CHARACTERIZATION OF THERMOPHILIC AMYLASE FROM AN OBLIGATE THERMOPHILE, THERMOACTINOMYCES VULGARIS

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Abstract: Amylase finds a wide range of applications in starch industries, i.e., baking, brewing, distillery. The wild-type (1227) and mutant strains (1261 and 1286) of Thermoactinomyces vulgaris were screened for the production of amylase using 1% soluble starch. The maximum production of amylase was observed after 12 h of incubation at 50°C in wild-type strain 1227 of T. vulgaris. The amylase was found to be thermostable, exhibiting its optimal activity at 75°C and at pH 6.0 in this obligate thermophile; and it preferred soluble starch as its substrate. Among the metal ions tested, Mn²⁺ was most stimulatory, while Hg²⁺ was most inhibitory to the activity of amylase. Thus, T. vulgaris amylase is a thermophilic metalloenzyme, requiring Mn²⁺ for its high-temperature catalysis, which can be exploited for amylase-based industries of diverse interests.

Keywords: Amylase, Metalloenzyme, Thermoactinomyces vulgaris, Thermophilic amylase

INTRODUCTION

Amylases are enzymes which hydrolyze starch molecules to give diverse products, including dextrans and progressively smaller polymers composed of glucose units (Windish and Mhatre, 1965). Amylases constitute a class of industrial enzymes, which alone form approximately 25% of the enzyme market (Burhan et al., 2003; Rao et al., 1998; Sidhu et al., 1997). This plays a vital role in many industrial processes such as sugar, textile, paper, brewing, and distilling industries. It is also used in food and pharmaceutical industries as a digestive aid (Sivaramakrishnan et al., 2006).

The enzymes from thermophilic microbes have been proved to be more useful in biotechnological applications. Thermophiles represent an obvious source of thermostable enzymes, being reasonable to assume that such character will confer their proteins a high degree of thermal stability. The Thermoactinomyces growing at high temperature is thus a good source of industrially important enzymes.

MATERIAL AND METHODS

Strains Used and Culture Conditions

A wild-type strain (Stock no. 1227) and two mutant strains (Stock nos. 1261, and 1286) of T. vulgaris, which were kindly supplied by Professor D.A. Hopwood, John Innes Centre, Norwich, U.K., were used for screening the production of extracellular amylase. The auxotrophic strains 1261 (nicotinamide thiamine) was streptomycin resistant. The other auxotrophic strains 1286 (thiamine) was streptomycin sensitive. The media and culture conditions, as described by Hopwood and Wright (1972) were used with certain modifications (Singh, 1980; Sinha and Singh 1980).

Screening of T. vulgaris Strains for the Production of Extracellular Hydrolytic Enzymes

Amylase activity was determined, using soluble starch (1%) as a substrate in Hopwood’s medium (Hopwood and Wright, 1972). Sterile modified Hopwood’s agar medium was poured on a sterile Petri-plate and allowed to solidify at room temperature. It was then inoculated with the wild-type (1227) and auxotrophic mutant strains of T. vulgaris, using 0.2 mm cork-borer and incubated for 24-48 h at 50°C. The plates were then stained with Lugol’s Iodine (2 g KI and 1 g I₂ crystal dissolved in 300 ml distilled water, filtered and stored in brown bottle). The formation of a halo zone surrounding the colony under blue black background was considered positive for amylase activity.

Culture Condition for Amylase Production

The Hopwood medium, supplemented with 3% starch as sole carbon source was used as a production medium. The 30 ml of sterilized liquid medium was inoculated with 1ml of spore suspension containing 10⁶ spores/ml (having O.D. of 0.65 at 600 nm). The contents were mixed thoroughly and incubated at 50°C. Mycelium was filtered out with Whatmann no. 1 filter paper, and the filtrate was used for assaying the amylolytic activity of T. vulgaris. In order to investigate the optimum conditions for maximum production of amylase, samples were taken at regular time intervals (i.e., after 4, 8, 12, 16, 20 and 24 h) and assayed at its growth temperature (50°C) for amylase production.

Assay for Amylase Activity

The amylase activity was assayed by the method of Bernfeld (1955) by estimating the reducing sugar (maltose) produced during starch hydrolysis using 3.5 dinitrosalicylic acid (DNS) as a coupling reagent. The reaction mixture consisted of 0.5 ml of 1% (w/v) soluble starch in 20 mM sodium acetate buffer (pH
5.0-6.0) and 0.5 ml of appropriately diluted enzyme solution was incubated at 50°C for 15 min. The reaction was stopped by adding 1 ml of 3.5 dinitrosalicylic acid (DNS) solution, followed by heating in a boiling water bath for 5 min. The contents were then cooled to room temperature, and after the addition of 10 ml distilled water, the absorbance was measured at 540 nm with UV-Visible Spectrophotometer (UV-1700 Pharma Spec, Shimadzu). The activity of 1 unit (U) of amylase was defined as that amount of enzyme liberating 1 mM of reducing sugar (with maltose as standard) per minute under standard assay conditions. Specific activity was expressed as enzyme units per mg of protein (U/mg protein). Protein concentration in enzyme solution was determined by the method of Bradford (1976).

Effect of Temperature on Amylase Activity
The optimum temperature, needed for maximal amylase activity, was determined by incubating the reaction mixture for 15 min at varying temperatures, starting from 50°C to 95°C.

Effect of pH on Amylase Activity
The effect of pH on the activity of the amylase was studied by performing the enzyme assay over a range of pH from 4.0 to 11.0, using 20 mM sodium acetate buffer (pH 4.0-6.0), 20 mM potassium phosphate buffer (6.5-8.0) and 20 mM glycine-NaOH buffer (8.5-11.0).

Substrate Specificity
The substrate specificity of the enzyme was checked by using different substrates such as soluble starch, corn starch and potato starch (at a 1% concentration each) separately in the reaction mixture and the residual activity was detected for each substrate under standard assay conditions.

Effect of Metal Ions on Amylase Activity
The effect of different metal ions on amylase activity was determined by the addition of metal ions in the form of their respective chlorides at a final concentration of 1 and 5 mM to the reaction mixture, and the assay was performed under standard conditions of temperature and pH. The tested ions included chloride salts of the following – Li⁺, Na⁺, K⁺, Mg²⁺, Ca²⁺, Ba²⁺, Cr³⁺, Mn²⁺, Fe³⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺ and Hg²⁺. The activity assayed in the absence of metal ions was taken as control.

Effect of Metal Chelating Compound (EDTA) on Amylase Activity
The effect of different concentrations of a metal chelating agent, ethylene-diaminetetraacetic acid (EDTA) on the specific activity of T. vulgaris amylase was studied. The enzyme assays were done under standard conditions without and with 5 mM of the stimulatory divalent cation (Mn²⁺).

RESULT

Amylase Activity Test
Screening for amylase producing ability of T. vulgaris strains was done by growing them in the medium, containing soluble starch (1%) as a substrate, based on the zone of hydrolysis. The presence of a clear halo zone around the colony of T. vulgaris after staining the starch with I₂-KI indicated the presence of amylase activity in the wild-type (1227) and mutant strains (1261 and 1286) after 48 h of incubation at 50°C (Fig 1).

Cultivation Conditions for Amylase Production in T. vulgaris
In order to investigate the optimal conditions for maximum production of amylase, samples from the growing cultures were taken at regular time intervals (i.e., after 4, 8, 12, 16, 20 and 24 h) and assayed for amylase production at their growth temperature (50°C). The maximum activity of amylase was observed after 12 h of growth in the wild-type strain 1227.

Fig 1. Screening of Thermactinomyces vulgaris wild-type strain 1227 (A) and its auxotrophic mutant strains 1261 (B) and 1286 (C) for the production amylase after 48 h of incubation at 50°C.
The amylase activity increased with the increase in the time interval till 12 h, exhibiting 10.678 U/mg protein in wild-type strain 1227 (Fig. 2). Thus, the maximum production of amylase was observed after 12 h of incubation at 50°C. Hence, production medium after 12 h was used as enzymatic source for further characterization of amylase from wild-type strain 1227 of *T. vulgaris*.

**Effect of Temperature on Amylase Activity**

The optimum temperature for amylase activity was determined by performing the assays at varying temperatures, starting from 50°C to 95°C. The optimum temperature of amylase of wild-type strain (1227) was found to be 75°C with a specific activity of 17.626 U/mg protein (Fig. 3).

**Effect of pH on Amylase Activity**

A pH range between 4.0 and 11.0 was used to study the effect of pH on the amylase activity. It was observed that the specific activity was found to be maximum at pH 6.0 showing 17.735 U/mg protein activity for wild-type strain 1227. This observation indicated that the pH optima of amylase was same (i.e. pH 6.0) in the wild-type strain 1227 of *T. vulgaris* (Fig. 4).
Substrate Specificity of Amylase

The substrate specificity of amylase was checked by using different substrates such as soluble starch, corn starch and potato starch at 1% concentration each in the reaction mixture, and the specific activity was determined under standard conditions. The specific activity of amylase was found to be maximum (17.474 U/mg protein) in wild-type strain (1227), when soluble starch was used as substrate (Fig. 5). Considering this, further characterization of enzyme was done using soluble starch as the substrate for enzyme assays.

Effect of Metal Ions on Amylase Activity

For studying the effects of metal ions on amylase activity, enzyme assays were done in the absence (control) as well as in the presence (1 mM and 5 mM) of different metal ions in the form of their respective chlorides.

In the wild-type strain (1227), Mn\textsuperscript{2+} was found to enhance the amylase activity by 18% with specific activity of 20.219 U/mg protein at 1 mM concentration and by 214% with specific activity of 53.709 U/mg protein at 5 mM concentration of Mn\textsuperscript{2+} with respect to control (17.128 U/mg protein). Co\textsuperscript{2+} also increased the activity of amylase by 14% with specific activity of 19.469 U/mg protein at 1 mM concentration and by 18% with specific activity of 20.143 U/mg protein at 5 mM concentration of Co\textsuperscript{2+}. The addition of Hg\textsuperscript{2+} decreased the activity of amylase by 15% with specific activity of 14.954 U/mg protein at 1 mM concentration and by 79% with specific activity of 3.512 U/mg protein at 5 mM concentration of this divalent cation with respect to control (17.128 U/mg protein). Also, at 5 mM concentration, Cr\textsuperscript{3+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+} inhibited the activity of amylase by 11% (15.208 U/mg protein), 40% (10.341 U/mg protein) and 18% (14.097 U/mg protein), respectively. Mn\textsuperscript{2+} (5 mM) was most stimulatory and Hg\textsuperscript{2+} was most inhibitory to the amylase activity of wild type strain of T. vulgaris (Fig. 6).
**Effect of Metal Chelating Compound (EDTA) on Amylase Activity**

In order to check the metal ion specificity of amylase, the effect of different concentrations of a metal chelating agent, ethylene-diaminetetraacetic acid (EDTA) on the specific activity of amylase of *T. vulgaris* was investigated in the absence and in the presence of 5 mM of the stimulatory divalent cation (Mn$^{2+}$). The increasing concentration of EDTA decreased the specific activity of amylase in the wild-type strain 1227 both in the absence as well as in the presence of Mn$^{2+}$. However, the specific activity of amylase was more in the presence of 5 mM Mn$^{2+}$ (Fig. 7).

In the wild-type strain 1227, the specific activity of amylase in control was found to be 17.416 U/mg protein and 49.913 U/mg protein in the absence and presence of Mn$^{2+}$ (5 mM), respectively. As the concentration of EDTA increased from 1 mM to 10 mM, the activity of amylase was found to decrease from 17.416 U/mg protein (in control) to 2.924 U/mg protein (at 10 mM EDTA); while in the presence of 5 mM of this divalent cation, the activity decreased from 49.913 U/mg protein (in control) to 16.243 U/mg protein (at 10 mM EDTA) (Fig. 7).

The effect of different concentrations of stimulatory divalent cation (Mn$^{2+}$) on the specific activity of *T. vulgaris* amylase was studied in the absence and presence of 10 mM of the metal chelating agent (EDTA).

In the wild-type strain (1227), the specific activity of amylase in control was found to be 17.306 U/mg protein and 3.163 U/mg protein in the absence and presence of metal chelating agent EDTA (10 mM), respectively (Fig. 8).

In the absence of EDTA, the specific activity increased with increasing concentration of Mn$^{2+}$; and at 10 mM of this divalent cation the activity reached 55.245 U/mg protein. But, in the presence of fixed concentration (10 mM) of EDTA, the specific activity increased upto 21.061 U/mg protein in the presence of same concentration (10 mM) of Mn$^{2+}$.
The increasing concentration of divalent cation (Mn$^{2+}$) increased the specific activity of amylase in the wild-type strain of T. vulgaris. However, metal chelating compound (EDTA) at 10 mM concentration in the absence of Mn$^{2+}$ decreased the amylase activity in the wild-type strain 1227, from 17.416 U/mg protein (in control) to 2.924 U/mg protein (Fig. 8). The stimulatory divalent cation (Mn$^{2+}$) recovered the activity of amylase from inhibition caused by 10 mM of EDTA. A concentration of 10 mM of Mn$^{2+}$ was able to recover about 38% amylase activity (Fig. 8).

Further, the observations pertaining to the inhibition of amylase of T. vulgaris strains by the metal chelating compound (EDTA) in the absence as well as in the presence of the stimulatory divalent cation (Mn$^{2+}$) indicated that, even 10 mM of EDTA was not able to inhibit the enzyme completely, and that too in the absence of Mn$^{2+}$. The presence of Mn$^{2+}$ in the assay mixture, on the other hand, was able to protect the enzyme against EDTA-dependent inhibition (Figs 8, 9). This indicated that Mn$^{2+}$ is firmly bound to the enzyme.

The data suggests that the thermophilic amylase of T. vulgaris exhibits a high degree of metal ion specificity and is, therefore, a metalloenzyme, requiring Mn$^{2+}$ for its enhanced rate of catalysis.

**DISCUSSION**

Temperature is an important factor which has a direct effect on the catalytic functions of enzymes. Many enzymes from a variety of thermophiles function optimally at elevated temperatures (Singleton and Amelunxen 1973; Zuber 1976). In the present investigation concerning the effect of temperature on the catalytic activities of amylase in the wild-type strain 1227 of T. vulgaris reveal that the enzyme is highly thermostable, exhibiting the optimal activities at 75°C in (Fig. 3). Mamo et al. (1999) also reported optimal temperature of 75-80°C for amylase from Bacillus sp. WN11. The optimum pH for amylase activities was found to be 6.0 in wild-type strain 1227, showing specific activity of 17.735 U/mg protein. The enzyme from Bacillus sp. WN11 was optimally active and stable at pH 5.5 (Mamo et al., 1999). α-Amylase from Thermomonospora curvata also exhibited its optimal activity at pH 5.5 to 6.0 (Glymph and Stutzenberger, 1977). Similar to these, the pH optimum (pH 5.0-6.0) of amylase of T. vulgaris was also in the acidic range. The use of liquefying amylases that are active relatively at lower pH could reduce the amount of acid used to lower the pH from a liquefying to saccharifying range of various industrialized enzymatic processes.

The enzymes are highly substrate-specific, and the substrate specificity of amylase varies from microorganism to microorganism. In general, amylase displays highest specificity towards starch, followed by amylase, amylopectin, cyclodextrin, glycogen and maltotriose. The amylase of wild-type strain 1227 of T. vulgaris showed maximum specific activity when soluble starch was used as the substrate (Fig. 5). Sivasakthi et al. (2012) used cassava as a substrate for the production of amylase by Aspergillus niger. Maximum activity was observed in the presence of 5 mM of Mn$^{2+}$, that enhanced the catalytic activity of amylase of T. vulgaris wild-type strain 1227 to 314 % (i.e., about 3.1-fold over the control) (Fig 6). Hg$^{2+}$ was found to inhibit the activity of amylase in the wild-type strain 1227 of T. vulgaris by 79 % (Fig 6). Similarly, Mn$^{2+}$-dependent activation of amylase has been reported in Halobacter sp. MMD047 (Shanmughapriya et al., 2009). Hg$^{2+}$ strongly inhibited the amylase activity by 73 % in Bacillus subtilis (Ashger et al., 2007). It was also found to inhibit the amylase activity in Halobacter sp. MMD047 (Shanmughapriya et al., 2009).
In order to elucidate the metal ion specificity of *T. vulgaris* amylase, investigations were carried out to observe the effect of the metal chelating compound (EDTA) in the absence as well as in the presence of the stimulatory divalent cation (Mn\(^{2+}\)). The increasing concentration of EDTA decreased the specific activity of amylase in the wild-type strain 1227 of *T. vulgaris* (Fig 7) both in the absence as well as in the presence of Mn\(^{2+}\). However, the specific activity of amylase was more in the presence of 5 mM Mn\(^{2+}\). Further, the observations pertaining to the inhibition of *T. vulgaris* amylase by the metal chelating compound (EDTA) in the absence as well as in the presence of the stimulatory divalent cation (Mn\(^{2+}\)) indicated that even 10 mM of EDTA was not able to inhibit the enzyme completely and that too in the absence of Mn\(^{2+}\). The presence of Mn\(^{2+}\) in the assay mixture, on the other hand, was able to protect the enzyme against EDTA-dependent inhibition in wild-type strain of *T. vulgaris* (Fig 8). This indicated that Mn\(^{2+}\) is firmly bound to the enzyme. Thus, the thermophilic amylase of *T. vulgaris* exhibits metal ion specificity and is, therefore, a metalloenzyme, requiring Mn\(^{2+}\) for its enhanced rate of catalysis. The effect of ethylene-diaminetetraacetic acid (EDTA) on α-amylase from alkaliphilic *Bacillus* sp. varies considerably, with some being unaffected in its presence at concentration as high as 100 mM (Hagihara et al., 2001). However, amylase of *Bacillus* sp. IMD 370 is completely inhibited by 1 mM EDTA (Kelly et al., 1995). The amyloytic activity of *Bacillus licheniformis* NH1 is strongly inhibited by the chelating agent (EDTA) at 5 mM concentration and about 75 % of its original activity was lost (Hmidet et al., 2009). It is concluded that amylase of *T. vulgaris* is a thermophilic metalloenzyme, requiring Mn\(^{2+}\) for its high-temperature catalysis, which can be exploited for amylase-based industries of diverse interests.

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