ELSEVIER

Journal of Biotechnology 60 (1998) 113–117

JOURNAL OF Biotechnology

# Characterization of a fungal amylase from *Mucor* sp. associated with the marine sponge *Spirastrella* sp.

B.R. Mohapatra [a], U.C. Banerjee [b,*], M. Bapuji [a]

[a] *Forest and Marine Products Division, Regional Research Laboratory (CSIR), Bhubaneswar-751 013, India*
[b] *Biochemical Engineering Research and Process Development Centre, Institute of Microbial Technology, Sector 39A, Chandigarh-160 036, India*

Received 22 May 1997; received in revised form 8 December 1997; accepted 9 December 1997

**Abstract**

A novel amylase was isolated from the *Mucor* sp. associated with the marine sponge *Spirastrella* sp., grown at 30°C. The enzyme has an optimum pH of 5.0 and an optimum temperature of 60°C. The half lives of the partially purified enzyme at 55 and 60°C were 120 and 50 min, respectively. The activation and deactivation energies of the partially purified enzyme were 46.60 and 157.05 kJ $mol^{-1}$, respectively. The enzyme activity was not affected by the addition of 3% NaCl, 10 mM $Ca^{2+}$ and 25 mM $Mg^{2+}$, but was strongly inhibited by EDTA. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Activation and deactivation energies; Amylase; *Mucor* sp.; *Spirastrella* sp.; Thermostability

## 1. Introduction

The marine biosphere is considered to be one of the richest of the earth's innumerable habitats, yet is one of the least well characterized (Cowan, 1997). The potential of marine organisms for commercial development and exploitation impinges on virtually every area of biotechnology. It is well known that the marine microorganisms produce a variety of industrially important metabolites (Faulkner, 1994), however, studies on the metabolites produced by the fungi closely associated with marine organisms are limited (Kobayashi and Masami, 1993). These studies are mostly focused on the production of biologically active compounds by the fungi while their capability of degrading a wide range of polymeric compounds have not been well exploited. Amylase plays an important role in the biogeochemical cycle of carbon and also has a wider application in the biotechnological based food, detergent and

* Corresponding author. Tel.: +91 172 690908; fax: +91 172 690585; e-mail: Uttam@koel.imtech.ernet.in

0168-1656/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
*PII* S0168-1656(97)00197-1

pharmaceutical industries. From the early 1980s, halophilic microorganisms have been considered as a group of extremophiles with a biotechnological potential similar to that of other extremophilic microorganisms. Exoenzymes from halophilic microorganisms with polymer-degrading capacity are of great commercial interest. There are a number of enzymes of this type, produced by some halobacteria, that have optimal activity at high salinities and could therefore be used in many harsh industrial processes where the concentrated salt solutions used would otherwise inhibit many enzymatic conversions. In addition, most halobacterial enzymes are considerably thermotolerant and remain stable at room temperature over long periods. The few halobacterial exoenzymes that have been reported include the amylases produced by *Halobacterium halobium* (Good and Hartman, 1970) and *Halobacterium sodomense* (Oren, 1983), the proteases from *Halobacterium salinarium* (Norberg and Hofsten, 1969) and *H. halobium* (Izotova et al., 1983), and the lipases from several halobacteria (Gonzalez and Gutierrez, 1970). In view of the potential uses of amylases and advantages of marine microorganisms, the study of this enzyme from various sources is desirable. This paper describes the characterization of partially purified amylase from the marine fungus associated with the sponge *Spirastrella* sp. and seems to be the first report from the fungal associate of a sponge from Andaman and Nicobar Islands, India.

## 2. Materials and methods

### 2.1. Microorganism and culture conditions

The marine fungus *Mucor* sp. was isolated from the sponge *Spirastrella* sp. and cultivated from the intertidal region of Havelock Island, Andaman Sea, India. The fungus was maintained on the culture medium consisting of (w/v) 1% soluble starch; 0.1% casein and 1.5% agar with 50% (v/v) aged sea water. For inoculation in liquid medium, some spores/hyphal suspension from a 5 day-old culture were transferred to sterile 50% (v/v) aged sea water. The suspension ($10^5$ spores $ml^{-1}$) was mixed and 5% (v/v) inoculum was added to a 250 ml flask containing 50 ml of the culture medium except agar. After incubation on an orbital shaker (30°C, 200 rpm) for 5 days, the mycelia were harvested. All the experiments were conducted in triplicate.

### 2.2. Preparation of amylase

The fungal culture was filtered through Whatman filter paper (No. 1) and the filtrate was passed through 0.45 $\mu$m Millipore filter. The final filtrate was treated as crude enzyme preparation. The crude enzyme was passed through DE-52 column pre-equilibrated with Tris/HCl buffer (10 mM, pH 7.2). Amylase activity was eluted from the column using the same buffer with a linear gradient of 0.1–0.8 mM KCl. Fractions containing amylase activity were pooled and used for further studies.

### 2.3. Estimation of amylase activity

$\alpha$-Amylase was assayed by the iodine method (Gogoi et al., 1987), one unit being defined as the amount of enzyme which hydrolyzed 1 mg starch in 1 min at 60°C.

### 2.4. Analysis of hydrolytic products

The reaction mixture containing 250 ml enzyme solution, 250 ml citrate buffer (0.2 M, pH 5.0) and 500 ml soluble starch (2 g $l^{-1}$) was incubated at 60°C for 60 min. The reaction was stopped by boiling the sample at 100°C in a water bath. The hydrolyzed products were determined by thin layer chromatography on precoated silica gel 'G' plate using the solvent system *n*-propanol:ethylacetate:water (4:5:1). The chromatograms were visualized by spraying with anilinephthalate reagent and heated at 100°C for 5 min. The hydrolysed products were identified by co-chromatography with authentic samples of glucose and maltose.

## 3. Results and discussion

Maximum growth and enzyme production were obtained when the initial pH of the culture medium was adjusted to 5.0 (data not shown). The optimum pH for maximum amylase activity (41.84 IU $ml^{-1}$) was found to be 5.0 (data not shown). This pH optimum value is within the range of values reported from other fungal sources, such as *Aspergillus niger* (Bhumibhamon, 1983; Vandersall et al., 1995), *Aspergillus candidus* (Kolhekar et al., 1985) and *Rhizopus nodosus* (Muthukumaran and Dhar, 1983) and lower than that of *Mucor pusillus* (Ogundero, 1979). At pH 6.0 and 7.0, the enzyme activity was 83 and 75%, respectively. The optimum temperature for the maximum amylase activity was 60°C (Fig. 1). At 50 and 65°C, the enzyme exhibited 75 and 29% of its maximum activity. This temperature optimum was higher than the values reported for *Aspergillus flavus* (Khoo et al., 1994), *Asergillus oryzae* (Ray and Majumdar, 1994) and *Aspergillus fumigatus* (Domingues and Peralta, 1993). The half lives of the partially purified enzyme at 55 and 60°C were 120 and 50 min, respectively (Fig. 2). At 65°C, the half life of the partially purified enzyme was 15 min, still faster loss of activity occurred at 70°C in the absence of substrate (data not shown). However, in the presence of substrate (soluble starch), the enzyme was stable up to 60°C which is the optimum temperature for maximum activity. It was also found that the amylase from the *Mucor* sp. isolate required 2% (w/v) NaCl for the maximum activity and even when the NaCl concentration was increased to 8–9%, the enzyme retained 57 and 48% of the maximum activity, respectively (Fig. 3). The amylases of marine origin thus seems to differ at least in their requirement of sodium chloride and in their ability to tolerate high salinity which may be beneficial for the different applications of amylases including saccharification of starch. Considerable efforts have been devoted to the selection of microorganisms producing amylases with new physical properties and tolerance to extreme conditions used in industrial processes (e.g. temperature, salts and pH). The activation and deactivation energies of the partially purified enzyme were 46.60 and

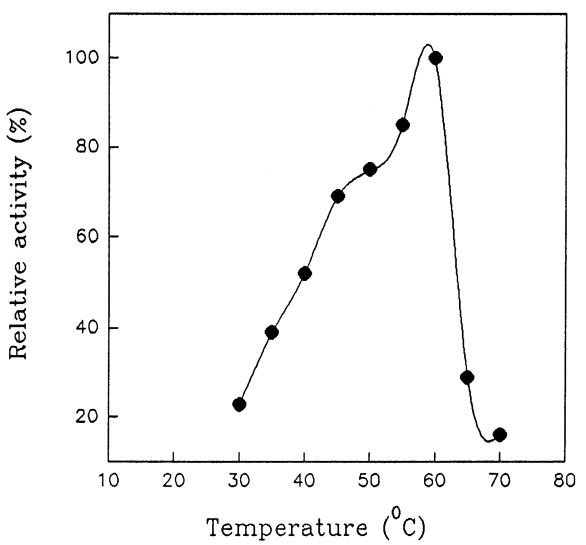


Fig. 1. Effect of temperature on amylase activity of *Mucor* sp. associated with the marine sponge *Spirastrella* sp. (The reaction mixture consisted of 500 $\mu$l soluble starch (2 g $l^{-1}$) in 0.2 M citrate buffer pH 5.0, 250 $\mu$l enzyme solution and 250 $\mu$l citrate buffer (0.2 M, pH 5.0). The reaction mixture was incubated for 10 min. One milliliter 1.5 N acetic acid and 1 ml iodine reagent [0.2% (w/v) iodine and 2.0% (w/v) potassium iodide] were added to stop the reaction.)

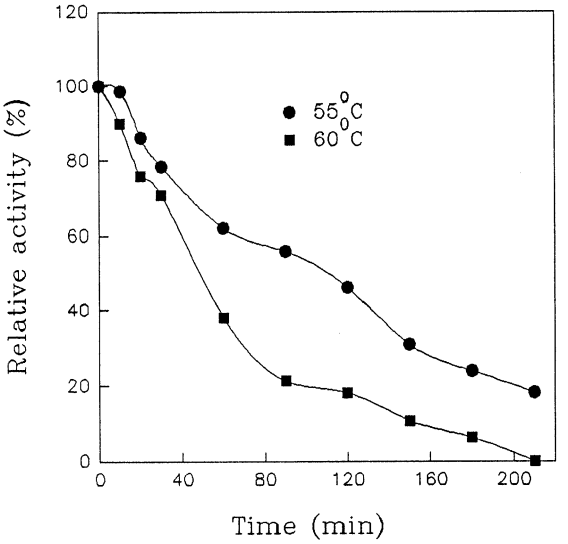


Fig. 2. Thermostability of amylase activity of *Mucor* sp. associated with the marine sponge *Spirastrella* sp. at 55°C (●) and 60°C (■).

157.05 kJ $mol^{-1}$, respectively (Fig. 4). Under optimal conditions (60°C, pH 5.0) amylase exhibited Michaelis–Menten type kinetics and its $K_m$ value was lower than reported earlier from *Aspergillus terreus* (Ali and Hossain, 1991). The *Mucor* sp. was grown on different carbohydrate substrates (0.5%) and D-glucose induced the highest amylase activity followed by starch, inulin and lactose (Table 1). The effects of divalent metal ions and EDTA are shown in Table 2. The metal ions such as $Cu^{+2}$, $Fe^{+2}$, $Ni^{+2}$ and $Hg^{+2}$ inhibited the partially purified enzyme at higher concentration (25 mM) while $Ca^{+2}$, $Co^{+2}$ and $Mn^{+2}$, inhibited to a lesser extent. In contrast, the enzyme was unaffected by $Mg^{+2}$ at 25 mM. EDTA strongly inhibited the activity at 5 mM. Similarly $Hg^{+2}$, $Cu^{+2}$, $Zn^{+2}$ and $Co^{+2}$ inhibited the amylase activity of species such as *A. terreus* (Ghosh et al., 1991), *A. flavus* (El-Abyad et al., 1994) and *A. oryzae* (Kong et al., 1991). The product of enzymatic hydrolysis of soluble starch after 60 min incubation was primarily glucose as confirmed by co-chromatography, suggesting that the enzyme preparation may be a glucoamylase (data not shown). To understand fully the nature

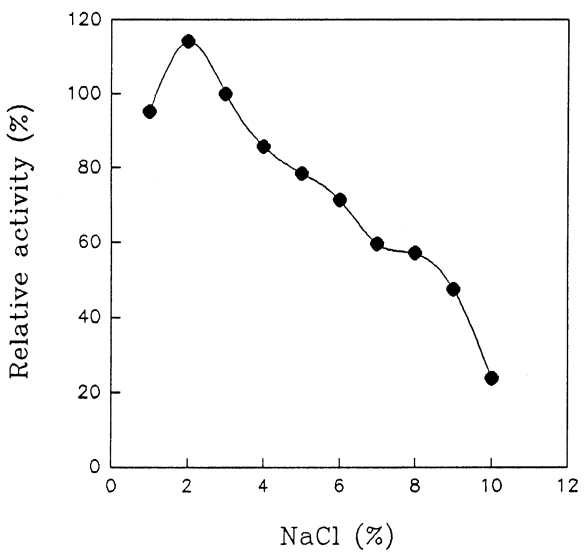


Fig. 3. Effect of NaCl on amylase activity of *Mucor* sp. associated with the marine sponge *Spirastrella* sp. (The reaction mixture consisted of 500 $\mu$l soluble starch (2 g $l^{-1}$) in 0.2 M citrate buffer pH 5.0, 250 $\mu$l enzyme solution and 250 $\mu$l citrate buffer (0.2 M, pH 5.0) having different concentrations of NaCl. The reaction mixture was incubated at 60°C for 10 min. One milliliter 1.5 N acetic acid and 1 ml iodine reagent [0.2% (w/v) iodine and 2.0% (w/v) potassium iodide] were added to stop the reaction).

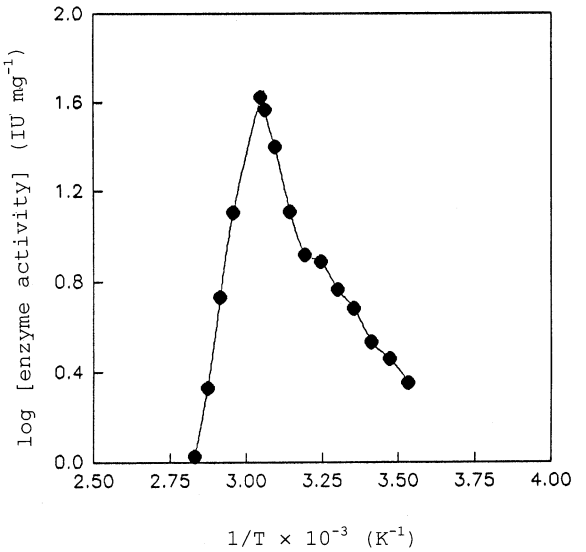


Fig. 4. Dependency of amylase activity of *Mucor* sp. associated with the marine sponge *Spirastrella* sp. on temperature (Arrhenius plot).

Table 1
Effect of various carbohydrates on amylase production

| Growth substrate (0.5%) | Amylase activity (IU $ml^{-1}$) |
|---|---|
| *Polysaccharides* | |
| Inulin | 18.09 |
| Soluble starch | 18.22 |
| *Disaccharides* | |
| Lactose | 18.09 |
| Sucrose | 13.33 |
| Maltose | 9.52 |
| *Monosaccharides* | |
| L-Arabinose | 1.90 |
| D-Xylose | 13.33 |
| D-Galactose | 5.71 |
| D-Glucose | 25.68 |
| D-Mannose | 2.86 |

The concentration of each carbohydrate was 0.5% (w/v). The carbohydrates were added to the 1 day-old culture growing on the medium consisting of 1% (w/v) soluble starch, 0.1% casein and 50% (v/v) aged seawater at 30°C. The amylase activity in the culture supernatant was assayed after 3 days.

Table 2
Effect of metal ions and EDTA on amylase activity

| Metal ions | Percentage activity retained | | | | | |
|---|---|---|---|---|---|---|
| | Concentration (mM) | | | | | |
| | 0.1 | 1 | 5 | 10 | 20 | 25 |
| $Ca^{+2}$ | 100 | 100 | 100 | 100 | 20 | 25 |
| $Co^{+2}$ | 100 | 91 | 100 | 100 | 87 | 80 |
| $Cu^{+2}$ | 100 | 100 | 93 | 89 | 40 | 16 |
| $Fe^{+2}$ | 100 | 100 | 90 | 25 | 22 | 7 |
| $Hg^{+2}$ | 93 | 93 | 83 | 63 | 44 | 34 |
| $Mg^{+2}$ | 100 | 100 | 100 | 100 | 100 | 100 |
| $Mn^{+2}$ | 95 | 93 | 93 | 81 | 78 | 78 |
| $Ni^{+2}$ | 100 | 100 | 100 | 100 | 55 | 25 |
| $Zn^{+2}$ | 100 | 100 | 82 | 84 | 64 | 69 |
| EDTA | 95 | 46 | 0 | 0 | ND | ND |

ND, Not determined.
The activity is expressed as a percentage of the activity in the absence of chemical and metal ions. The enzyme was preincubated with a metal ion at 37°C for 5 min. Separate blanks with individual metal ions were prepared.

and properties of the amylase isolated from this fungus and to determine whether this amylase posses new specificities, it will be essential to isolate, purify and characterize this enzyme.

## Acknowledgements

One of the authors (BRM) would like to thank the Department of Ocean Development, Government of India, Delhi for financial assistance.

## References

Ali, S., Hossain, Z., 1991. Characterization of glucoamylase from *Aspergillus terreus*. J. Appl. Bacteriol. 71, 144–146.

Bhumibhamon, O., 1983. Production of amyloglucosidase by submerged culture. Thai J. Agric. Sci. 16, 173–184.

Cowan, D.A., 1997. The marine biosphere: a global resource for biotechnology. Trends Biotechnol. 15, 129–131.

Domingues, C.M., Peralta, R.M., 1993. Production of amylase by soil fungi and partial biochemical characterization of amylase of a selected strain (*Aspergillus fumigatus Fresenius*). Can. J. Microbiol. 39, 681–685.

El-Abyad, M.S., El-Shanshoury, A.R., Hafez, M., 1994. Purification and characterization of the glucoamylase produced by a strain of *Aspergillus flavus*. Microbios 80, 7–15.

Faulkner, D.J., 1994. Marine natural products. Nat. Prod. Rep. 11, 355–394.

Ghosh, A., Chatterjee, B., Das, A., 1991. Purification and characterization of glucoamylase by *Aspergillus terreus*. J. Appl. Bacteriol. 71, 162–169.

Gogoi, B.K., Bejbaruah, R.L., Pillai, K.R., Baruah, J.N., 1987. Production, purification and characterization of an $\alpha$-amylase produced by *Saccharomycopsis fibuligera*. J. Appl. Bacteriol. 63, 373–379.

Gonzalez, C., Gutierrez, C., 1970. Presence of lipase among species of extremely halophilic bacteria. Can. J. Microbiol. 16, 1165–1166.

Good, V.A., Hartman, P.A., 1970. Properties of the amylase from *Halobacterium halobium*. J. Bacteriol. 104, 601–603.

Izotova, L.S., Strongin, A.Y., Chekulaeva, L.N., Sterkin, V.E., Ostoslavskaya, V.I., Lyublinskaya, L.A., Timokhina, E.A., Spenavov, V.M., 1983. Purification and properties of serine protease from *Halobacterium halobium*. J. Bacteriol. 155, 826–830.

Khoo, S.L., Amirul, A.A., Kamaruzaman, M., Nazalan, N., Azizan, M.N., 1994. Purification and characterization of $\alpha$-amylase from *Aspergillus flavus*. Folia Microbiol. 39, 392–398.

Kobayashi, J., Masami, I., 1993. Bioactive metabolites of symbiotic marine microorganisms. Chem. Rev. 93, 1753–1769.

Kolhekar, S.R., Mahajan, P.B., Ambedkar, S.S., Borkar, P.S., 1985. Purification and characterization of glucoamylase from a higher yielding mutant of *Aspergillus candidus* Link Var. *aureus*. Appl. Microbiol. Biotechnol. 22, 181–186.

Kong, X., Wang, J., Cui, Y., Jiang, L., 1991. Purification and properties of $\alpha$-amylase from *Aspergillus oryzae*. Weishengwu Xuebao 31, 274–280.

Muthukumaran, N., Dhar, S.C., 1983. Purification and properties of a glucoamylase fraction from the culture filtrate of *Rhizopus nodosus*. Ital. J. Biochem. 32, 239–253.

Norberg, P., Hofsten, B.V., 1969. Proteolytic enzymes from extremely halophilic bacteria. J. Gen. Microbiol. 55, 251–256.

Ogundero, V.W., 1979. Amylase and cellulase activities of thermophilic fungi causing deterioration of tobacco products in Nigeria. Mycopathologia 69, 131–135.

Oren, A., 1983. A thermophilic amyloglucosidase from *Halobacterium sodomense*, a halophilic bacterium from the Dead Sea. Curr. Microbiol. 8, 225–230.

Ray, L., Majumdar, S.K., 1994. Enzymatic hydrolysis of starch to glucose and immobilization of amyloglucosidase in calcium alginate gel. Res. Ind. 39, 109–112.

Vandersall, A.S., Cameron, R.G., Nairn, C.J., Yelenosky, G., Wodzinski, R.J., 1995. Identification, characterization and partial purification of glucoamylase from *A. niger*. Prep. Biochem. 25, 29–55.