

Production of Extracellular Protease by a Brazilian Strain of *Beauveria bassiana* Reactivated on Coffee Berry Borer, *Hypothenemus hampei*

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ABSTRACT

Studies were carried out on extracellular protease production by *Beauveria bassiana* CG432 in liquid medium containing glucose and yeast extract. *B. Bassiana* presented active growth after lag period of 24 h., produced 80% of the total of the extracellular protease activity in 48 h which was maximum on the 5th culture day. The extracellular protease presented optimum activity at 60°C, was stable up to 1M Cl⁻, maintained the stability during 15 day at 4°C and -18°C, but was not stable if frozen repeatedly.

Key words: Protease activity, *Beauveria bassiana*, enzyme stability

INTRODUCTION

The entomopathogenic fungus *Beauveria bassiana* is commonly isolated from dead insects in the environment (Boucias and Pendland, 1998; Cottrell and Shapiro-Ilan, 2003) and its spores are frequently used as biological control agent against pest infestation in agriculture (MacLeod, 1954; Furlong and Pell, 2001). During multiplication on the insect, the conidia produce different virulence factors, identified as extracellular enzymes, especially proteases characterized as chymotrypsin-like serine proteinases with activity on casein, hide protein azure (HPA), azocoll, elastin Congo Red and collagen substrates (Chrzanowska et al., 2001), chitinases (Samsinakova et al., 1971; St-Leger et al., 1986) and lipases (Giraldo-Cardozo et al., 2001).

The production of extracellular enzymes was related to the infection process of the insects because it allowed the penetration of the germ tube across the cuticle which is formed by nearly 70% of proteins (Hepburn, 1985), chitin and lipids (Bidochka and Khachatourians, 1992), and the growth of the fungus through the use of hemolymph nutrients (Shimizu et al., 1993). The entomopathogenic fungus can diversify the production of virulence factors in response to the different insects (Moino et al., 1998). Inducer substrates have been tested in order to increase the protein content and to release the extracellular proteases to make easier the studies on kinetic activity and structure and to correlate with virulence levels of the fungal strain in bioassays on insects. Neves and Hirose (2005) observed that the Brazilian *B. bassiana* strain CG432 bioassayed on coffee berry borer, *Hypothenemus hampei*,

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presented better conidia production and more than 70% of mortality, with 10^8 conidia/mL concentration among 61 Brazilian strains when reactivated on the same insect. Previous studies showed that extracellular proteases produced by this strain were active at pH 4.5, 7.0 and 9.5, suggesting the production of at least three different types of proteases (Stürmer et al., 2003/2004).

There are many papers about protease production and their characteristics, but more information is necessary about *B. bassiana* strains adapted to Brazilian conditions. Thus, the objective of this study was to determine the culture time for the maximum production of extracellular proteases by *B. bassiana* CG432 reactivated on coffee berry borer and the effect of some reaction and storage conditions on the activity and stability of these enzymes.

MATERIALS AND METHODS

Fungal strain

B. bassiana CG432 was isolated from adult insects of the family Membracidae (Homoptera), Rio Grande do Norte State, northeast, Brazil, supplied to the Entomopathogen Bank in the Department of Agronomy at Londrina State University by the Brazilian Corporation for Agricultural Research – EMBRAPA/Cenargen.

Conidium reactivation

The fungus conidia was maintained in Petri dishes containing the solid culture medium for *B. bassiana*: 20g agar, 10g anhydrous D-glucose, 5g yeast extract, 1.58g NaNO_3 , 1.05g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1g KCl; 0.6g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.36g KH_2PO_4 , distilled water to one liter (Alves, 1998), at 25°C, 12h photophase, for ten days. Media produced conidia were sprayed on *H. hampei* alive adults previously treated with 0.5% sodium hypochlorite solution placed on Petri dishes and incubated at 25°C until insect death. Newly emerged conidia from the insect were subcultured not more than four times, at ten days intervals in solid culture medium and used to prepare the reactivated inoculum suspension containing 10^8 conidia/mL (Neves and Hirose, 2005).

Fungal growth conditions

The fungus was cultivated at 28°C, 150 rpm during for nine days in 250 mL erlenmeyers flasks, with 100 mL of liquid culture medium (as above culture medium) without agar with 1% of the suspension of 10^8 conidia/mL as the inoculum, in duplicate. One un-inoculated culture medium was used as control. At 24 h, a group of the three flasks (duplicate and control) was filtrated through previously weighed Whatman No 1 filter papers and the filtrate, called crude extract (CE), was dialysed against 5 mM phosphate buffer pH 7 at 4°C, served as the enzyme source for determination of the extracellular protease activity and the soluble protein content.

Biomass determination

Biomass was determined from the residue material on the filter paper. The biomass obtained from the 1st to the 9th day of cultivation was washed with two aliquots (50 mL each) of distilled water and dried at 70°C until constant weight.

Extracellular protease assay

Extracellular protease was determined (Ito, 2003), using 200µl aliquots of CEs obtained from the first to the ninth day of culture were incubated at 37°C for 30 minutes with 100µl of the 50mM phosphate buffer pH 7 and 250µL bovine serum albumin 5mg/mL dissolved in the same buffer. The reaction was interrupted with 250µL 10% TCA and centrifuged at 1100g for 15 minutes. The protease activity was considered as the absorbance lecture at 650 nm corresponding to the released soluble peptide content of the supernatant (Hartree, 1972). Each 0.1 increase in absorbance was considered as one unit of protease activity.

Effect of temperature and ions Cl^- on protease activity

Temperature and Cl^- effect on protease activity was determined using the CE obtained after 5 days of cultivation in 50 mM phosphate buffer pH 7 at temperatures from 20 to 70°C. The effect of 0.1 to 1.0 M NaCl concentration was determined at 37°C in 50 mM acetate, phosphate and Glycine-NaOH buffer at pH 4.5, 7 and 9.5, respectively (Stürmer et al., 2003/2004).

Protease stability on storage conditions

Protease stability was evaluated by determining the protease activity of CE obtained on the 5th

cultivation day after stay storing at 4°C or frozen at -18°C for 30 days. The effect of successive freezing and thawing of the sample was also evaluated 6 times (each 5 days) during the same time period.

RESULTS AND DISCUSSION

Protease production and fungal growth

The highest extracellular protease activity by *B. bassiana* CG432 (Fig. 1A) was observed on the 5th day (Abs 650 nm 0.489) but around 80% of this activity was obtained after 2nd day of the cultivation. After the 5th day, there was an abrupt

reduction of the activity until the 9th day. Variable time courses of extracellular protease production have been related in literature, reflecting the variability in protease production on different media. The stimulatory effects of a series of 24 nitrogen sources including inorganic, organic nonprotein, proteins and complex natural media on the production of proteases in submerged cultures demonstrated that the maximum amount of protease into the maize meal, yeast extract, ground maize and wheat bran broth was released 3 days after inoculation (Kucera, 1971).

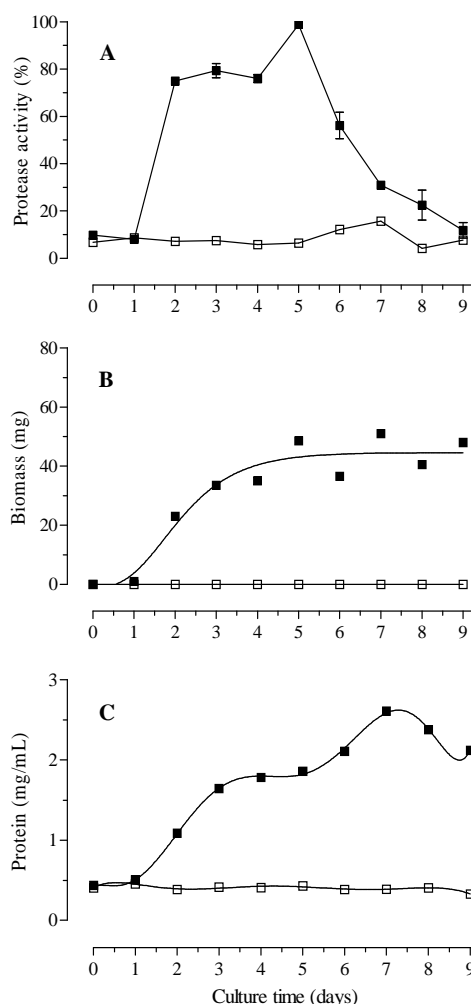


Figure 1 – Growth curve of *Beauveria bassiana* CG432. Fungi culture (■); no inoculated medium (□). Time course of protease production (A); biomass weight (B); protein concentration (C).

Others studies that used inducer substrates to produce proteases from *B. bassiana*, stopped the cultures in between 3rd to 6th day of the cultivation as 0.2% lyophilized porcine blood plasma and 0.5% ground larval of *Apis mellifera* with higher release at 3rd day by *B. Bassiana* 278 (Chrzanowska *et al.*, 2001), 1% gelatin at 3rd and 4th day by *B. bassiana* GK2016 (Bidochka and Khachatourians, 1987 and 1988), 1% gelatin and casein, at 4th day by *B. bassiana* 11892A (Urtz and Rice, 2000), ground migratory grasshopper cuticle, at 5th day by *B. bassiana* GK2016 and GK2018 (Bidochka and Khachatourians, 1990, 1991, 1993), colloidal chitin, at 6th day (Havukkala *et al.* 1993), and liquid medium with cuticle ground from the own coffee berry borer, *Hypothenemus hampei* at 5th to 7th days after the inoculation (Giraldo-Cardozo *et al.*, 2001).

The biomass production (Fig. 1B) and the concentration of soluble protein (Fig. 1C) of the culture medium increased after 2nd day and coincided with 80% of the total of the extracellular protease production, after a small lag period of 24 h shorter than obtained by two isolates of *B. Bassiana* (Arcas *et al.*, 1999). These results could be related with the production of complex proteolytic enzymes during the reactivation of the inoculum on coffee berry borer alive, using liquid medium without inducer substrates such as the cuticle (Havukkala *et al.* 1993; Urtz and Rice,

2000) or extract from insects (Bidochka and Khachatourians, 1990, 1991, 1993; Giraldo-Cardozo *et al.*, 2001).

The active growth continued until the 5th day when the enzyme was released in the culture medium reaching the maximum extracellular protease activity. After that, the biomass production was stable when the protease release was reduced (Fig. 1A and 1B). These results confirmed the importance of the extracellular protease activity on the substrate utilization during the multiplication cellular phase of this fungus as related to other microorganisms (Fu-Chu *et al.*, 2004). The concentration of soluble protein of the culture medium was highest on the 7th day probably related with biomass (Fig. 1C).

The previous reactivation of the fungus on insects proposed in this work presented the advantage of less laborious laboratory work to produce the inoculum than to prepare different substrates and less cultivation time, considering that 80% of the total activity was obtained on 2nd day.

Temperature effect

The extracellular proteases were more active between 40 and 60°C (Fig. 2). This result was better than those by Bidochka and Khachatourians (1987), with optimum temperature between 37 to 42°C.

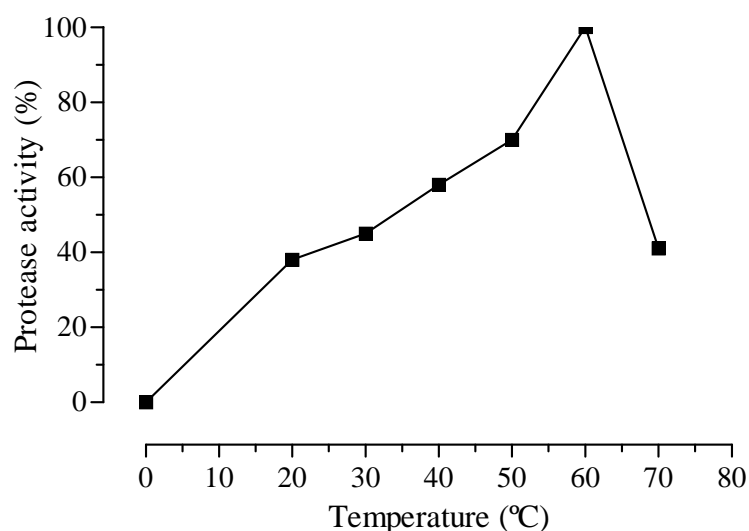


Figure 2 – Effect of the temperature on the extracellular protease activity produced by *Beauveria bassiana* CG432.

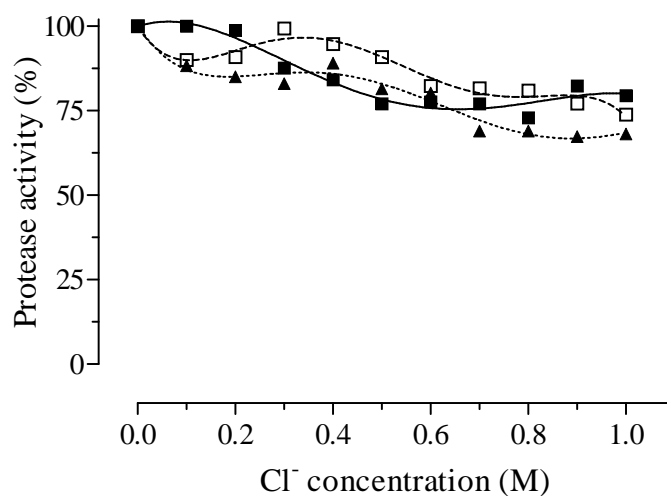


Figure 3 – Effect of chloride ion concentration at pH 4.5 (□), 7.0 (■) and 9.5 (▲) pH on the extracellular protease activity produced by *Beauveria bassiana* CG432.

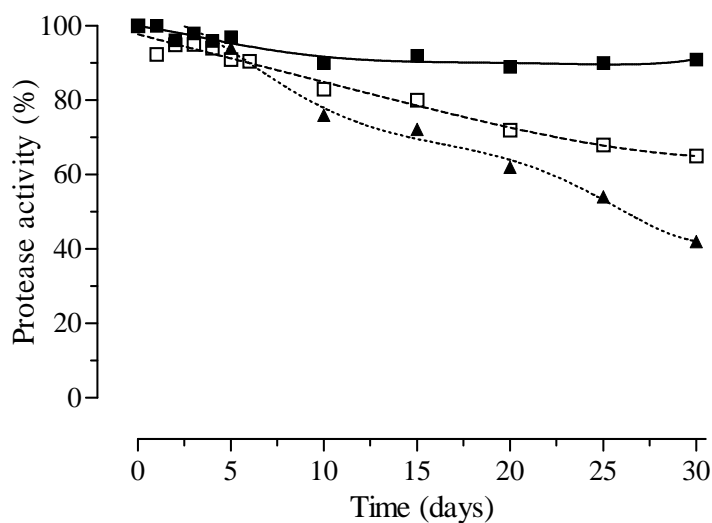


Figure 4 – Stability of extracellular protease produced by *Beauveria bassiana* CG432 stored at 4°C refrigeration (□), -18°C freezing (■) and submitted to repetitive freezing/thawing (▲).

Cl⁻ ion concentration effect

The CE proteases remained active when analyzed in increasing concentrations of chloride ions of 0.1 to 1.0 M at pH 4.5, 7.0 and 9.5 (Fig. 3). This could be important information, because chloride ions

are frequently used on buffer analysis and purification laboratory procedures.

Protease stability in storage

It was observed that the proteases maintained above 90% of their activity up to the 30th day under freezing and above 80% until the 15th day under refrigeration. The activity fell down to 50% at the 5th times of freezing/thawing (Fig. 4). Thus, repetitive freezing of the extracts should be avoided.

At least, the extracellular proteases produced in these conditions presented optimum temperature activity at 60°C, without interferences from Cl⁻ ion and was stable for many days on refrigeration or freezing conditions. This fact could be very important in purification procedures. Also, proteases produced under same conditions were stable during 10 days at 25°C (Stürmer *et al.*, 2003/2004) being too important on insect procedures for bioassay when used as bioinsecticide.

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RESUMO

A cepa brasileira do fungo entomopatogênico *Beauveria bassiana* CG432 foi cultivada em meio de cultivo líquido contendo glucose e extrato de levedura a 28°C, 150 rpm, durante 9 dias utilizando 1% de inóculo constituído por 10⁸conídios/mL pré-ativados em insetos vivos de broca-do-café. Avaliou-se diariamente o crescimento do fungo através da quantificação da biomassa e a atividade de proteases extracelulares no filtrado do cultivo pela determinação de

peptídeos solúveis liberados pela hidrólise de soro albumina bovina a 37°C durante 30 minutos. O fungo cresceu ativamente após reduzido período lag de 24 horas, produziu 80% da atividade total de proteases em 48 horas e atingiu produção máxima em 5 dias de cultivo, sem adição de substratos indutores no meio de cultivo. As proteases apresentaram atividade ótima a 60°C, estabilidade até 1M de íons cloreto, mantiveram mais de 80% de atividade durante 15 dias a 4°C e -18°C, porém foram sensíveis a congelamentos repetitivos.

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