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Production, purification and characterization of a thermostable β-1,3-glucanase (laminarinase) produced by *Moniliophthora perniciosa*

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ABSTRACT

The enzyme glucanase from *Moniliophthora perniciosa* was produced in liquid medium and purified from the culture supernatant. A multivariate statistical approach (Response Surface Methodology – RSM) was employed to evaluate the effect of variables, including inducer (yeast extract) and fermentation time, on secreted glucanase activities *M. perniciosa* detected in the culture medium. The crude enzyme present in the supernatant was purified in two steps: precipitation with ammonium sulfate (70%) and gel filtration chromatography on Sephacryl S-200. The best inducer and fermentation time for glucanase activities were 5.9 g L^{-1} and 13 days, respectively. The results revealed three different isoforms (GLUI, GLUII and GLUIII) with purification factors of 4.33, 1.86 and 3.03, respectively. The partially purified enzymatic extract showed an optimum pH of 5.0 and an optimum temperature of 40°C. The enzymatic activity increased in the presence of KCl at all concentrations studied. The glucanase activity was highest in the presence of 0.2 M NaCl. The enzyme showed high thermal stability, losing only 10.20% of its specific activity after 40 minutes of incubation at 90°C. A purified enzyme with relatively good thermostability that is stable at low pH might be used in future industrial applications.

Key words: glucanase, *Moniliophthora perniciosa*, production, kinetic characterization, purification, heat stability.

INTRODUCTION

The hemibiotrophic basidiomycete *Moniliophthora perniciosa* (Aime and Phillips-Mora 2005), the causative agent of witches' broom disease in *Theobroma cacao*, is responsible for major crop losses in South American and Caribbean cocoa plantations (Kilaru and Hasenstein 2005).

The disease shows two distinct stages: a biotrophic (intercellular monokaryotic mycelium) and a necrotrophic/saprotrophic phase (intracellular dikaryotic mycelium). The biotrophic mycelium depends on the host tissue for nutrients to sustain its growth and development.

A typical fungal cell wall is composed of chitin, glucans and proteins, and it can be degraded by chitinases and glucanases (Lin et al. 2007, Perez et al. 2002, Schirmbock et al. 1994). These enzymes are widely distributed among fungi, and some have been purified (Donzelli and Harman 2001).

β-glucan, a linear polysaccharide, is a principal cell wall component of cereal grains, including barley, rye,

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rice and wheat, and most of microorganisms in which it has multiple functions, including a role in nutrition. Structurally, β-glucans are comprised of β-1,3-, β-1,6- and β-1,4-linked D-glucopyranosyl units (Planas 2000, Parrish et al. 1960). β-1,3-glucanase is an enzyme with strict cleavage specificity for β-1,3-linkages (Planas 2000).

The β-1,3-glucanases are classified as exo-β-1,3-glucanase (EC 3.2.1.58) and endo-β-1,3-glucanase (EC 3.2.1.39) according to their mode of hydrolysis. Hydrolysis of 1,3-β-linkages in the polysaccharide chain results in the release of short oligosaccharides. Exo-β-1,3-glucanases remove glucose units from β-1,3-D-glucan by hydrolyzing terminal β-1,3-D linkages in a consecutive manner and produce D-glucose as the sole product (Pitson et al. 1993).

Enzymes that catalyze depolymerization or selective modification of cereal β-glucans are of biotechnological and biomedical importance because they have shown a significant potential application in malting and brewing processes (Konig et al. 2002, Mannonen et al. 1997), biological control of plant pathogens (De La Cruz et al. 1995), fungal and yeast cell wall degradation (Pitson et al. 1993), animal feed (Konig et al. 2002) and medical fields (Pang et al. 2005, Li et al. 2007).

To evaluate *M. perniciosa* as an industrial source of glucanase, the present study evaluated the influence of fermentation time and inducer on the activity of secreted glucanase from the fungus, using response surface methodology (RSM). Furthermore, the study describes the production, purification and characterization of β-1,3-glucanase (laminarinase) from *Moniliophthora perniciosa* in saprotrophic phase mycelium.

MATERIALS AND METHODS

CHEMICALS

Laminarim and bovine serum albumin were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All other chemicals used were of high-quality analytical grade.

MICROORGANISM

Moniliophthora perniciosa (CCMB 0257) was obtained from the Collection of Cultures of Microorganisms of Bahia (CCMB, Feira de Santana, Brazil). The fungus was maintained on potato dextrose agar at 28°C in an incubator (IGO 150 Cell Life – Jouan).

CULTURE CONDITIONS OF *Moniliophthora perniciosa*

Moniliophthora perniciosa was grown on potato dextrose agar for 10 days at 28°C to produce β-1,3-glucanase. Then, discs of fungal mycelium, with 1 cm in diameter, were taken from the solid culture, transferred into 100 mL erlenmeyer flasks containing liquid culture media with the following components dissolved in distilled water: wheat bran, 40.0 g L^{-1}; yeast extract, 3.0-9.0 g L^{-1}; K_2HPO_4 $3H_2O$, 1.0 g L^{-1}; $MgSO_4$ $7H_2O$, 0.2 g L^{-1} and KCl, 0.2 g L^{-1}. The incubation was carried out as described in the experimental design; flasks were heated at 28°C for 7 to 21 days and shaken at 120 rev min^{-1} in a rotary shaker. The liquid culture medium, on which *M. perniciosa* was grown, was filtered and centrifuged (Centrifuge 5804R – Eppendorf, São Paulo, Brazil) at 8,000 g for 15 min at 4°C, and the supernatant, containing the proteins from *M. perniciosa*, was used as a crude enzymatic extract (Hua et al. 2007).

DOEHLERT EXPERIMENTAL DESIGN FOR ENZYME PRODUCTION

Fermentation time and inducer concentration are among the most important parameters that affect the production of enzymes are fermentation time and inducer concentration (Giese et al. 2005). In this work, yeast extract was used as an inducer. To predict production of enzymes under any conditions of fermentation time and yeast extract concentration in the experimental domain, RSM was used. The Doehlert experimental design contained two variables, yeast extract and fermentation time, and three replicates at the center of the domain lead to a total of 9 experiments (Table I). Measurements were performed in triplicate to verify the best culture medium conditions to induce the production of β-1,3-glucanase. Yeast extract was studied at five concentrations, ranging from 3 to 9 g/L, and fermentation time was studied at three time points, ranging from 7 to 21 days. The experimental errors were evaluated from replication of a central point. The experimental data was processed using STATISTICA software. All the experiments in this step were carried out in random order.

Statistica version 7.0 was used to estimate the lack of fit of the model to the experimental data through analysis of variance (ANOVA).

TABLE I
Doehlert matrix used for the optimization of 1,3-glucanase production from *Moniliophthora perniciosa*.

Experiment	Inductor concentration (g/L)	Time (days)	Enzyme activity (μmol glucose/mL/min)
1	3 (–1)	14 (0)	0.1873
2	4.5 (–0.5)	7 (–0.866)	0.1875
3 (C)	6 (0)	14 (0)	0.2026
4 (C)	6 (0)	14 (0)	0.2013
5 (C)	6 (0)	14 (0)	0.2007
6	7.5 (0.5)	7 (–0.866)	0.1750
7	9 (1)	14 (0)	0.1913
8	4.5 (–0.5)	21 (0.866)	0.1722
9	7.5 (0.5)	21 (0.866)	0.1766

(C): central point; coded values are presented in parentheses.

Enzyme Purification

The purification procedure for β-1,3-glucanase from *M. perniciosa* involved the following two steps:

Step 1: Ammonium sulfate precipitation

The crude enzymatic extract previously obtained was brought to 70% saturation by adding solid ammonium sulfate and centrifuged at 10,000 × g for 10 min after standing for 1 h. The precipitate, with high laminarinase activity, was resuspended in phosphate citrate buffer (pH 6.2) in the ratio of 1:3 (w/v) and stored at low temperature.

Step 2: Sephacryl S-200 chromatography

The crude enzymatic extract was applied to a Sephacryl S-200 (Amersham Bioscience, USA) column (48 × 1.2 cm) previously equilibrated with sodium phosphate buffer (0.05 mol L^{-1}, pH 7.0), which was also used to elute the product. Fractions of 1.5 mL were collected and assayed for glucanase activity. Fractions showing β-1,3-glucanase activity were pooled and concentrated by ultrafiltration using an Amicon PM 10 membrane, which has a molecular weight cut-off of 10 kDa.

Protein Determination and Enzyme Analysis

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. The enzyme activity of the purified β-1,3-glucanase was measured using laminarin (from *Laminaria digitata*, Sigma) as the substrate. Laminarin was dissolved in 0.05 mol L^{-1} citrate buffer (pH 6.2). The reaction mixture contained 100μL of 0.1% laminarin and 100μL of enzyme solution. The mixture was incubated at 50°C for 15 min. The reaction was stopped by the addition of 200μL of 1% 3,5-dinitrosalicylic acid, and the mixture was boiled for 15 min. The amount of reducing saccharides released from laminarin was measured spectrophotometrically (Cary 50 UV-Visible spectrophotometer, Varian Inc., São Paulo, Brazil) at 540 nm according to precedent (Miller 1959). One unit (U) of β-1,3-glucanase activity was defined as the amount of the enzyme required to release one microgram of glucose per minute under the above conditions.

Heat Stability and Optimal pH and Temperature

The optimal pH of β-1,3-glucanase activity was assayed at pH 5.0, 6.0, 7.0, 8.0, and 9.0. Blanks lacking glucanase were used in each determination.

To determine the optimum temperature, β-1,3-glucanase activity was tested under standard assay conditions in the temperature range of 30-90°C. The temperatures were controlled using a circulating water bath.

To test the stability of the enzyme at elevated temperature, the samples of β-1,3-glucanase in test tubes of uniform size were incubated in water at different temperatures (50, 60, 70, 80 and 90°C) and different amounts of time (0, 10, 20, 30, 40, 50 and 60 min). After heating, the tubes were cooled in an ice bath, and the residual activity measurement was carried out at a pH of 6.2 and at a temperature of 50°C.

Effect of Cations

The effect of Na^+ and K^+ on β-1,3-glucanase activity was investigated. The following concentrations of both NaCl and KCl were used: 0.2, 0.4, 0.6, 0.8 and 1 mol L^{-1}. β-1,3-glucanase activity was determined using the assay previously described, which uses laminarin as a substrate. An activity reading of 100% is obtained when no reagent is added.

Replications and Statistical Analysis

The extractions and chromatographic isolations were repeated at least three times. Average values of triplicates (which differed by <5%) were calculated.

RESULTS

Production of β-1,3-glucanase

A Doehlert matrix for two variables was applied to find the optimum conditions for the β-1,3-glucanase production from *Moniliophthora perniciosa*. The experimental results obtained with the Doehlert design for these variables together with the coded and real values for the variables studied are shown in Table II.

Below, Equation 1 illustrates the relationship between these two variables and the response R, in which R is the enzyme activity, IC is the inducer concentration and T is the fermentation time:

$$\begin{aligned} R = {} & 0.108(\pm 0.008) + 0.011IC(\pm 0.002) \\ & -0.0014IC^2(\pm 0.0001) + 0.0091T(\pm 0.0006) \\ & -0.00043T^2(\pm 0.00002) + 0.00040IC \times T(\pm 0.00006) \end{aligned} \quad (1)$$

The corresponding surface response and level curves graphics for this equation are shown in Figures 1 and 2.

ANOVA has been applied using the experimental data to evaluate the quadratic model fitted. A test based on the Fisher distribution (F-test) has indicated that the fitted equation is statistically significant ($F = 24.65 > 9.01$). A lack-of-fit sum of squares ($F = 16.02 < 18.51$) indicates that there is good agreement between the model's predicted response and the experimental values studied for each variable. Lagrange criterion applied to this equation indicates that the critical point is characterized as a maximum. The derivatization of this general equation as δIC and δT results in the following two new equations:

$$\delta R = 0.011 - 0.0028IC + 0.00040T = 0 \quad (2)$$

$$\delta R = 0.0091 - 0.00086T + 0.00040IC = 0 \quad (3)$$

The results showed that fermentation times longer than 18 days and shorter than 7 days caused a reduction in the amount of β-1,3-glucanase produced by *M. perniciosa*. Concentrations of yeast extract greater than 9 g L^{-1} and less than 3 g L^{-1} caused a decrease in the production of β-1,3-glucanase. Concentrations of yeast extract between 4 and 8 g L^{-1} and fermentation times between 8 and 17 days showed the highest concentrations of β-1,3-glucanase. The optimum activity of 0.2 μmol min^{-1} mL^{-1} was obtained using yeast extract concentration of 5.9 g L^{-1} and a fermentation time of 13 days (Figs. 1 and 2).

Purification of β-1,3-glucanase

The β-1,3-glucanase enzyme was purified by aqueous of *M. perniciosa* as described previously. After chromatography on Sephacryl S-200, it resulted in the separation of three peaks with β-1,3-glucanase activity, named GLUI, GLUII and GLUIII, with 4.33, 1.86 and 3.03-fold purification, respectively (Table III and Fig. 3).

Heat Stability and Optimal pH and Temperature

The β-1,3-glucanase from *M. perniciosa* was active between pH 5.0 and pH 9.0, with maximum specific activity at pH 5.0 (Fig. 4). The effect of temperature on the specific activity of laminarinase is shown in Figure 5. The maximum activity was obtained at 40°C. The effect of temperature on the residual specific activity of glucanase is shown in Figure 5. β-1,3-glucanase retained 99.01, 95.61 and 92.38% of original activity after 50 min of incubation at 60, 70 and 80°C, respectively, and lost 10.20% of its specific activity after 40 min of incubation at 90°C (Fig. 6).

Effect of Cations

The effects of Na^+ and K^+ ions on glucanase activity are shown in Figures 7 and 8. In the presence of KCl, the glucanase activity was higher in the concentration of 0.40 mol L^{-1}, showing an increase of 25% in the activity (Fig. 7), in relation to other analyzed concentrations. In the presence of NaCl, the glucanase activity was higher in a 0.2 mol L^{-1} solution, showing an in-

TABLE II
Analysis of variance for data presented in Table I.

	SS	df	MS	F_{cal}	F_{tab}
Regression	0.00112757	5	0.00022551	24.65	9.01
Residual	0.00002745	3	0.00000915		
Lack-of-Fit	0.00002440	1	0.00002440	16.02	18.51
Pure Error	0.00000305	2	0.00000152		
Total SS	0.00115502	8			

SS – sum of squares; df – degree of freedom; MS – mean square; F_{cal} – calculated F value; F_{tab} – tabulated F value.

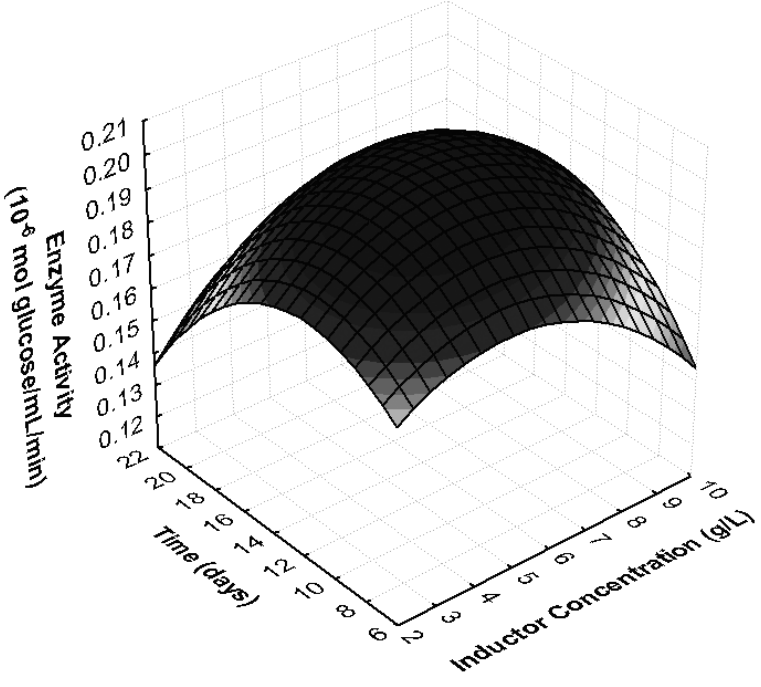

Fig. 1 – Response surface for inductor concentration *versus* incubation time.

TABLE III
Extraction and purification of β-1,3-glucanase.

Purification step	Activity Units (AU)	Protein (mg mL^{-1})	Specific activity (AU mg^{-1} protein)	Purification factor
Ammonium sulfate precipitation	0.088	6.0×10^{-2}	1.47	1
Sephacryl S-200 – GluI	0.035	5.5×10^{-3}	6.36	4.33
Sephacryl S-200 – GluII	0.041	1.5×10^{-2}	2.73	1.86
Sephacryl S-200 – GluIII	0.025	5.6×10^{-3}	4.46	3.03

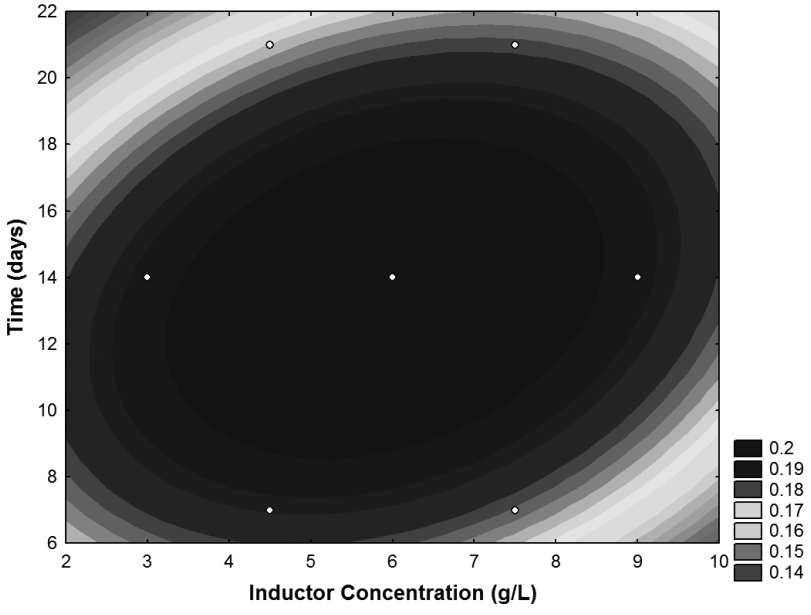

Fig. 2 – Isoresponse contour plot of inducer concentration *versus* fermentation time for β-1,3-glucanase.

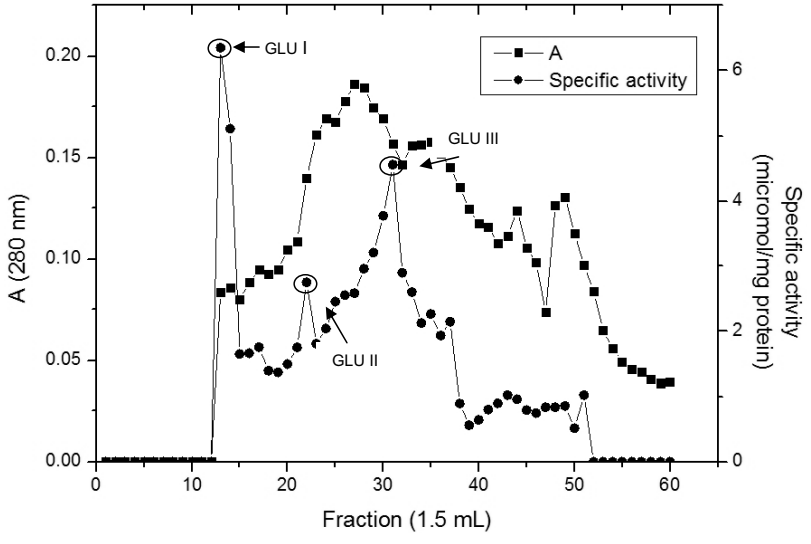

Fig. 3 – Purification of β-1,3-glucanase from *M. perniciosa* on Sephacryl S-200 column: (■) protein absorbance at 280 nm; (●) specific activity.

crease of 8.83% in the activity (Fig. 8), also in relation to other concentrations studied for the same compound.

DISCUSSION

β-1,3-glucanase is one of the most important cell wall lytic enzymes. It has been purified from a number of fungal species in which the enzyme differs in its physiological properties, molecular structure, and molecular weight (McQuilken and Gemmell 2004). It was also speculated that the activity of the enzyme may vary due to the β-glucan structure and the type of linkage (Vazquez-Garciduenas et al. 1998). β-1,3-glucanase has been purified from many plant species, usually by multi-step procedures that employ ion exchange columns followed by gel filtration and hydrophobic interaction.

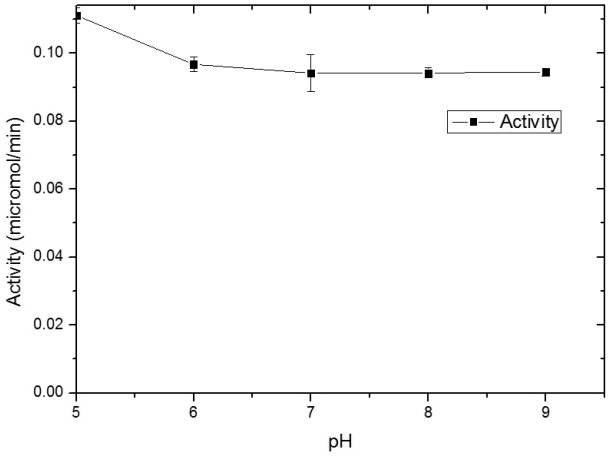

Fig. 4 – Effect of pH on the activity of the purified β-1,3-glucanase from *M. perniciosa*. The pH values were adjusted with the following buffer systems: citrate (5.0-6.0), phosphate (7.0) and Tris-HCl (8.0-9.0). The enzyme activity was measured at 50°C for 15 min.

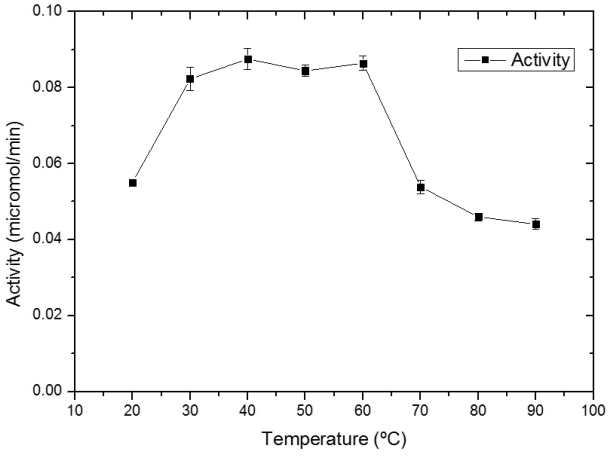

Fig. 5 – Effect of temperature on the activity of the purified β-1,3-glucanase from *M. perniciosa*. All reactions were carried out in 0.05 mol L^{-1} of citrate buffer (pH 6.2) for 15 min. The temperatures tested ranged from 30-90°C.

No previous studies could be found that describe the purification or characterization of a β-glucan degrading enzyme from *M. perniciosa*. The extracellular β-1,3-glucanases in the supernatant of the cell culture of *M. perniciosa* were partially purified and obtained in three isoforms, in which the optimal pH and temperature were 4.0 and 40°C, respectively. The isozymes showed high thermal stability, losing only 10.20% of their specific activity after 40 min of incubation at 90°C.

Murray et al. (2001) obtained a purification fold of 23.00 when they purified glucanase from *Talaromyces emersonii* by gel filtration, Hydrophobic Interaction Chromatography, and anion exchange. Boyce and Walsh (2007) obtained a purification fold of 9.00 when they purified glucanase using various purification steps. The β-1,3-glucanase from *Periconia byssoides* was purified by HiTrap Q XL, resulting in a purification fold of 4.50.

Studying the influence of metal ions on the activity of the α-1,3-glucanase showed that K^+ cations had an activating effect that was greater than that seen with Na^+ cations.

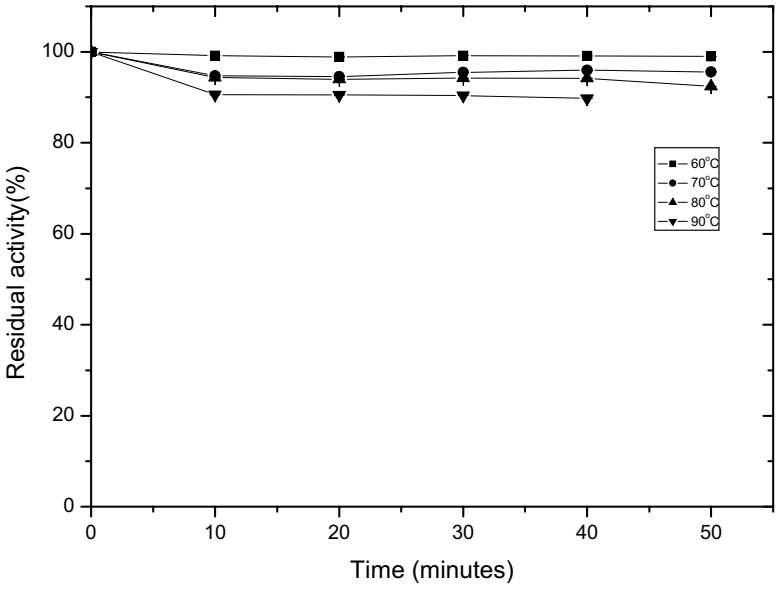

Fig. 6 – Effect of stability of the purified β-1,3-glucanase from *M. perniciosa*. All reactions were carried out in 0.05 mol L^{-1} citrate buffer (pH 6.2) in the range of 60-90°C for 15 min. The percentage of residual β-1,3-glucanase activity was determined under standard assay conditions.

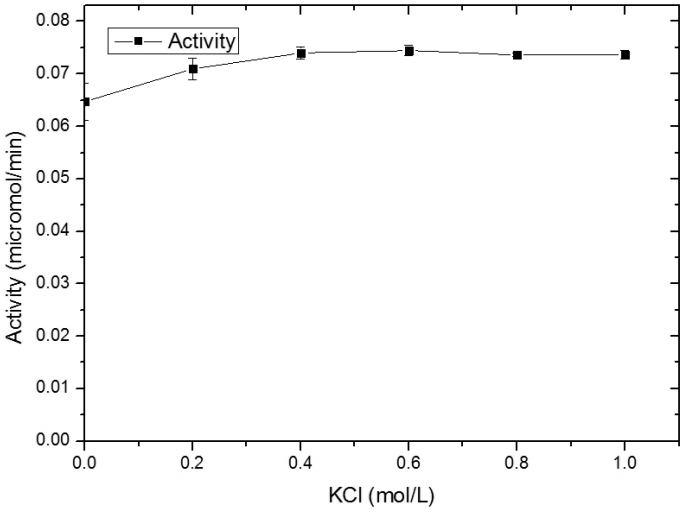

Fig. 7 – The effect of KCl on β-1,3-glucanase activity in the range of 0.2-1 mol L^{-1}. All reactions were carried out in 0.05 mol L^{-1} citrate buffer (pH 6.2) at 50°C for 15 min.

Notario et al. (1976) found that the β-1,3-glucanases are acidic proteins, and our results agree with theirs. Similar values of pH have been reported for β-1,3-glucanases from other fungi, including *Trichoderma harzianum* (Marco and Felix 2007) and *Trichoderma asperellum* (Bara et al. 2003).

Optimal temperatures of 55-60°C, 45°C, and 65°C were reported for β-glucanases from *Bacillus polymyxa* (Gosalbes et al. 1991), *B. pumilis* (Suzuki and Kancho 1976) and *B. brevis* (Louw et al. 1993), respectively. Similar values have been reported, but the optimum temperature of *M. perniciosa* was lower when compared to

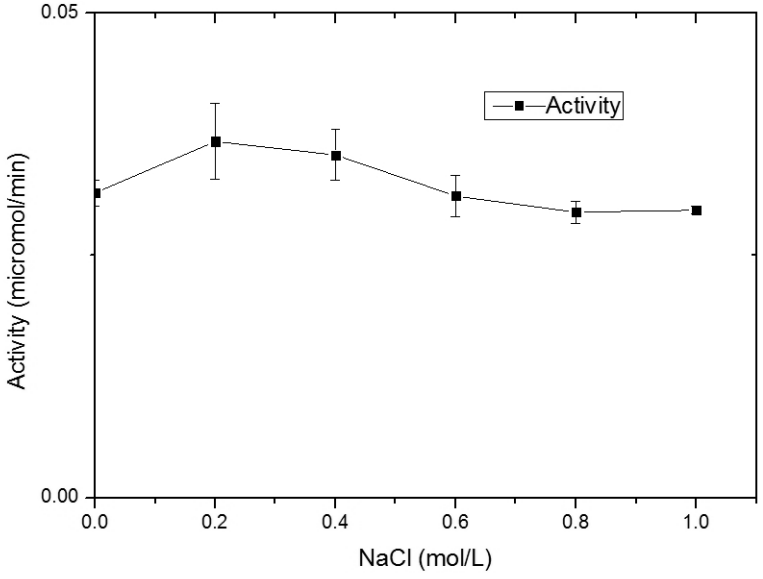

Fig. 8 – The effect of NaCl on β-1,3-glucanase activity in the range of 0.2-1 mol L^{-1}. All reactions were carried out in 0.05 mol L^{-1} citrate buffer (pH 6.2) at 50°C for 15 min.

most others (Nagata et al. 1990, Fontaine et al. 1997, Bara et al. 2003, Marco and Felix 2007, Li et al. 2007), and higher in relation to *T. harzianum* (Théodore and Panda 1995).

Murray et al. (2001) have also pointed out that the thermal stability of the *T. emersonii* enzyme is noteworthy because the estimated $t_{1/2}$ values at 70°C and 80°C were 136 min and 25 min, respectively, while 15% of the original activity remained after 15 min at 100°C in the absence of substrate. The β-1,3-glucanase of *Agaricus brasiliensis* was stable up to 50°C and showed a half-life of 30 min when incubated at 55°C (SHU et al. 2006). Low thermal stability was found at 45, 50 and 60°C in *Trichoderma harzianum* (Marco and Felix 2007), and half-life values of 55, 21.5 and 5 minutes were found for the same enzyme in *Chaetomium thermophilum*, when incubated at 65, 70 and 80°C. This demonstrates the superiority of β-1,3-glucanase from *M. perniciosa* in comparison with the thermal stability of other fungi studied by other authors. Thus, β-1,3-glucanase could be effective at the standard temperatures typically used for industrial processes.

The results of this study have shown that *M. perniciosa* could produce β-1,3-glucanase in the submerged culture, which contains three isoforms. The β-1,3-glucanases showed high thermostability and increased activity in the presence of KCl, with concentrations varying from 0.1 to 1.0 M. More studies are necessary to investigate the production of glucanases from *M. perniciosa*, to explore the possibility of molecular targets as a disease control for witches' broom, and to apply this enzyme in industrial processes.

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RESUMO

A enzima glucanase de *Moniliophthora perniciosa* foi produzida em meio líquido e purificada a partir do sobrenadante da cultura. A metodologia de superfície de resposta (MSR) foi usada para avaliar os efeitos das variáveis, incluindo indutor (extrato de levedura) e tempo de fermentação, na atividade da glucanase de *M. perniciosa* detectada no meio de cultura. A enzima presente no sobrenadante foi purificada em duas etapas: precipitação com sulfato de amônio (70%) e cromatografia de filtração em gel em Sephacryl S-200. A produção da enzima glucanase foi maior na concentração de 5,9 g L^{-1} de extrato de levedura e 13 dias de fermentação. Os resultados mostraram três diferentes isoformas (GLUI, GLUII e GLUIII) com fatores

de purificação de 4,33, 1,86 e 3,03, respectivamente. O extrato enzimático parcialmente purificado mostrou um pH ótimo de 5,0 e uma temperatura ótima de 40°C. A atividade enzimática aumentou na presença de KCl em todas as concentrações estudadas. A atividade da glucanase foi maior na presença de NaCl 0,2 M. A enzima apresentou alta estabilidade térmica, perdendo apenas 10,20% de sua atividade específica após 40 minutos de incubação a 90°C. Os resultados de termoestabilidade e a atividade em baixo pH mostraram que a enzima glucanase de *M. perniciosa* tem características promissoras para futuras aplicações industriais.

Palavras-chave: glucanase, *Moniliophthora perniciosa*, produção, caracterização cinética, purificação, estabilidade térmica.

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