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Isolation and characterization of β-amylase from *Penicillium nigricans*

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ABSTRACT

A β-amylase-producing fungus was isolated from forest soil by culture plate method with starch as the sole source of carbon and identified as *Penicillium nigricans.* The failure to develop a clear zone around the fungal colony after staining with iodine *in situ* indicated the absence of α-amylase in the isolate. The β-amylolytic nature was further confirmed by the presence of maltose as the major end product of starch breakdown following thin layer chromatography. The isolate showed maximum enzyme activity on the 23rd day of cultivation. The partly purified enzyme showed highest activity at 60° and pH 5.0. About 70% of the enzyme activity was retained within the range pH 3.5-7.0. The calcium ion had an enhancing effect whereas Na^+, Mn^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+} and Ag^+ ions moderately inhibited the β-amylase activity. The β-mercaptoethanol had an inhibitory effect on the enzyme. The enzyme was completely inhibited by ethylenediaminetetraacetic acid (EDTA) suggesting the isolated enzyme to be a metalloenzyme. The enzyme showed highest activity towards starch followed by amylose and did not show any cross specificity towards cellulose. Absence of substrate cross specificity and ability to hydrolyse waste starches made the enzyme candidate for industrial applications.

KEYWORDS

Isolation;
β-amylase;
Penicillium nigrican;
Metalloenzyme;
Waste starches.

INTRODUCTION

Enzymes are among the most important products obtained for human need through microbial sources. A large number of industrial processes in industrial, environmental and food biotechnologies utilize enzymes at some stage or the other. The utilization of starch for industrial applications has increased considerably in the last decades since new starch derived products, and the genetic engineering of microbes that produce them are all of importance to food, chemical and pharmaceutical industries. This has intensified the need to search for novel microorganisms and enzymes with commercial potential[1].

β-Amylase (EC 3.2.1.2) is an important starch hydrolyzing enzyme for food plant sources. It reported

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from various bacteria, actinomycetes and fungi. The commercial production of β-amylase and its ability to biodegrade waste starches could be effectively exploited for maltose production at a normal cost with simultaneous utilization of starchy wastes, which would otherwise contribute to environmental pollution[2,3].

Although an important work has been reported using various fungal strains, there is still a need of extensive and continuous isolation for new β-amylase-producing fungi with better properties and suitable for commercial exploitation. The present study deals with the isolation of a β-amylolytic fungus, the characterization of the partially purified enzyme and simultaneous evaluation of its industrial applicability.

MATERIALS AND METHODS

Isolation and identification the β-amylase producing fungus

A fungal strain was isolated from the forest soil sample by serial dilution and by plating on Czapek agar plates containing starch as a sole carbon source. The Czapek agar medium contained (g/L) starch (30.0), $NaNO_2$ (3.0), K_2HPO_4 (1.0), $MgSO_4.7H_2O$ (0.5), KCl (0.5), $FeSO_4.7H_2O$ (0.01) and agar (15), pH 7.0. The culture was purified through subculturing on Czapek solid medium. The colonies were finally transferred to potato dextrose agar (PDA) slants and maintained at 4°C. The isolate was identified on the basis of morphological and microscopic features[4]. The isolate was further identified by authentic authority (Agarkar Research Institute, Pune).

Growth and time course study for optimal β-amylase production

The fungus was grown on PDA slants for 120 h at 37 ºC. A loopful of *P. nigricans* culture was used to inoculate 100 ml Czapek medium (without agar) in 250-ml Erlenmeyer flasks. The incubation was carried out at 37 ºC for 25 days. The enzyme activity of the culture broth was recorded every day at regular intervals. Growth was measured by weighing the dried mycelial mat (at 60 ºC for 80 min) on dried pre-weighed filter paper (Whatman No.1).

Enzyme extraction and assay

The culture broth was filtered through preweighed filter paper and the filtrate was used as crude β-amylase. For further characterization, the crude enzyme was centrifuged using cooling centrifuge (REMI) at 10,000 x g for 10 min (at 4 °C). The clear supernatant was partially purified by 60% ammonium sulfate precipitation followed by dialysis against 0.016M acetate buffer (pH 4.8). The assay mixture (0.5 ml) containing an equal volume of properly diluted enzyme and 1% (w/v) starch in 0.016M acetate buffer (pH 4.8) was incubated at 60 °C for 30 min[5]. The reducing sugar produced was measured colorimetrically at 540 nm[6]. Controls were prepared with heat-inactivated enzyme using maltose as standard. One unit of the enzyme activity was defined as the amount of enzyme that catalyses the liberation of reducing sugar equivalent to 1 µmole of maltose per min per ml under the assay conditions.

Chromatography of enzyme hydrolyzed products

Chromatography of β-amylase hydrolyzed products was studied by thin-layer chromatography (TLC)[7]. Enzyme (0.5 ml) was incubated overnight with 0.5 ml of 1% starch dissolved in 0.2 M acetate buffer (pH 5.0). Sample was spotted on the chromatographic plate. 1% glucose, starch, and maltose standards were also applied. Carbohydrates were detected by staining with aniline-diphenylamine phosphoric acid reagent.

Characterization of partially purified β-amylase

The effect of pH on enzyme activity was studied over a range of pH 3.5 to 7, using buffers of 0.2M strength each: citric acid buffer (pH 2.0-3.5), acetate buffer (pH 4.0-5.5) and phosphate buffer (pH 6.0-7.5). The effect of temperature was determined by performing the standard assay procedure at optimum pH with a temperature range of 0 °C to 90 °C. The effect of various additives was investigated by treating enzyme with β-mercaptoethanol, EDTA and various cations at 1mM dissolved in 0.016M acetate buffer (pH 5) at 60 °C for 30 min. The affinity of the enzyme towards various substrates (starch, amylose, cellulose, maize, rice, rice husk, wheat and wheat bran) was tested by treating each substrate with properly diluted enzyme at 60 °C for 30 min. The reducing sugar produced was measured as described earlier.

RESULTS AND DISCUSSION

Isolation and β-amylase production by *Penicillium nigricans*

A positive β-amylase producing fungus was isolated from a forest soil by culture plate technique with starch as the sole source of carbon. The isolate was identified as *Penicillium nigricans* based on morphological and microscopic characteristics and further confirmed by National Fungal Culture collection of India, Agarkar Research Institute (Pune). The extracellular β-amylase production was assessed in Czapek media using starch as the substrate. The failure to develop a clear zone around the fungal colony after staining with iodine *in situ* indicates the absence of α-amylase in the strain[8]. The culture showed maximum activity on 23rd day as compared to maximum activity by *Malbranchea sulfurea* strain on 9th day[9], *Emericella nidulans* strain on 6th day[10] and by *Syncephalastrum racemosum* on 4th day[3].

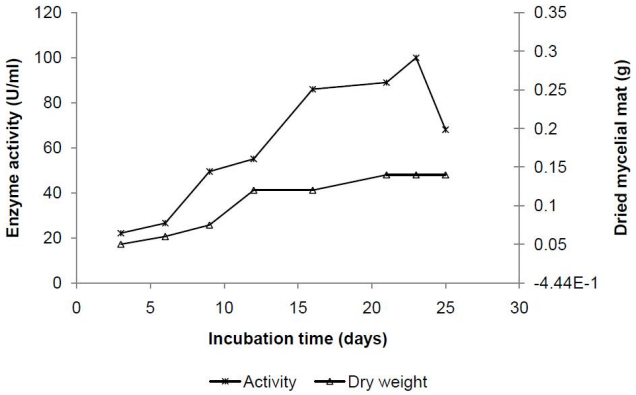

Figure 1 : Kinetics of growth with subsequent β-amylase production in *Penicillium nigricans*.

Chromatography of enzyme hydrolyzed products

The β-amylolytic nature was confirmed by the presence of maltose as the major end product of starch breakdown following thin layer chromatography. This result also ruled out the possibility of presence of other amylolytic enzymes like α-glucosidase, glycoamylase and amyloglucosidase where glucose should be the major end product[11].

β-amylase characterization

The partially purified β-amylase showed maximum enzyme activity at pH 5.0 (Figure 2) and 60 °C (Figure 3). β-amylase from *P. nigricans* is thus a thermostable enzyme. About 70% of the enzyme activity could be retained within the range at pH 3.5-7.0. This optimal activity and stability in the acidic range and high temperature makes the enzyme suitable for industrial use. Likewise, maximum -amylase activity at 60 °C at pH 5.0 with *Syncephalastrum racemosu was recorded*[3].

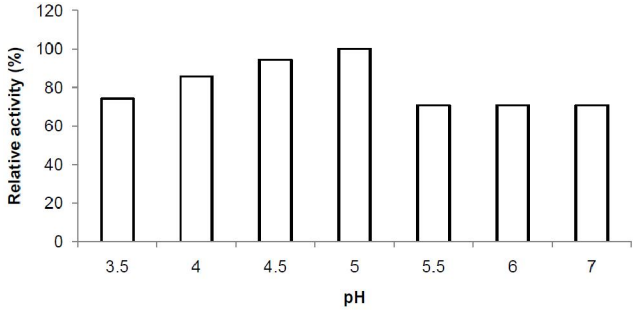

Figure 2 : Effect of pH on -amylase activity.

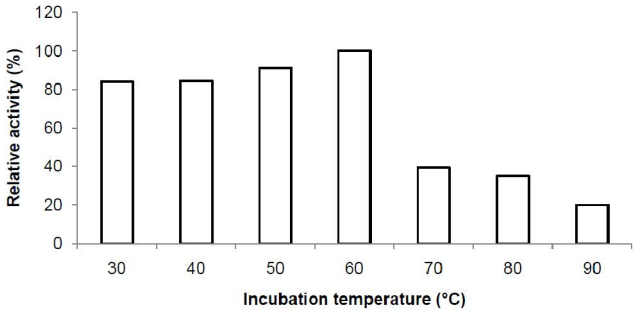

Figure 3 : Effect of temperature on β-amylase activity.

The effects of metal ions and other additives tested (Figure 4) showed that among the metal ions tested, calcium ion had an enhancing effect. Similarly, Ray and Chakraverthy[3] reported stimulation of β-amylase by Ca^{2+} from *Syncephalastrum racemosum*. Ca^{2+} might thus be required for stabilization and maintenance of the enzyme conformation. The β-amylase activity was moderately inhibited by Na^{+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+} and Ag^{+}. The β-mercaptoethanol had an inhibitory effect on the enzyme, indicating that cysteine could be playing a major role in enzyme folding pattern and its importance in the active site. The enzyme was completely inhibited by a metal chelating agent (EDTA) suggesting the isolated enzyme to be a metalloenzyme. The β-amylase isolated from a halophilic soil isolate (*Halobacillus* sp. LY9) was also a metalloenzyme[12].

Influence of β-amylase towards various substrates was investigated (Figure 5). The enzyme showed highest activity towards starch followed by amylose. This

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behavior was also observed with amylases of *Aspergillus terreus* NA-170 mutant[13]. The isolated enzyme did not show any cross specificity towards cellulose. The partially purified enzyme digested the indigenous starches, of which wheat starches showed the best results followed by rice starch (Figure 5). This differential rate of digestion could be attributed to the existence of different crystalline granules in starch, which in turn might depend upon the botanical source[1]. The ability of the enzyme to biodegrade indigenous starches and the absence of other contaminating amylolytic activities made it candidate for use in saccharification industries to produce a specific sugar. This will be facilitated by its wide pH tolerance range and higher temperature preferences.

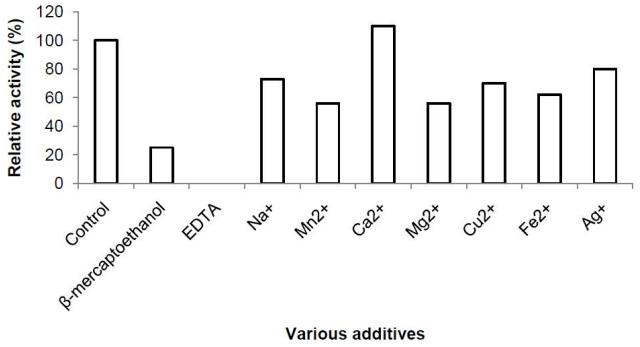

Figure 4 : Effect of various additives on β-amylase activity from *Penicillium nigricans*.

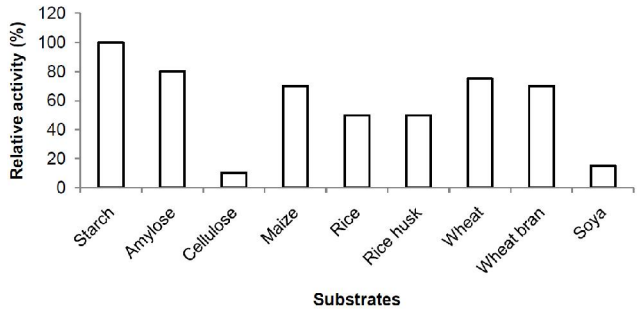

Figure 5 : β-amylase activity of *Penicillium nigricans* towards various substrates (1%, w/v).

CONCLUSION

As an extensive search continues to find a promising β-amylolytic fungal strain, the isolated high β-amylase-producing *Penicillium nigricans* can be successfully used for commercial overproduction of enzyme. In addition, its ability to biodegrade waste starches can be effectively exploited for maltose production at a low cost, with simultaneous utilization of starch wastes, and this will reduce environmental pollution.

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