable purified α-amylase was observed at 50ºC. Moreover, Odibo and Ulbrich-Hofmann, (2001) concluded that the optimum temperature for the enzymes were 60 ºC for α-amylase and 70ºC for glucoamylase, respectively. These result are parallel to report of silva *et al*., (2013) that optima of pH and temperature were 6.0 and 65 °C for *Aspergillus niveus*. Similarly, Adeniran and Abiose (2011) also conclude that optimal thermal activity of the amyloglucosidase between 50 and 60°C for *Aspergillus niger*. The result of present study indicated that the obtained amylase could be used at any temperature between 40 and 65°C in biotechnological applications.

CONCLUSION

In the present investigation, we have partial purified and characterized a novel amylase from soil isolated fungal strain of *Aspergillus niger*. The obtained amylase reveals maximum activity at higher temperature (60°C) with stability at longer time. The ability of the amylase to digest waste pod biomass of *pithecellobium dulce* as sustrate, and high stability and activity in a high range of temperature suggest that the enzyme is applicable in digestion of raw starches in food industries, and it may be useful in industrial process carrying out higher temperature. Moreover, the lower Km values allow for faster and easier industrial processes, so it can be concluded that its industrial potential is high.

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