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SHORT COMMUNICATIONS

Characterization of a β-amylase from *Propionicimonas* sp. ENT-18 ectosymbiont of *Acromyrmex subterraneus brunneus*

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Abstract The last decade has seen an increase in the industrial use of amylase, mainly due to its role in the production of anhydrous ethanol through the hydrolysis of corn starch. Most amylases in use nowadays are isolated from microorganisms, indicating their potential as a source of amylases for industrial applications. Since most microbial diversity remains unknown, microbes associated with previously undiscovered niches can provide an untapped source of new molecules. We investigated the diversity of actinobacteria associated with an underexploited niche and have identified a number of rare actinobacteria. We further aimed to characterize the amylase produced by one such actinobacterium, *Propionicimonas* sp. ENT-18, an ectosymbiont associated with the integument of *Acromyrmex subterraneus brunneus*. Our data indicate that this actinobacterium produces a high-molecular-weight β-amylase (170.4 kDa), with maximum production at the end of the log phase (96 h at 28°C) when cultured in Starch-Czapek broth. The maximum specific activity (50 mU/mg protein) was obtained at pH 5.5 at 30°C. The β-amylase produced was partially purified by ice-cold acetone precipitation, with a recovery of 65% activity. Although this enzyme has not shown any special trait (e.g., thermal or pH stability) for industrial applications, this is the first report of a β-amylase-producing *Propionicimonas*.

Keywords Actinomycete · Leaf-cutter ant ectosymbiont · Amylolytic enzyme · Acid amylase

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Introduction

Actinomycetales is the most studied and exploited bacterial order because of its vast capacity to produce useful biotechnological molecules (Bérdy 2005). The potential of microorganisms within this group as sources of bioactive molecules has prompted a number of screening programs for new molecules (Goodfellow and Fiedler 2010). Several such screening programs have focused on the selection of new species of lytic enzyme-producing actinobacteria (Stamford et al. 2001), mainly because of their high capacity to produce a wide range of enzymes with different characteristics. Nowadays, screening for novel depolymerases (e.g., amylases) has been renewed because of their high biotechnological potential for biomass degradation (van der Maarel et al. 2002), but especially because of their critical role in obtaining anhydrous ethanol from grains or starchy materials (Wheals et al. 1999).

Amylase production (mainly α-amylase and γ-amylase) is a common characteristic of many genera of Actinobacteria (Stamford et al. 2002). Furthermore, some genera of these filamentous bacteria are thermophilic and have been reported as producers of pH and thermo-stable

amylases, which are desirable traits for industrial application (Collins et al. 1993; Stamford et al. 2001).

In our previous investigation (Zucchi et al. 2011) of the diversity of actinobacteria associated with the integument of the leaf-cutter ant *Acromyrmex subterraneus brunneus*, we isolated a close relative of *Propionicimonas paludicola* (Akasaka et al. 2003), a recently discovered group of bacteria that awaits investigation of its biotechnological potential. Although many species of actinobacteria associates of *Acromyrmex* sp. (mainly *Pseudonocardia*) have been identified by wide 16S rRNA sequencing (Mueller et al. 2008; Sen et al. 2009) and culture-dependent approaches (Cafaro and Currie 2005; Kost et al. 2007; Zucchi et al. 2011), little is known of the biotechnological potential of these actinobacteria (Haeder et al. 2009; Oh et al. 2009). Since there are no reports on the amylolytic activity of *Propionicimonas*, we aimed to characterize the amylase produced by *Propionicimonas* sp. ENT-18 in order to investigate its potential for industrial applications.

Materials and methods

The actinobacterium strain *Propionicimonas* sp. ENT-18 used in this study was previously isolated from the integument of *Acromyrmex subterraneus brunneus* (GenBank accession no. GQ304819; Zucchi et al. 2011), and a stock culture has been maintained in ISP-2 broth (Shirling and Gottlieb 1966) at 4°C.

Amylase production was stimulated by inoculating the selected isolate (ENT-18) in Starch-Czapek broth (SC; Stamford et al. 2001). A pre-inoculum was prepared using two plugs (0.5 cm) of an ENT-18 stock culture in conical flasks containing 50 mL ISP-2 broth (Shirling and Gottlieb 1966) and incubated at 28°C for 3 days. After this period, aliquots of 1 mL were inoculated into three conical flasks containing 50 mL SC broth, and incubated for up to 5 days at 28°C. Each flask was considered as one replicate. The enzyme produced was sampled at 24 h intervals by taking 5 mL from the culture flask for each replicate. The supernatant containing the amylase was separated from the cellular fraction after centrifugation (15,000 *g*, 15 min, 4°C), and later used to test for amylase activity.

The growth of ENT-18 was calculated after daily determination of the mycelial dry weight until the 5th day of incubation in SC broth. The mycelium was removed by vacuum filtration, and the filter paper containing the actinobacteria mycelia was dried to constant weight in an oven at 110°C. The growth of ENT-18 was expressed in milligrams of dry mass/50 mL culture medium. At the same sampling points, the pH of the culture was measured. This experiment was carried out in triplicate (one 50 mL flask=one replicate).

The pH range for growth of ENT-18 was also determined in ISP-2 broth. A 1 ml pre-inoculum was inoculated in 50 mL ISP-2 broth over a range of pH (from 4.0 to 12.0) obtained with the aid of HCl or NaOH solutions, and incubated at 28°C for 7 days. The biomass produced was evaluated as previously described. This experiment was performed in duplicate.

Amylase activity was measured using 100 μL of 1.0% starch (w/v) as substrate, incubated with 100 μL of the crude enzyme extract. The reaction was interrupted with the addition of 200 μL of 3,5-dinitrosalicylic acid (DNS) (Noelting and Bernfeld 1948), heated at 100°C for 5 min; 1.6 mL water was added prior to quantification by spectrophotometry at 550 nm. The activity was calculated using a standard curve of glucose as a reference. One unit of enzyme activity (U) was defined as 1 μmol of substrate hydrolyzed/minute. This experiment was carried out in triplicate, with independent samples for each determination.

Maltase (EC 3.2.1.20) activity was tested by using 100 μL of an 80% acetone precipitate of the crude enzyme extract mixed with 100 μL maltose (20 mM) in a pH range from 3.0 to 10.5 at 30°C following Dubowsky (1962). Tubes were heated at 100°C for 10 min, and the absorbance was measured at 630 nm to verify the hydrolysis of maltose. A control assay without enzyme (amylase or maltase) was carried out to detect any spontaneous hydrolysis of the substrate.

The optimum pH of the amylase produced from isolate ENT-18 was determined in 50 mM citrate-phosphate (pH 4.0–7.0), 50 mM phosphate (pH 7.0–8.0), 50 mM TRIS-HCL (pH 8.0–9.0) and 50 mM glycine-NaOH (pH 9.0–10.5) buffer systems. The optimum temperature was determined after incubation of the enzyme extracts with 100 μL of 1.0% starch (w/v), at optimum pH and in a temperature range from 30°C to 50°C. This experiment was carried out in triplicate, with independent samples for each determination.

The protein concentration of samples was determined following Bradford (1976), using the commercial reagent Coomassie Plus™ Protein Assay (Pierce), and bovine serum albumin as the standard, according to the manufacturer's instructions.

Different volumes of cold acetone (−20°C) were added to the enzyme extract, for sequential protein precipitation. The supernatant was separated into the following saturation ranges: 0–20%, 20–40%, 40–60%, and 60–80% acetone. At each acetone addition, samples were homogenized by inversion, incubated at −20°C for 10 min, and the protein was harvested after centrifugation (10,000 *g*, 10 min, 4°C). Samples were dried completely at room temperature and the pellet was resuspended in the original initial volume of water.

The product originated by the hydrolysis of starch in the presence of the ENT-18 enzyme extract was identified by thin-layer chromatography (TLC) by comparing against

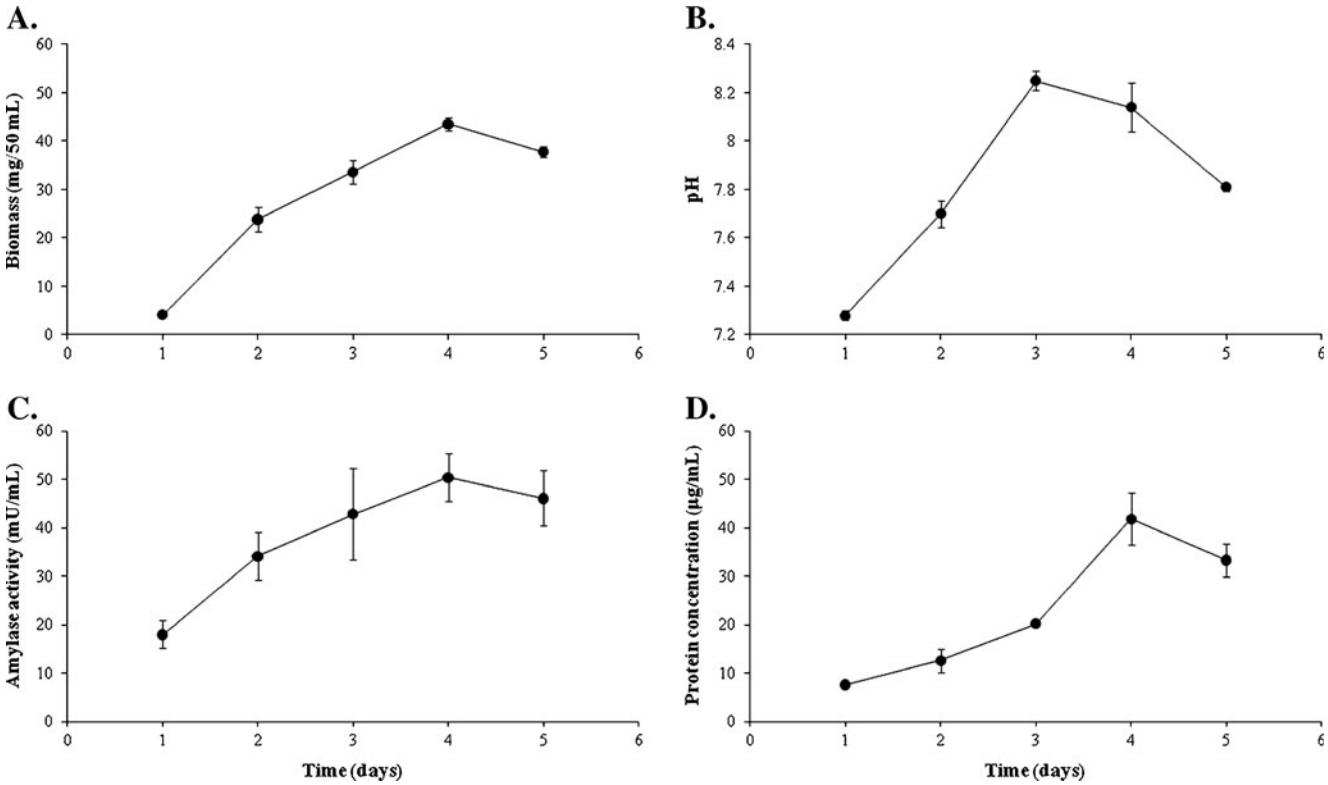

Fig. 1a–d *Propionicimonas* sp. ENT-18 growth in Starch-Czapek (SC) broth and amylase production. **a** Biomass (mg/50 mL medium). **b** Medium pH. **c** Amylolytic activity (mU/mL). **d** Protein concentration (µg/mL). *Error bars* Standard error

standard solutions of 100 mM of glucose and maltose (100 mM). Hydrolysis was performed at 30°C with 10 µL of a 60% acetone precipitate of the crude extract and 10 µL of 1.0% starch solution (w/v), pH 5.5 for 12 h. The sample was dried, resuspended in 2 µL of water, and applied onto a silica gel-F_{254} TLC plate (Macherey-Nagel®). The TLC plates were resolved using a solution of acetonitrile:water (85:15) as a carrying solvent, and sugars were revealed after spraying the plate surface with 5% sulfuric acid, followed by heating at 100°C until spots could be clearly identified.

The molecular weight of the β-amylase produced by *Propionicimonas* sp. ENT-18 was estimated by using native polyacrylamide gel electrophoresis (Laemmli 1970) in a refrigerated system at 4°C. The sample (14 µL) was added to loading buffer (4 µL of 100% glycerol and 2 µL of 0.5% bromophenol blue), loaded on a 7.5% polyacrylamide gel, and run at 5.0 mA. The gel was then blotted against a 7.5% polyacrylamide gel containing 0.1% starch, incubated for 12 h at room temperature in 10 mM citrate-phosphate buffer (pH 5.5), and stained in a 3% potassium iodide solution. The molecular weight of the amylase was determined by comparing the R_f with those of protein standards loaded in a mirror 7.5% PAGE gel stained with the GelCode® Blue Stain reagent (Pierce).

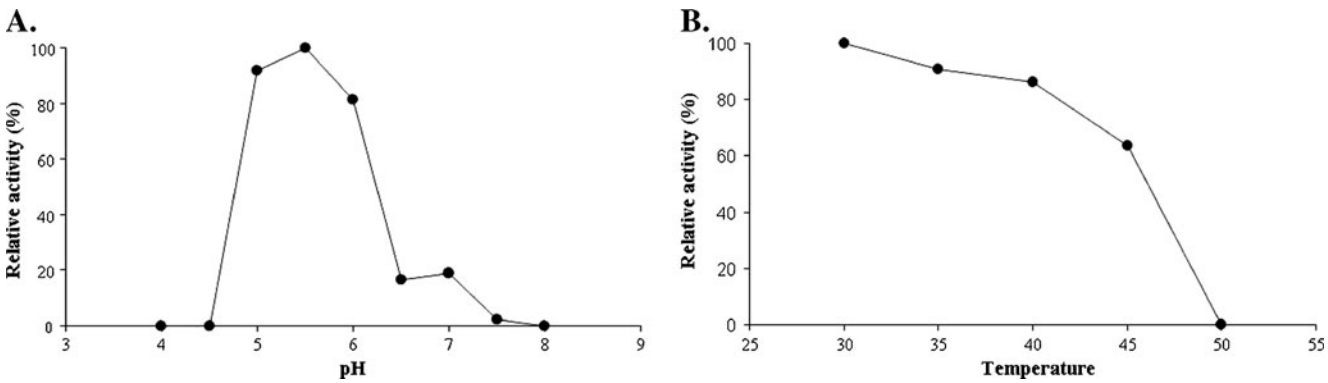

Fig. 2 Effect of pH (**a**) and temperature (**b**) on amylase activity from *Propionicimonas* sp. ENT-18

Table 1 Purification of β-amylase from *Propionicimonas* sp. ENT-18 using precipitation by ice-cold acetone

Fraction (% acetone)	Protein (mg/mL)	Activity (mU/mL)	Specific activity (mU/mg protein)	Purification (fold)	Yield (%)
Crude extract	38.53±0.61	63.67±3.02	1.66±0.06	1.0	100.00±0.00
0–20	8.46±0.61	4.70±1.05	0.57±0.08	0.3	7.84±1.67
20–40	15.22±0.03	9.15±1.51	0.61±0.11	0.4	14.34±2.34
40–60	8.29±0.17	27.47±1.13	3.31±0.14	2.0	43.13±2.05
60–80	10.15±2.34	nd[a]	nd	nd	nd

[a] Non-detectable

Results

ENT-18 was tolerant to a broad range of pH, growing in conditions ranging from pH 5.0 to 11.0. The maximum growth was registered at pH 7.0 and pH 8.0; and the optimum pH (7.9) was given by the equation $y = -15.522x^2+246.18x - 818.59$ ($r^2=0.99$). The results obtained for biomass production in SC broth demonstrated that ENT-18 reached the end of the log phase (43.5 mg) after 96 h of incubation (Fig. 1a). The pH of the medium also varied during cell growth, peaking after 3 days (pH 8.14), and gradually decreasing to 7.8 on the 5th day of culture (Fig. 1b). The maximum amylolytic activity at pH 5.5 (50.4 mU/mL) occurred at pH 8.1 after 4 days of incubation (Fig. 1c), which coincided with the maximum biomass production and the highest protein concentration (Fig. 1d). All these parameters were highly correlated (Supplementary material 1).

The amylase produced by *Propionicimonas* sp. ENT-18 was active between pH 5.0 and 7.0 (Fig. 2a). At lower (4.5) and higher (8.0) pH values, the enzyme was completely inactivated. The optimum pH observed for this enzyme was 5.5, but enzyme activity was only 4% lower at pH 5.0. The optimum temperature for ENT-18 amylase was observed at 30°C (Fig. 2b). Enzyme activity was reduced at higher temperatures, reaching 86.0% of the relative activity at 40°C. The enzyme remained active up to 45°C, and no detectable activity was seen at 50°C.

The specific activity after 60% saturation with acetone was equal to 3.31 mU/mg protein, which led to a two-fold purification from the crude extract. The acetone purification used allowed the recovery of 65.31% of the amylase activity found in the crude extract (Table 1).

A well-defined spot with an R_f identical to the maltose standard, and a faint spot with an R_f identical to the glucose standard could be identified by TLC after a 12 h hydrolysis reaction of starch with the 60% ice-cold precipitated fraction of the whole extract. The presence of maltose as the major hydrolysis product indicates that the 170.4 kDa amylase produced by ENT-18 is a β-amylase (EC 3.2.1.2) (Fig. 3). The absence of any enzyme activity of the ENT-18 extract when using maltose as a substrate, strongly supports the characterization of this enzyme as a β-amylase.

Discussion

Amylases, obtained mainly from microorganisms, are used in a number of industrial processes (Wheals et al. 1999). Because of their high versatility and profitability for industrial application, many efforts have been made to identify new species of bacteria capable of producing amylases with desirable traits (e.g., thermal and pH stability) (Stamford et al. 2002).

In general, amylases can be stable in a pH range from 4.0 to 11.0 (Hamilton et al. 1999), and most amylases are stable between 5.0 and 8.0 (Fogarty 1983). However, there are also reports of amylases that are stable at lower pH (Coronado et al. 2000). pH stability is one of the most important traits for industrial application, because the pH will usually change during the fermentation process (Ray and Nanda 1996). The ENT-18-produced amylase showed an optimum pH at 5.5, and was stable from pH 5.0 to 7.0. Although this optimum pH value is similar to that reported for *Streptomyces griseus* (Wako et al. 1979), the narrow pH range shown could restrict the use of this enzyme in industrial processes. Nevertheless, the highest amylase production occurred when the pH of the medium was

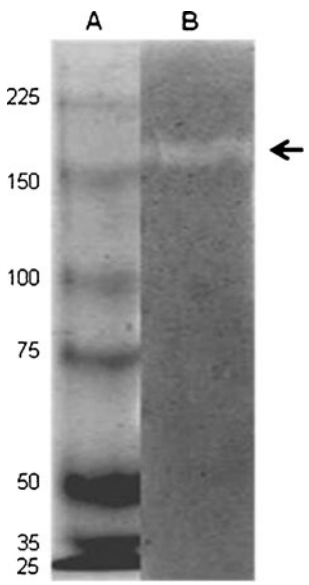

Fig. 3 Determination of the molecular weight of the β-amylase produced by *Propionicimonas* sp. ENT-18. Lanes: *A* Molecular weight ladder (Broad Range Protein, Promega, Cat. # v8491), *B* zymography in a soluble starch-containing gel of the β-amylase (*arrow* 170.4 kDa)

above 8. Although this may seem contradictory, similar results have been reported by others (Stamford et al. 2001, 2002), and understanding the regulatory mechanisms involved in the production of amylase was not the goal of our experiments. Furthermore, the strong correlation between enzyme production and the pH of the medium (r= 0.93), suggests that this latter parameter should be taken into account if the interest is to use the microorganism (instead of the purified enzyme) for starch fermentation.

The optimum temperature of the ENT-18-produced amylase was relatively low (30°C), although 60% of the activity was still retained at 45°C. Some thermophilic actinobacteria have been reported to produce amylases active at higher temperatures (Stamford et al. 2002). However, according to these latter authors, at least for *Streptosporangium*, the behavior for this enzyme is considered uncommon. In *Propionicimonas* sp., the growth temperature ranged from 10 to 40°C, with an optimum temperature around 35°C (Akasaka et al. 2003). The amylase optimum temperature found for ENT-18 agrees with the report by Vihinen and Mantsala (1989), who stated that the optimum temperature for amylase activity is generally related to the temperature for microorganism growth. ENT-18-produced amylase was still active after 6 months when kept in storage conditions (−20°C) (data not shown).

The amylase produced by ENT-18 was classified as a β-amylase, and because no enzyme activity was detected when using maltose as a substrate, we conclude that the identification of glucose as a product of hydrolysis of starch by the ENT-18 extract was probably due to the direct activity of β-amylase on starch, as reported by others (McCleary and Codd 1989; Friedberg and Rhodes 1986). Although the molecular weight found for ENT-18 amylase is unusually high (170.4 kDa), it is within the range previously described for bacterial β-amylases (for review, see Ray and Nanda 1996). This class of enzyme has been used extensively in the food industry, and also has some potential use in the brewery, pharmaceutical, and cosmetic industries, mainly because of its capacity to release large amounts of maltose (Ray and Nanda 1996). On the other hand, β-amylases are not usually used in anhydrous ethanol production because of their inability to efficiently hydrolyze starch to glucose (Brethauer and Wyman 2010).

In conclusion, the β-amylase produced by *Propionicimonas* sp. ENT-18 did not demonstrate any characteristic desired by industries (e.g., thermal and pH stability). However, this is the first report of a β-amylase produced by a member of this genus.

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