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Partial biochemical characterization of a thermostable chitinase produced by *Streptomyces owasiensis* isolated from lichens of the Amazonian region

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The current study aims to identify *Streptomyces* spp., isolated in the Amazon region and capable of producing chitinase as well as to partially characterize the enzyme. Optimum temperature and pH, thermal and pH stabilities and the behavior of chitinase were determined as compared to other substances. *Streptomyces owasiensis* was the best chitinase producer in submerged culture fermentation using chitin 1% (w/v) at 140 rpm for 96 h at 34°C. The enzyme showed optimal activity at pH 7.0 and stability at the assessed pH variations. It also showed optimal temperature of 80°C and 180 min of thermostability between 30 and 90°C. The enzymatic activity was potentiated in the presence of various ions, especially Fe^{2+}, the same occurred in the presence of the anionic surfactant sodium dodecyl sulfate (SDS), but it suffered inhibition influence by EDTA. The chitinase produced by *S. owasiensis* showed characteristics of industrial relevance and it highlights the first report on enzyme production from the species isolated in the Amazon.

Key words: N-acetyl-D-glucosamine, chitin, chitinase, actinomycetes, *Streptomyces owasiensis.*

INTRODUCTION

The Amazonian region has an immense biodiversity, however, much of its species and their phylogenetic relationships are unknown, mainly the microbiological diversity and its interactions (Souza et al., 2004). Micro-organisms present a wide biochemical diversity which, along with the possibility of using genetic engineering

to modify its genetic makeup, which results in an excellent biological source of biomolecules producer for both industrial and pharmaceutical interest including enzymes (Neves et al., 2006).

Streptomyces spp. is a genus that stands out in secondary metabolites production. In accordance with González et al. (2005), they are a group of Gram positive bacteria characterized by the formation of aerial mycelium on solid medium and by spore formation. These microorganisms may be found in different environments such as water, plants and in association with lichens. The biochemical characteristics of the chitinase enzyme produced by the genus *Streptomyces* spp. are of great industrial interest, especially for the use in the environmental field and in biocontrol (Bon et al., 2008).

Chitin is a linear polymer of β-1,4 linked N-acetyl-D-glucosamine (GlcNAc), considered as the second most abundant polysaccharide in nature. It is commonly found in the structure of fungal cell walls and in the exo-skeleton of arthropods, crustaceans and nematodes (Dahiya et al*.,* 2006; Rattanakit et al., 2007). Chitinases are enzymes that can hydrolyze chitin by catalyzing the cleavage of linkages β-1,4 between GlcNAc residues (Alcazar-Fuoli et al., 2011).

Chitinolytic enzymes have several applications, among which, the preparation of chito-oligosaccharides and N-acetyl-D-glucosamine are of pharmaceutical importance. These enzymes are also used in protoplast isolation from fungi and yeast, fungi biocontrol, in the treatment of waste containing chitin and in the control of malaria transmission (Dahiya et al., 2006).

However, chitinase obtainment through microbiological pathways has shown to be promising in recent years. *Chitiolyticbacter meiyuanensis* SYBC-H1 (Hao et al., 2012); *Serratia marcescens* (Zarei et al., 2011); *Streptomyces* sp. TH-11 (Hoang et al., 2011) and *Micrococcus* sp*.* AG84 (Annamalai et al*.*, 2010) are among the microorganisms used for the production of the enzyme.

The present study aimed to select and identify a strain of *Streptomyces* spp. producing chitinase as well as to partially characterize the enzyme.

MATERIAL AND METHODS

Microorganisms

The current study assessed thirty strains of *Streptomyces* spp. stored in the culture collection of the Department of Parasitology, Federal University of Amazonas (DPUA), Brazil. For the activation and maintenance of microorganisms, we employed the ISP-2 culture medium, and the inoculum was standardized to a concentration of 10^8 colony forming units (CFU) mL^{-1} by spectrophotometry (Libra S22, Biochrom, Cambridge - England).

Screening the best producer of chitinase

The medium used for screening and producing the enzyme was composed by 1% chitin powder (Sigma, St. Louis, EUA), 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ (Wang et al*.,* 2010), incubated in an orbital shaker at 140 rpm for 96 h at 34°C. The liquid resulting from the fermentation was filtered by means of qualitative filter paper, thus the crude enzymatic extract was obtained and used for all analytical determinations described in the present study.

Identification of the microorganism

The identification at the level specie of the isolate that showed the best production of the enzyme chitinase was carried out by polymerase chain reaction (PCR). The DNA of strain was extracted using the purification kit of genomic DNA (Promega Corporation, Madison, EUA) according to the manufacturer's instructions. Amplification of 16S ribosomal DNA gene was performed by PCR using universal primers for eubacteria FD1 (5'-AGAGTTTGATCCTGGCTCAG3') and RD1 (5'AGGAGGTGATCCAGCC-3') (Weisburg et al., 1991). The reaction mixture consisted of 10 to 50 ng of DNA, 5 pmol of each primer: 200 mM dNTP, 1.5 mM $MgCl_2$, 1X buffer, 1 U Taq DNA polymerase Platinum (Invitrogen Life Technologies) to a final volume of 25 ul. The reaction was performed with 5 min of denaturation at 94°C, 25 cycles were performed for 1 min at 94°C, 30 min at 52°C and 2 min at 72°C. These cycles were followed by a final elongation period of 10 min at 72°C. The amplification product was sequenced and the resulting sequence was compared with all sequences available in GenBank using BLAST software of the National Center for Biotechnology Information (NCBI).

Assay of chitinolytic activity and determination of total protein

The colloidal chitin is used as substrate in the detection of enzyme activity in accordance with the protocol by Harighi et al. (2007). The determination of the chitinase activity was performed according to the method by Waghmare and Ghosh (2010), although with some changes. The reaction mixture was composed of 1 mL of colloidal chitin 1% (w/v), 0.5 mL of sodium phosphate buffer 25 mM, pH 7.4 and 0.5 mL of the crude enzyme extract incubated at 37°C for 30 min. The reducing sugars were detected by applying the method described by Miller (1959). The calibration curve was performed by using N-acetylglucosamine, as per the standard, at concentrations of 0 to 5 mg mL^{-1}. One chitinase activity unit was defined as the proper amount of enzyme in order to release 1 µmol of N-acetylglucosamine per minute.

A commercial kit that employs the bicinchoninic acid (Pierce, Rockford, USA) using bovine serum albumin as standard in different concentrations was applied to determine total protein. The measurements of protein concentration were expressed in mg mL^{-1}.

Optimum temperature and pH, thermal and pH stabilities

Incubation temperatures of 30, 40, 50, 60, 70, 80 and 90°C were

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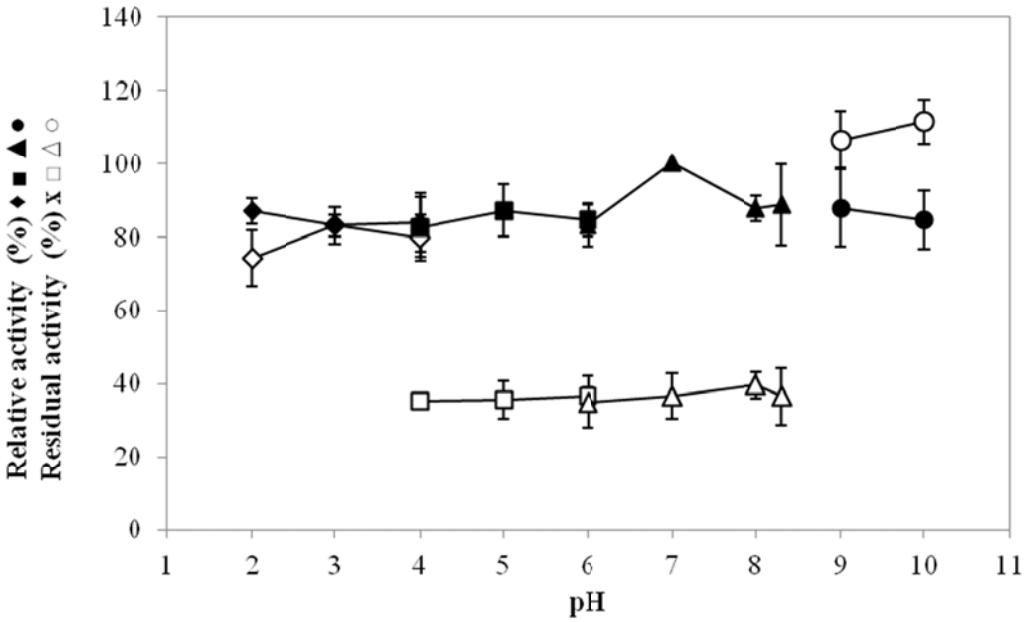

Figure 1. Optimum pH (closed symbols) and pH stability (open symbols), after 180 min of chitinase produced by *S. owasiensis* in the buffers: Glycine-HCl (♦ ◊) (pH 2.0, 3.0, 4.0); acetate (■ □) (pH 4.0; 5.0; 6.0); phosphate (▲ Δ) (pH 6.0; 7.0; 8.0; 8.3); carbonate-bicarbonate (● ○) (pH 9.0, 10.0).

used to analyze the optimum temperature of chitinolytic activity. The enzymatic activities were expressed as relative activity (%). The thermal stability was measured by incubating the enzyme extract at the indicated temperatures, over a total of 180 min. The enzymatic activity was expressed as residual activity (%). To determine the optimum pH of the enzyme activity, we used the following buffers: at 100 mM glycine-HCl (pH: 2.0; 3.0; 4.0), acetate (pH: 4.0; 5.0; 6.0), sodium phosphate (pH: 6.0; 7.0; 8.0; 8.3) and carbonate-bicarbonate (pH: 9.0; 10.0), and the enzyme activities were expressed as relative activity (%). The pH stability of the enzyme was determined by using the same buffers applied in the investigation of the optimum pH, aliquots were taken every 30 min, totaling 180 min. The enzymatic activity was expressed as residual activity (%).

Effect of ions and inhibitors

The effect of different metal ions and other substances on the chitinolytic activity of the enzyme was measured by subjecting the enzymatic extract to solutions containing substances and ions (5 mM), and this mixture was maintained at room temperature (±25°C) for 60 min, and then, chitinase activity was measured. The following ions were evaluated: Zn^{2+}; Mg^{2+}; Mn^{2+}; Fe^{2+}; K^{+}; Cu^{2+}; Ca^{2+}; Ni^{2+}; Ba^{2+}; Pb^{2+}. Ethylenediaminetetraacetic acid (EDTA), iodoacetic acid and sodium dodecyl sulfate (SDS) were also tested substances. The results were expressed as relative activity (%).

RESULTS AND DISCUSSION

Identification of the microorganism and producer of chitinase

The best microorganism producer of chitinase was identified as *Streptomyces owasiensis* by 16S DNA gene sequencing. The specific activity of chitinase produced by this strain was 2.923 U mg^{-1} and a total protein of 0.065 mg mL^{-1}. The chitinase production evidenced in the present study stands out as the first report regarding the production of extracellular enzymes from the species *S. owasiensis.*

Effect of pH on enzyme activity and stability

The chitinase showed optimal activity in neutral pH range and stability in all the assessed conditions. Thus, the optimal chitinolytic activity occurred at pH 7.0 (sodium phosphate buffer), proving to be stable up to pH 10.00, showing a linear behavior after 180 min of testing (Figure 1).

Han et al. (2009) evaluated a chitinase enzyme produced by *Streptomyces* sp. DA11 and found pH 8.0 as optimum for enzyme catalytic activity, although the enzymatic stability was maintained up to pH 11.0, suggesting a very similar behavior to that observed by the current study. Thus, the chitinase enzyme isolated from *S. owasiensis,* in the present study, has shown better enzymatic activity in neutral and moderately basic pH range versus the chitinase produced by *Streptomyces* sp. DA11. According to Yuli et al. (2004), neutral chitinase can be used for many purposes in the pharmaceutical industry, especially in the production of chitooligosaccharides. Taking into account the biochemical characteristics exhibed by chitinase of *S.*

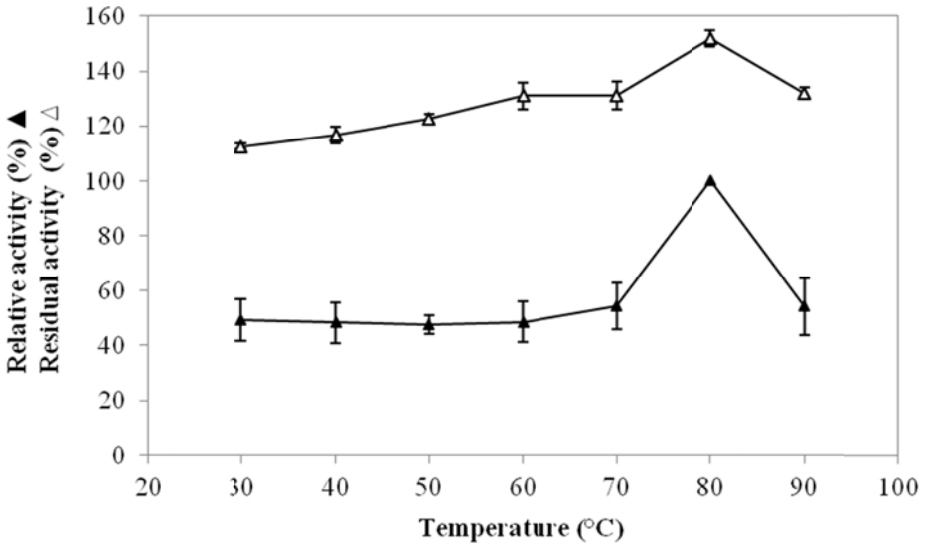

Figure 2. Optimum temperature (■) and temperature stability (●), after 180 min on the activity of enzymatic extract containing chitinase produced by *S. owasiensis.*

owasiensis, it seems to be attractive to the industrial production of this enzyme.

Optimum temperature and thermal stability

As shown in Figure 2, the optimum temperature of chitinase characterized in this study was 80°C. Furthermore, the enzyme was able to keep residual activity above 100% when it was submitted to variable temperatures as well as to exposure time of 180 min (Figure 2). It was observed that the optimum temperature for the enzyme catalysis is different from the vast majority of common enzymes. The optimum of the chitinase occurred at 80°C and the enzyme was stable throughout the period of study at different temperatures, such temperature tolerance features are unusual and of great industrial interest. Regarding a thermostable chitinase obtained by *Bacillus* sp. 13.26, Yuli et al. (2004), have found that the optimum temperature of the enzyme was 60°C, keeping the stability for 300 min at 70°C. According to Bruins et al. (2001), the thermostability can be considered as the outcome of an evolutionary strategy that requires both intrinsic factors that are directly associated with the structure of the molecule (which provide the rigidity and flexibility of the molecule) and the extrinsic factors that contribute to the stabilization of proteins in a particular medium, including some solutes, molecular chaperones, and the binding of substrate. In this respect, the discovery and study of thermostable enzymes is a promising research work with a wide commercial scope, which interest is additionally increased because of the fact that the elaboration of many industrial products is commonly developed under high temperatures in order to reduce the risk of contamination, especially microbial.

Effect of ions and inhibitors in the chitinolytic activity of *S. owasiensis*

As can be seen in Table 1, the presence of some substances in the reaction mixture can suppress or enhance the enzyme activity. The majority of the ions evaluated in the current study stimulated the catalytic activity of chitinase produced by *S. owasiensis* with significant prevalence in the addition of Fe^{2+}. This Fe^{2+} enhancing characteristic was also observed in chitinase produced by *Micrococus* sp. AG84 (Annamalai et al., 2010). Taken together, the results presented in both studies suggest that the increased enzymatic activity in the presence of Fe^{2+} or Cu^{2+} may be related to the residues of aspartic and glutamic acid found in the primary sequence of these chitinases (Annamalai et al., 2010), since the glutamic acid residues are structurally involved in the catalytic process of this enzyme.

An enzyme inhibitor, according to Marques and Yamanaka (2008), is a substance that is capable of specifically interfering with an enzymatic reaction by reducing or even slowing down the catalytic process. The influence of several ions and substances on the chitinase enzymatic activity was assayed. Table 1 shows that the chitinolytic activity underwent inhibition in the presence of the chelating agent EDTA. Another substance which enhanced the chitinolytic activity was the anionic surfactant SDS, thus an increase of 59% was noticed in relative activity as compared to the control sample (Table 1). Intriguingly, this result is opposite to results

Table 1. Effect of different ions and other substances on the activity of an enzymatic extract containing chitinase produced by *S. owasiensis.*

Ions	Relative activity(%)
Control	100±0
Zn^{2+}	148±12
Mg^{2+}	107±2
Mn^{2+}	171±5
Fe^{2+}	234±5
K^{+}	129±9
Cu^{2+}	141±9
Ca^{2+}	164±2
Ni^{2+}	120±8
Ba^{2+}	117±6
Pb^{2+}	99±2
Substances	
EDTA	62±6
Iodoacetic acid	100±10
SDS	159±5

that had been previously reported by Wang et al. (2009), Han et al. (2009) and Kim et al. (2003) because in these cases, the presence of SDS in the reaction mixture inhibited the chitinolytic activity. On the other hand, the simultaneous addition of chelating agent EDTA negatively affected the chitinolytic activity, showing a 38% decrease in the relative activity as compared to the control (Table 1). Similar results of inhibition of chitinase in the presence of EDTA were obtained by Wang et al. (2009) with a decrease of 43% (Wang et al., 2008), 75% (Han et al., 2009) and with 57% reduction in relative activity, respectively.

Conclusion

In summary, the *S. owasiensis* selected in the current study demonstrated the ability to produce an extracellular chitinase with properties which showed thermostability and increased efficiency in the neutral pH range, which makes this chitinase an enzyme of interest for both scientific study and pharmaceutical industry.

Conflict of Interests

The authors have not declared any conflict of interests.

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