UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS BIOLÓGICAS
PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

Produção e caracterização parcial da enzima galactose oxidase de *Fusarium acuminatum*

Dayane Alberton

Maringá
2005

Dayane Alberton

Produção e caracterização parcial da enzima galactose oxidase de *Fusarium acuminatum*

Dissertação apresentada ao Curso de Pós-graduação em Ciências Biológicas (área de concentração – Biologia Celular) da Universidade Estadual de Maringá para a obtenção do grau de Mestre em Ciências Biológicas.

Maringá
2005

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

A334p

Alberton, Dayane
Produção e caracterização parcial da enzima galactose oxidase de *Fusarium acuminatum* / Dayane Alberton. – Maringá, PR : [s.n.], 2005.
40 f. : il.

Orientador : Profª. Drª. Ione Parra Barbosa Tessmann; co-orientadora : Profª. Drª. Rosane Marina Peralta
Dissertação (mestrado) - Universidade Estadual de Maringá . Pós-Graduação em Ciências Biológicas, 2005.

1. Galactose oxidase(enzima). 2. *Fusarium acuminatum*(fungo). I. Título. II. Universidade Estadual de Maringá. Centro de Ciências Biológicas.

CDD 21.ed.660.6

Zenaide Soares da Silva CRB 9/1307

Orientadora: Prof[a]. Dr[a]. Ione Parra Barbosa Tessmann

Co-orientadora: Prof[a]. Dr[a]. Rosane Marina Peralta

AGRADECIMENTOS

Agradeço à Prof[a]. Dr[a]. Ione Parra Barbosa Tessmann, pela orientação segura e eficiente, e pela amizade, sem as quais este trabalho não teria sido realizado.

Agradeço à Prof[a]. Dr[a]. Rosane Marina Peralta, pela co-orientação deste trabalho.

Agradeço ao Programa de Pós-graduação em Ciências Biológicas, por proporcionar, por meio de seus professores e funcionários, o ambiente propício e o estímulo para o desenvolvimento do espírito científico.

Agradeço ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão de bolsa que possibilitou a realização deste trabalho.

Agradeço ao Prof. Dr. Carlos Kemmelmeier, pela doação de isolado, e ao Prof. Dr. Dauri José Tessmann, pela ajuda com as análises estatísticas.

Agradeço aos meus companheiros de laboratório: Elainy Patrícia Lino Gasparotto e Gleison Ricardo de Biazio, pelo apoio racional e emocional.

Agradeço a Alvina Chaves, Márcia Fabrício e Armando

ABSTRACT

The enzyme galactose oxidase belongs to the family of oxidases with one copper radical. It is a monomeric protein with 68 kDa and it has in the active site two important components to the catalysis: one copper ion and one tyrosil radical, that is formed by a thioeter bond between the amino acid residues Cys 228 and Tyr 272. This enzyme is produced in an inactive precursor form, which has an amino terminal pro-sequence of 17 amino acids residues and in which is absent the tyrosil radical. In the presence of copper and molecular oxygen, this enzyme promotes its own maturing by the removal of the amino terminal pro-sequence and by the formation of the thioeter bond. The enzyme active mature form is able to catalyze the oxidation of two electrons from primary alcohols to the correspondent aldehydes, with concomitant reduction of molecular oxygen to hydrogen peroxide.

Although the biological role of the galactose oxidase is unknown, the chemical characteristics of its molecule and its interesting catalytic mechanism are considered as appeals to its biotechnological and biomedical use. This enzyme has several substrates, is specific for the C6 hydroxyl group of the galactose and derivatives, has a structural simplicity, and a considerable stability, being active in considerably high concentrations of urea. Due to these properties, this enzyme has applications in: galactose and lactose concentration determination in dairy industry and in clinical analysis, histochemistry studies, carbohydrate synthesis, transformation of polysaccharides in paper strength additives, and cancer diagnosis.

The enzyme galactose oxidase is secreted by few species of filamentous fungi. The most studied producer microorganism is *Fusarium graminearum*. The extra cellular enzyme of this fungus has been extensively characterized and used in the applications of

the galactose oxidase. Although *F. graminearum* is the known microorganism with the major galactose oxidase enzyme productivity ability, its production level is still considered low and the secreted enzyme has limitations in some of its biochemical and kinetic characteristics, that compromises its effective use. Because of this, it would be interesting the search for other microorganisms with higher producer potential and that could secrete an enzyme with better biochemical and kinetic characteristics.

Another galactose oxidase enzyme producer microorganism, recently identified, is *Fusarium acuminatum*. The production and the biochemical properties of the enzyme secreted by this fungus were not yet studied. Considering this, the objective of this work were to study the production of the enzyme galactose oxidase by *F. acuminatum* in submerse fermentation and to study some of the biochemical properties of the enzyme from this fungus.

The production of the enzyme galactose oxidase by *F. acuminatum* was first identified in a mineral liquid medium proposed to produce galactose oxidase using *F. graminearum*. Modifications in this medium were proposed to optimize the production of the enzyme by *F. acuminatum*. An inoculum of 2% (vol/vol) in a medium with initial pH of 7.0 was identified as the best for enzyme production. Increments in the culture medium buffer system concentration did not alter the production. The same production level were obtained when glucose, galactose, soluble starch, or sucrose were used as carbon source, however, there was a decrease in the production when lactose or sorbose were used. The ions Cu^{2+}, Mg^{2+}, and Mn^{2+} are essential for enzyme production, but small variations in the concentration of the Cu^{2+} ion did not alter the production. When *F. acuminatum* was grown in the absence of copper, an apoenzyme, which could be activated by incubation with the ion Cu^{2+}, was produced. At last, the presence of Ca^{2+} ion in the culture medium seems to have a positive effect in the enzyme production.

The results of the biochemical analysis performed with the enzyme galactose oxidase of the *F. acuminatum* culture medium have revealed that the optimum pH of the enzyme is 7.5; the best temperature of action is 30ºC; the molecule is thermo stable in temperatures up to 50ºC; and it is able to oxidize the following substrates: dihydroxyacetone, D-(+)-raffinose, α-D-(+)-melibiose, D-(+)-galactose, and methyl-α-D-galactopyranoside.

The results of the polyacrylamide gel electrophoresis analyses have shown that the enzyme galactose oxidase from *F. acuminatum* has a lower molecular weight and is less basic than the *F. graminearum* enzyme. Because of its lower basicity, the enzyme from *F. acuminatum* could be of better application in histochemistry studies. A less basic protein is less inhibited or inactivated by macromolecular contaminants and is more reactive with glycoconjugates attached on the cell membranes.

In conclusion, this work has delineated some aspects of the galactose oxidase enzyme production by *F. acuminatum* and has pointed out directions for future experiments. In addition, some of the *F. acuminatum* galactose oxidase enzyme studied biochemical characteristics have demonstrated interesting structural and chemical differences between this enzyme and the enzyme from *F. graminearum* that should be better investigated.

RESUMO

A enzima galactose oxidase pertence à família das oxidases com um radical de cobre. Ela é uma proteína monomérica com 68 kDa e no sítio ativo possui dois componentes importantes para a catálise: um íon cúprico e um radical tirosil, formado por uma ligação tioéter entre os resíduos de aminoácidos Cys 228 e Tyr 272. Esta enzima é produzida em uma forma precursora inativa, que contém uma pró-seqüência de 17 resíduos de aminoácidos na extremidade aminoterminal e na qual está ausente o radical tirosil. Na presença de cobre e oxigênio molecular, a enzima promove seu amadurecimento pela remoção da pró-seqüência e pela formação da ligação tioéter. A forma madura e ativa é capaz de catalisar a reação de oxidação de dois elétrons de álcoois primários para aldeídos correspondentes, com concomitante redução do oxigênio molecular para peróxido de hidrogênio.

Embora o papel biológico da galactose oxidase seja desconhecido, as características químicas de sua molécula e o seu interessante mecanismo catalítico são de grande apelo para o seu emprego biotecnológico e biomédico. Essa enzima tem vários substratos, é estereoespecífica para o grupo hidroxila do C6 da galactose e derivados, tem uma simplicidade estrutural e uma considerável estabilidade, sendo ativa em concentrações consideravelmente altas de uréia. Devido a essas propriedades, a galactose oxidase tem aplicações em: dosagens de galactose e lactose em indústrias de laticínios e em análises clínicas, estudos de histoquímica, síntese de outros carboidratos, transformação de polissacarídeos em aditivos que dão resistência a papéis e detecção precoce de câncer.

A enzima galactose oxidase é secretada por algumas espécies de fungos filamentosos. O microorganismo secretor mais estudado é o *Fusarium graminearum*. A enzima extracelular desse fungo tem sido extensivamente caracterizada e utilizada nas

aplicações da galactose oxidase. Embora o *F. graminearum* seja o microorganismo conhecido com maior capacidade produtora da enzima galactose oxidase, a sua produção ainda é considerada baixa e a enzima secretada apresenta limitações em algumas das suas características bioquímicas e cinéticas, que comprometem a sua efetiva utilização. Por isso, seria interessante a pesquisa de outros microorganismos com maior potencial produtor da enzima galactose oxidase e que pudessem secretar uma enzima com características bioquímicas e cinéticas melhores.

Um outro microorganismo produtor da enzima galactose oxidase, recentemente identificado, é o *Fusarium acuminatum*. A produção e as propriedades bioquímicas da enzima produzida por este microorganismo ainda não foram estudadas. Considerando o exposto, os objetivos deste trabalho foram estudar a produção da enzima galactose oxidase por *F. acuminatum* em fermentação submersa e estudar algumas propriedades bioquímicas da enzima deste fungo.

A produção da enzima galactose oxidase pelo *F. acuminatum* foi primeiramente identificada em um meio de cultura líquido mineral proposto para a produção de galactose oxidase por *F. graminearum*. Modificações neste meio foram propostas para otimizar a produção da enzima por *F. acuminatum*. Um inóculo de 2% (vol/vol) em um meio com pH inicial de 7,0 foi identificado como o melhor para a produção da enzima. Aumentos na concentração do sistema tampão do meio de cultivo não alteraram a produção. Os mesmos níveis de produção da enzima foram obtidos quando glicose, galactose, amido solúvel ou sacarose foram utilizados como fonte de carbono, no entanto, houve um decréscimo na produção quando lactose e sorbose foram utilizadas. Os íons Cu^{2+}, Mg^{2+} e Mn^{2+} são essenciais para a produção da enzima, mas pequenas alterações na concentração do íon Cu^{2+} do meio não alteraram a produção. Quando o *F. acuminatum* foi crescido na ausência de cobre, uma apoenzima, que foi ativada por incubação com o íon Cu^{2+}, foi produzida.

Por último, a presença do íon Ca^{2+} no meio de cultivo parece ter um efeito positivo na produção da enzima.

Os resultados das análises bioquímicas realizadas com a enzima galactose oxidase do meio de cultura de *F. acuminatum* revelaram que o pH ótimo da enzima é 7,5; a melhor temperatura de ação da enzima é 30ºC; a molécula é termoestável em temperaturas inferiores a 50ºC; e é capaz de oxidar os seguintes substratos: diidroxiacetona, D-(+)-rafinose, α-D-(+)-melibiose, D-(+)-galactose e metil-α-D-galactopiranosídeo.

Os resultados das análises de eletroforese em gel de poliacrilamida mostraram que a enzima galactose oxidase de *F. acuminatum* possui um peso molecular menor e é menos básica do que a enzima de *F.graminearum*. Por ser menos básica, a enzima de *F. acuminatum* pode ser melhor aplicada em estudos de histoquímica. Uma proteína menos básica é menos inibida ou inativada por contaminantes macromoleculares e é mais reativa com glicoconjugados ligados às membranas celulares.

Em conclusão, este trabalho delineou alguns aspectos da produção da enzima galactose oxidase por *F. acuminatum* e apontou diretrizes para futuros estudos de produção. Em adição, algumas das características bioquímicas estudadas da enzima galactose oxidase de *F. acuminatum* demonstram interessantes diferenças estruturais e químicas da enzima de *F. graminearum*, as quais deveriam ser melhor investigadas.

APRESENTAÇÃO

Esta dissertação de mestrado é apresentada na forma do seguinte artigo científico:

ALBERTON, D.; PERALTA, R.M.; AND BARBOSA-TESSMANN, I.P. Production of Galactose Oxidase from *Fusarium acuminatum* in Submerged Fermentation and its Partial Characterization. Trabalho submetido ao periódico científico Process Biochemistry.

Running title: Galactose oxidase from *Fusarium acuminatum*

Key words: galactose oxidase, *Fusarium acuminatum*, *Fusarium*

Abstract

Extra-cellular galactose oxidase production by *Fusarium acuminatum* using submerged fermentation was studied. Glucose (1.0% w/v) was used as sole carbon source. Maximum galactose oxidase production (approximately 4.0 U/ml) was obtained when fermentation was carried out at 25°C, with orbital shaking (100 rpm), with an initial medium pH of 7.0, for 96 h, using an inoculum of 2% (v/v) made of a homogenized four days old liquid culture, and in the presence of copper, manganese, and magnesium. The optimum pH of the enzyme was 7.5, the best temperature of action was 30^{o}C, at pH 7.0, and the enzyme was stable at temperature up to 50^{o}C, at pH 7.0. The enzyme was negatively charged at pH 8.9 and had an apparent MW of 60 kDa.

1. Introduction

The enzyme galactose oxidase (GO) (D-galactose: oxygen 6-oxidoredutase, EC 1.1.3.9.) is a radical copper oxidase that catalyzes the transfer of two-electron from hidroxylic substrates, including galactose, to molecular oxygen, with formation of hydrogen peroxide and an aldehyde [1,2,3], according to the following reaction:

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$$

Some interesting characteristics of this enzyme are: high stereospecificity, broad substrate tolerance, and strict selectivity of primary alcohols oxidation. These characteristics guarantee the several biotechnological and biomedical applications of this enzyme, which are: lactose and galactose concentration determination in milk

characteristics and the production technology processes of the enzyme from *F. acuminatum* are completely unknown. Considering the several applications of the enzyme GO, the problems associated with its production and purification, and the great number of research going on about *F. graminearum* GO production, characterization, and enzyme engineering, it was assumed that the enzyme from *F. acuminatum* should also be studied. Taking this in consideration, the goal of the present study was to optimize the culture conditions that influence galactose oxidase production by *F. acuminatum* in submerged fermentation and to investigate some biochemical properties of the secreted enzyme.

2. Materials and methods

2.1. Reagents

D-(+)-Raffinose pentahydrate, α-D-(+)-melibiose hydrate, and methyl-α-D-galactopyranoside were purchased from Across Organics. L-(-)Sorbose was bought from Fluka BioChemika. β-Alanine and 1,3-dihydroxyacetone dimmer were purchased from Aldrich. Gum guar, acrylamide, N,N'-methylene-bis-acrylamide, β-mercaptoethanol,

ammonium persulfate, glycine, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, Trizma base, TEMED, peroxidase, molecular weight markers, Tween 80, and *o*-dianisidine were obtained from Sigma. EDTA dissodium was bought from Promega. All other reagents were of analytical grade or better.

2.2. Microorganism and maintenance

F. acuminatum UnB 356 was a kind gift from Dr. J.C. Dianese (Plant Pathology Department, University of Brasília, Brasília, DF, Brazil). This strain of *F. acuminatum* was previously identified as a galactose oxidase producer [20]. The original galactose oxidase producer strain of *F. graminearum* [24,25] was kindly provided by Dr. C. Kemmelmeier (Departamento de Bioquímica, Universidade Estadual de Maringá, Maringá, PR, Brazil) and used as a control. These isolates are being maintained in potato dextrose agar (PDA) slants with trimestral transfer and in stocks under mineral oil [26].

2.3. Inoculum preparation and submerged fermentation

A fragment (1 cm^3) from a fresh PDA slant culture was aseptically transferred and smashed in 125 ml Erlenmeyer flasks containing 25 ml of the liquid medium described by Markus et al. [27] but modified to obtain 100 mM of phosphate buffer (solution A: 15.14 mM $(NH_4)_2SO_4$, 12.5 mM NH_4NO_3, 0.1% yeast extract, 42 mM KH_2PO_4, 58 mM Na_2HPO_4, with pH corrected to 7.0 with HCl or NaOH; solution B: 1.62 mM $MgSO_4 \cdot 7H_2O$, 12 µM $MnSO_4 \cdot H_2O$, 10 µM $CuSO_4 \cdot 5H_2O$). Solution A and B were sterilized by autoclaving (120^oC, 1 atm, 20 min), separately, and then combined aseptically. Before inoculation, the medium was supplemented with a solution of D-glucose (sterilized by autoclaving at 100^oC, without pressure, 20 min) to a final concentration of 1%.

Cultures were grown for 4 days, at 25ºC, on a rotary shaker at 100 rpm, in dark, and, then, homogenized through passage first in a sterile inox sieve and second in a sterile syringe with needle (40×12). The homogenized culture was used as inoculum (2% vol/vol) to other 125 ml flasks containing 25 ml of the same liquid medium. The subcultures were grown for 96 hours or for the indicated time in the same conditions, and then, filtered through filter paper. The culture filtrates were utilized for enzymatic analysis. When pointed out, the

mycelia biomass was estimated by drying the mycelia at 50ºC until constant weight.

2.4. Enzyme assay

Galactose oxidase activity was assayed by the peroxidase/*o*-dianisidine colorimetric method [28,29], based on the estimation of the colored oxidized *o*-dianisidine. The assay mixture comprised: 0.5 ml of the culture filtrate (pure or diluted), 0.1 ml of 0.5 M D-galactose, as substrate, and 1.4 ml of the following reaction solution: 0.04 mg/ml (6 U/ml) peroxidase (Sigma cat. P-8125), 0.2 mg/ml (previously dissolved in methanol 2 mg/ml) *o*-dianisidine (Sigma cat. D-3252), and 50 mM phosphate buffer (29 mM Na_2HPO_4 and 21 mM NaH_2PO_4), pH 7.0. The assay mixture was incubated for 10 minutes at 30ºC before reading the absorbance. A control assay mixture without galactose was used for each sample to calibrate the spectrophotometer. One enzyme unit was defined as the amount of enzyme that causes absorption of 1.0 at 460 nm in the assay condition [29].

2.5. Optimization of culture conditions

Different variables were tested to determinate the optimum conditions for GO enzyme production. To evaluate if the inoculum size could improve the enzyme production, the subcultures were inoculated with 1%, 2%, and, 6% (vol/vol) of the inoculum. The effect of the culture medium initial pH on the enzyme production was tested by adjusting the 100 mM phosphate buffer of the culture medium to obtain pH 6.0 (87.7 mM KH_2PO_4, 12.3 mM Na_2HPO_4) or 7.0 (as above). The effect of the culture medium phosphate buffer concentration on enzyme production was also analyzed. The influence of different carbon sources (1%) was studied by replacing glucose with galactose, lactose, soluble starch, sucrose, and sorbose. To determine the importance of the presence of Cu^{2+}, Mg^{2+}, and Mn^{2+} in the basal medium for enzyme production, a medium without those ions was prepared and used for the inoculum preparation. This same medium was used for the subcultures with the following additions: 1) Cu^{2+}, Mg^{2+}, and Mn^{2+}, as a control; 2) Cu^{2+} only; 3) Mg^{2+} and Mn^{2+} only; 4) Cu^{2+} (5 µM), Mg^{2+}, and Mn^{2+}; and 5) Cu^{2+} (20 µM), Mg^{2+}, and Mn^{2+}. When not indicated, concentrations were the same of the basal medium. The subculture filtrates without Cu^{2+} or without Mg^{2+} and Mn^{2+} had these ions added and were incubated for 24 hours, at 25^{o}C, under

orbital shaking (100 rpm), in dark, to evaluate the production of apoenzyme. Other reagents including: 11 µM $CaCl_2 \cdot 2H_2O$, 11 µM $CoCl_2 \cdot 6H_2O$, 11 µM $FeCl_3$, 11 µM $ZnSO_4 \cdot 7H_2O$, 11 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 25 mM KCl, and 1.6 mM H_3BO_3 were also used in the culture medium to test their importance for enzyme production. The importance of the presence of a surfactant during or after the culture for enzyme production was estimated by subculturing the microorganism with 0.2% Tween 80 or by the addition of this component (0.2%) after the subculture, with incubation for 30 minutes, before filtration. The effect of the cultivation temperature on the enzyme production was assessed by subculturing the microorganism at 25°C or 30°C.

2.6. Optimal pH and temperature, thermal stability, and substrates

Due to the *F. acuminatum* GO low-level production, the enzyme was not purified and the biochemical characterization was performed in the culture filtrates. The enzyme optimal pH determination was carried out by changing the phosphate buffer pH in the enzyme assay. To check the enzyme best action temperature, the enzyme assay was performed in different temperatures. To determine the thermal stability of the enzyme, the filtrate was incubated for 30 minutes in different

temperatures, and, then, submitted to the enzyme assay. To verify the range of activity toward different substrates, the enzyme assay was conducted with the substitution of the 0.1 ml of 0.5 M D-(+)-galactose by 0.1 ml of 0.5M D-(+)-glucose, 0.5 M D-(+)-raffinose, 1% guar gum, 0.5 M lactose monohydrate, 0.5 M 1,3-dihydroxyacetone dimer, 0.5 M α-D-(+)-melibiose, and 0.5 M methyl-α-D-galactopyranoside.

2.7. PAGE analysis

For the electrophoresis analysis, the proteins of the filtrate of two or three subculture flasks of *F. acuminatum* and *F. graminearum* were precipitated by addition of ammonium sulfate to 90% saturation. The precipitated proteins were centrifuged at 20.000*g* for 10 minutes at room temperature. The pellet was resuspended in 10 mM phosphate buffer, pH 7.0, and dialyzed once against 10 mM phosphate buffer containing 0.4 mM $CuSO_4$ and 0.5 mM EDTA, and two more times with the same buffer but omitting each time the EDTA and $CuSO_4$ [29].

PAGE analysis was carried out in two ways. In the first one, 12.5 µl of the dialyzed proteins were added to 12.5 µl of the sample dilution buffer (0.44 M potassium acetate, pH 6.8; 0.1 mg/ml basic fuchsin; and 10% glycerol) and loaded on a 7.5% polyacrylamide running gel, pH 4.3, with a 5% stacking gel, pH 6.8, in the β-alanine-acetic acid buffer

system of Reisfeld et al. [30]. In the second one, the methodology described by Laemmli [31] for denaturing gels was followed, but omitting SDS and β-mercaptoethanol. For that, 12.5µl of the dialyzed proteins were added to 12.5 µl of the sample dilution buffer and loaded on a 7.5% polyacrylamide running gel, pH 8.9, with a 4.5% stacking gel, pH 6.7, in the Tris-glycine buffer system. Both gels were developed with the *o*-dianisidine/peroxidase/phosphate buffer mixture described above containing 25 mM galactose, for 2 hours, at room temperature.

2.8. Non-denaturing SDS-PAGE analysis

The methodology described by Laemmli [31] was followed. Twelve and half µl of the sample dilution buffer without β-mercaptoethanol were added to 12.5 µl of the dialyzed protein, prepared as above. This mixture was loaded, without boiling, on a 10% polyacrylamide running gel, pH 8.9, with a 4.5% stacking gel, pH 6.7, in the Tris-glycine buffer system. Molecular weight markers (Sigma cat. M 3788) were treated in the same way. Each gel was developed by incubation with the enzyme reaction as described in the PAGE analysis. Some gels were also developed by the silver staining [32],

after the enzymatic reaction, for visualization of the molecular weight markers.

2.9. Statistical analysis

Statistical analyses were done by calculation of the average and standard deviation of the results. When indicated, data were submitted to the procedure ANOVA and compared by the Tukey test ($p<0.05$) using the SAS program (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. Media optimization for growth and galactose oxidase production

An inoculum level of 1-6% was used in the cultivation medium to establish the effect of inoculum size on enzyme production by *F. acuminatum*. The higher yields in the enzyme production, about 6 U/ml of filtrate, were obtained with the smallest inoculum sizes (Fig. 1). The lower enzyme production with the largest inoculum size (6%) may be due to the excessive initial growth. Even though the initial growth was higher with higher inoculums, the final mycelia yields were similar in all

used inoculums sizes. Markus et al. [27] have also found that a *F. graminearum* slowly growing mycelia produced more GO enzyme than a rapidly growing one. Thus, in all subsequent experiments, an inoculum of 2% was used because it gave a good enzyme yield and the fungi grow log phase was reached within 24 hours of culture. A culture time of 96 hours was also adopted in the subsequent experiments because the enzyme is produced at almost maximum level without culture deterioration (Fig. 1).

The initial pH of the culture medium had an effect on GO production by *F. acuminatum*. The amount of GO enzyme released in the medium was higher when the microorganism was grown in a medium which initial pH was 7.0 and was lower when the initial pH was 6.0 (Fig 2a). Shatzman and Kosman [33] have found similar results for GO production by *F. graminearum*.

As the medium initial pH had an effect on enzyme production and there is probable variation in pH during the fungi growth, it was thought that a more concentrated pH buffer system could improve the enzyme production. To test that, the liquid medium was prepared with the following phosphate buffer (KH_2PO_4 and Na_2HPO_4) concentrations: 100, 125, 150, 175, and, 200 mM, pH 7.0. The statistical analysis of the results (Fig. 2b) demonstrates that there was no influence of the phosphate buffer concentration on enzyme production. However, the

dried biomass weight was higher when higher concentrations of the phosphate buffer were used. An increased cell density associated with either higher concentration of the carbon source in the culture medium or medium pH is reported to have a detrimental effect on GO enzyme production by *F. graminearum* [27,33]. In all subsequent experiments, the culture medium was prepared to have 100 mM phosphate buffer, pH 7.0, because this condition has promoted the lowest microorganism growth associated with a good enzyme production.

The effect of different carbon sources added to the liquid medium on the production of GO by *F. acuminatum* was also investigated. Several alternative carbon compounds were added to the medium in substitution of glucose. The statistical analyses of the results (Table 1) have shown that the GO enzyme production was not different when glucose, galactose, sucrose, or soluble starch were used as carbon sources. However, when sorbose or lactose were used, there was a decrease in GO production (Table 1). Moreover, in the medium containing lactose, there was also a decrease in the microorganism growth (Table 1). In agreement with our results, glucose, galactose, and sucrose are reported to be good carbon sources for GO production by *F. graminearum* and *G. fujikuroi* [19,25,27], and lactose is also reported to decrease the GO production by *F. graminearum* and *G. fujikuroi* [19,25]. However, the reports in the literature for

soluble starch and sorbose as carbon sources for GO production by *F. graminearum* [27] are in opposition of the results found for *F. acuminatum*

Figure 3b. The addition of Cu^{2+} but not of Mg^{2+} and Mn^{2+} was able to promote the appearing of an enzymatic activity, indicating the presence of an activated enzyme. This result indicates that Mg^{2+} and Mn^{2+} are necessary for enzyme production but not for its activation.

The addition of Ca^{2+} in the culture medium had a slightly positive effect on *F. acuminatum* GO enzyme production (Table 2). Conversely, calcium is reported to be an inhibitor of GO production by *F. graminearum* [25]. Further tests with different concentrations of Ca^{2+} need be conducted for clarification of its role on *F. acuminatum* GO enzyme production. The addition of Co^{2+}, Mo^{2+}, and Zn^{2+} had a negative effect on *F. acuminatum* GO enzyme production and Mo^{2+} also caused a decrease in fungal growth (Table 2). Zinc is also reported to decrease the *G. fujikuroi* GO production [19], however, it is reported to have a stimulatory effect on *F. graminearum* GO production [24]. Addition of ferric ion in the culture medium did not cause any change on *F. acuminatum* GO enzyme production, but it did promote a decreased in fungal growth (Table 2). The addition of potassium to the culture medium to replace the potassium removed from the medium when the phosphate concentration was changed did not cause any improvement on the *F. acuminatum* GO production (Table 2). The addition of the surfactant Tween 80 in the culture medium during or after fungal growth did not contribute for an increase

in the enzyme production, although it did increase the cellular density (Table 2).

The amount of enzyme secreted when *F. acuminatum* was cultivated at 25 or 30^{o}C was not different (results not shown).

3.2. Optimal pH and temperature, thermal stability, and substrates

Data shows that the *F. acuminatum* GO enzyme optimum pH and temperature, under the used conditions, were 7.5 and 30^{o}C, respectively (Fig. 4a and b). The reported optimum pH for the *F. graminearum* GO is 6.7-7.3 [12,24,34]. The somewhat higher optimum pH of the *F. acuminatum* GO enzyme could be explained due probable differences in electric charges between those molecules. The *F. acuminatum* GO enzyme was thermo stable in temperatures bellow 50ºC (Fig. 4c). A sharp decrease in activity was observed above 50^{o}C. The T_{50} (the temperature at which the enzyme loses 50% of its activity following incubation for 30 minutes), calculated from the obtained polynomial regression curve equation, was 61.3ºC (Fig.4c). The *F. acuminatum* GO thermal stability was lower than the thermal stability of the *F. graminearum* GO, that is reported to have a T_{50} of 67^{o}C [21]. Possible reasons for the reduced thermo stability of the *F. acuminatum*

GO could be due to improper formation of disulfide bonds or lack of glycosylation.

One of the most considerable characteristics of the galactose oxidase from *F. graminearum* is its broad substrate specificity [1]. The results presented in Table 3 demonstrate that the *F. acuminatum* GO was able to oxidize all tested *F. graminearum* GO enzyme substrates [1,12,13]. The best substrate for the *F. acuminatum* GO appears to be dihydroxyacetone what is in agreement with the reports for the *F. graminearum* GO [1]. The activity of the enzyme on D-(+)-raffinose, α-D-(+)-melibiose, methyl-α-D-galactopyranoside, lactose, and guar gum indicates that it could oxidize galactose derivatives with substitutes at the carbon-1site. In contrast, the lack of activity of the enzyme on glucose indicated that the stereo configuration of the –OH at the carbon-4 is important for its activity and that the enzyme is specific for galactose and derivatives. The *F. graminearum* GO has this same specificity [1,22].

3.3. Electrophoresis analysis

The results obtained in the PAGE analysis in pH 4.3 (Fig. 5a) have revealed that the *F. acuminatum* GO run more slowly than the *F. graminearum* GO enzyme, indicating probable differences in electric

charge and molecular weight between these two enzymes. The electric charge difference became evident when the enzymes are run in pH 8.9 toward the negative pole (Fig. 5b). In this condition, only the enzyme from *F. graminearum* has entered in the running gel. This result confirms that the *F. graminearum* GO is positively charged at pH 8.9 (pI = 12) [34] and that the enzyme from *F. acuminatum* is not. A highly basic GO protein can be inhibited and inactivated by macromolecular contaminants and can be less reactive with glycoconjugates attached on the cell membranes [12]. One GO protein with decreased basicity has these problems diminished and could be useful for the histological applications of this enzyme.

To compare the MW of the GO produced by *F. acuminatum* and *F. graminearum*, these enzymes were separated in a non-denaturing SDS-PAGE (Fig. 6). In spite of not precise, because of the non-denaturing used conditions, the approximate MW of the proteins that have shown GO activity was calculated in a gel that contained non-denatured molecular weight markers and that was first developed with the reactive mixture and later silver stained (not shown). The *F. graminearum* GO band had an approximate MW of 66 kDa, what is in agreement with the data found in the literature [34,35,36]. However, the *F. acuminatum* GO band had an approximate MW of 60 kDa. The

differences in the MW could be explained in possible differences in size or glycosylation of the proteins.

4. Acknowledgements

This work was funded by: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil (Grant no. 471808/01-6); Fundação Araucária – Secretaria da Ciência e Tecnologia do Governo do Estado do Paraná, Brazil (Grant n°. 420); and International Foundation for Science (IFS), Sweden (Grant n°. F/3343-1) and Organization for the Prohibition of Chemical Weapons (OPCW), The Netherlands. D. Alberton was supported with a scholarship from CNPq. The authors thank Dr. D. J. Tessmann for the help with the statistical analyses.

5. References

[1] Whittaker JW. Galactose oxidase. Adv Prot Chem 2002; 60: 1-49.

[2] Whittaker JW. Free radical catalysis by galactose oxidase. Chem Rev 2003; 103: 2347-63.

[3] Rogers MS, Dooley DM. Copper-tyrosyl radical enzymes. Curr Op Chem Biol 2003; 7: 189-96.

[4] Tkáč J, Šturdík E, Gemeiner P. Novel glucose non-interference biosensor for lactose detection based on galactose oxidase-peroxidase with and without co-immobilised β-galactosidase Analyst 2000; 125: 1285-9.

[5] Adányi N, Szabó EE, Váradi M. Multi-enzyme biosensors with amperometric detection for determination of lactose in milk and dairy products. Eur Food Res Technol 1999; 209: 220-6.

[6] Rypins EB, Sankary H, Wynn MJ. Bedside micro-method for measuring effective hepatic blood flow, with use of first-order galactose clearance pharmacokinetics. Clin Chem 1985; 31: 1557-9.

[7] Karube I, Kimura J, Yokoyama K, Tamiya E. Integrated microbiosensors for clinical diagnosis. Ann N Y Acad Sci. 1990; 613: 385-9.

[8] Franke D, Machajewski T, Hsu CC, Wong CH. One-pot synthesis of L-fructose using coupled multienzyme systems based on rhammulose-1-phosphate aldolase. J Org Chem 2003; 68: 6828-31.

[9] Andreana PR, Xie W, Cheng HN, Qiao L, Murphy DJ, Gu QM, Wang PG. In situ preparation of β-D-1-O-hydroxylamino carbohydrate polymers mediated by galactose oxidase. Org Lett 2002; 4: 1863-6.

[10] Basu SS, Dotson GD, Raetz CRH. A facile enzymatic synthesis of uridine diphospho-[^{14}C]galacturonic acid. Anal Biochem 2000; 280: 173-7.

[11] Liu XC, Dordick, JS. Sugar-containing polyamines prepared using galactose oxidase coupled with chemical reduction. J Am Chem Soc 1999; 121: 466-7.

[12] Mazur, AW. Galactose oxidase. Selected properties and synthetic applications. ACS Symposium Series 1991; 466: 99-110.

[13] Chiu CW, Jeffcoat R, Henley M, Peek L. Aldehyde cationic derivatives of galactose containing polysaccharides used as paper strength additives. US Patent 5554745 (1996).

[14] Schulte BA, Spicer SS. Light microscopic histochemical detection of sugar residues in secretory glycoproteins of rodent and human tracheal glands with lectin-horseradish peroxidase conjugates and the galactose oxidase-Schif sequence. J Histochem Cytochem 1983; 31: 391-403.

[15] Roberts GP, Gupta SK. Use of galactose oxidase in the histochemical examination of mucus-secreting cells. *Nature* 1965; 207: 425-6.

[16] Vucenik I, Gotovac J, Druzijanic N, Shamsuddin AM. Usefulness of galactose oxidase-Schiff test in rectal mucus for screening of colorectal malignancy. Anticancer Res 2001; 21: 1247-56.

[17] Chagpar A, Evelegh M, Fritsche HA Jr, Krishnamurthy S, Hunt KK, Kuerer HM. Prospective evaluation of a novel approach for the use of a quantitative galactose oxidase-Schiff reaction in ductal fluid samples from women with breast carcinoma. Cancer 2004; 100: 2549-54.

[18] Said IT, Shamsuddin AM, Sherief MA, Taleb SG, Aref WF, Kumar D. Comparison of different techniques for detection of Gal-GalNAc, an early marker of colonic neoplasia. Histol Histopathol 1999; 4: 351-7.

[19] Aisaka K, Terada O. Production of galactose oxidase by *Gibberella fujikuroi*. Agric Biol Chem 1981; 45: 2311-16.

[20] Barbosa-Tessmann IP, Da Silva DA, Peralta RM, Kemmelmeier C. A new species of *Fusarium* producer of galactose oxidase. J Basic Microbiol 2001; 41: 143-8.

[21] Sun L, Petrounia IP, Yagasaki M, Bandara G, Arnold FH. Expression and stabilization of galactose oxidase in *Escherichia coli* by directed evolution. Protein Eng 2001; 14: 699-704.

[22] Xu F, Golightly EJ, Schneider P, Berka RM, Brown KM, Johnstone JA, Baker DH, Fuglsang CC, Brown SH, Svendsen A, Klotz AV. Expression and characterization of a recombinant *Fusarium* spp. galactose oxidase. Appl Biochem Biotechnol 2000; 88: 23-32.

[23] Whittaker MM, Whittaker JW. Expression of recombinant galactose oxidase by *Pichia pastoris*. Protein Expr Purif 2000; 20: 105-111.

[24] Cooper1 as251(0)-0.2239021153..31034(C)-2.0deC1-6.605331034(C)-2.0aE6

[30] Reisfeld RA, Lewis UJ, Williams DE. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 1962; 195: 281-3.

[31] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-5.

[32] Blum H, Beier H, Gross HJ. Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 1987; 8: 93-9.

[33] Shatzman AR, Kosman DJ. Regulation of galactose oxidase synthesis and secretion in

Table 2

Effect of the presence of different ions and Tween 80 in the galactose oxidase production by *F. acuminatum* UnB 356.

Ions and Surfactant	Enzymatic Activity (U/ml of filtrate)	Dried Mycelial Biomass (g/25 ml of medium)
Control	2.92 ± 0.45^{ab}	0.18 ± 0.02^{b}
Ca^{2+}	3.85 ± 0.39^{a}	0.18 ± 0.01^{ab}
Fe^{3+}	2.98 ± 0.50^{ab}	0.16 ± 0.03^{b}
BO_3^{3-}	2.89 ± 0.31^{ab}	0.19 ± 0.02^{ab}
Zn^{2+}	2.30 ± 0.60^{b}	0.18 ± 0.00^{ab}
Mo^{2+}	2.11 ± 0.19^{b}	0.17 ± 0.02^{b}
Co^{2+}	2.01 ± 0.21^{b}	0.19 ± 0.02^{ab}
K^{+}	2.73 ± 0.53^{ab}	0.19 ± 0.01^{ab}
Tween 80	2.82 ± 0.45^{ab}	0.22 ± 0.01^{a}
Tween 80 (30 min)	3.32 ± 0.66^{ab}	0.20 ± 0.01^{ab}
Variation Coefficient	16.3%	8.1%

Values are means and standard deviation of the results obtained in the analyses performed independently in the filtrates of three culture flasks.
Means followed by identical letters are not significantly different according to Tukey's Test ($\alpha = 0.05$).

Table 3
Activity of the enzyme galactose oxidase from *F. acuminatum* UnB 356 toward different substrates

Substrates	Enzymatic Activity (U/ml of filtrate)
D-(+)-Galactose	3.81 ± 0.06
Dihydroxyacetone	4.83 ± 0.23
D-(+)-Glucose	0.00 ± 0.00
Guar gum	0.06 ± 0.03
Lactose	0.05 ± 0.02
α-D-(+)-Melibiose	3.47 ± 0.04
Methyl-α-D-galactopyranoside	3.69 ± 0.07
D-(+)-Raffinose	4.19 ± 0.18

Values are means and standard deviation of the results obtained in the analyses performed independently in the filtrates of three culture flasks.

Figure legends

Fig. 1. Effect of inoculum size on galactose oxidase production by *F. acuminatum*. At each 24 hours, three flasks for each inoculum size were filtered and analyzed independently. Values are means and standard deviations of the obtained results (U/ml 1% ●, 2% ▲, 6% ■; g/25 ml 1% ○, 2% △, 6% □).

Fig. 2. (a) Effect of initial pH on galactose oxidase production by *F. acuminatum*. Data is the average and standard deviation of the analyses conducted independently in three culture flasks (U/ml at pH 6.0 ▲ and pH 7.0 ●; g/25 ml at pH 6.0 △ and pH 7.0 ○). (b) Effect of the phosphate buffer concentration on galactose oxidase production by *F. acuminatum*. Data is the average and standard deviation of the analyses conducted independently in four culture flasks (U/ml □; g/25 ml ◆). Means followed by identical letters are not significantly different according to Tukey's Test ($\alpha = 0.05$).

Fig 3. (a) Influence of the presence of Cu^{2+}, Mg^{2+}, and Mn^{2+} on galactose oxidase production by *F. acuminatum* UnB 356. Three flasks were cultivated for each treatment and analyzed independently (U/ml □; g/25 ml ◆). Results are the obtained average and standard deviation. Means followed by identical letters are not significantly different according to Tukey's Test ($\alpha = 0.05$). (b) Effect of copper addition on apoenzyme activation. Results are the obtained average and standard deviation of the analyses performed independently in the filtrate of three culture flasks that were incubated with the missing element for 24 hours.

Fig. 4. Optimum pH (a), best temperature of action (b), and thermal stability (c) of the galactose oxidase enzyme from the culture medium of *F. acuminatum*. Results are the average and standard deviation of the analyses performed independently in the filtrate of three culture flasks.

Fig. 5. Polyacrylamide gel electrophoresis analysis (PAGE). Proteins precipitated from the culture medium filtrate of *F. graminearum* and *F.*

acuminatum were developed in a polyacrylamide gel with pH 4.3 (a) and pH 8.9 (b). Migration was from top to bottom. The gel was revealed with the enzyme reaction containing peroxidase, *o*-dianisidine, and galactose.

Fig. 6. Non-denaturing SDS-PAGE analysis of the GO precipitated from the culture medium of *F. acuminatum* and *F graminearum*. The gel was revealed with the enzyme reaction containing peroxidase, *o*-dianisidine, and galactose. The position of the molecular weight markers was identified in a gel run in parallel and treated first with the enzyme reaction and second with the silver staining.

Figure 1

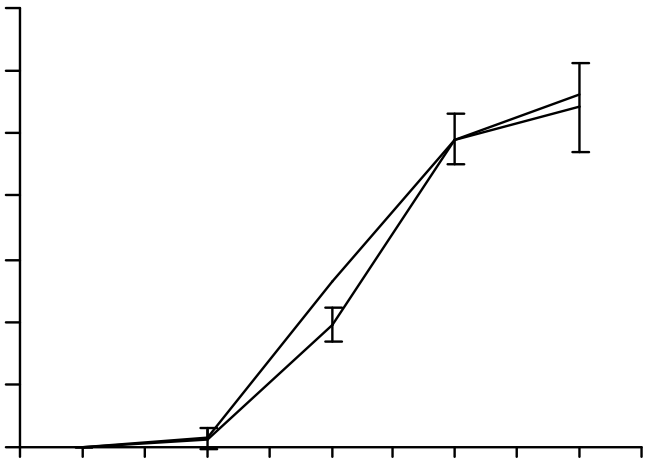
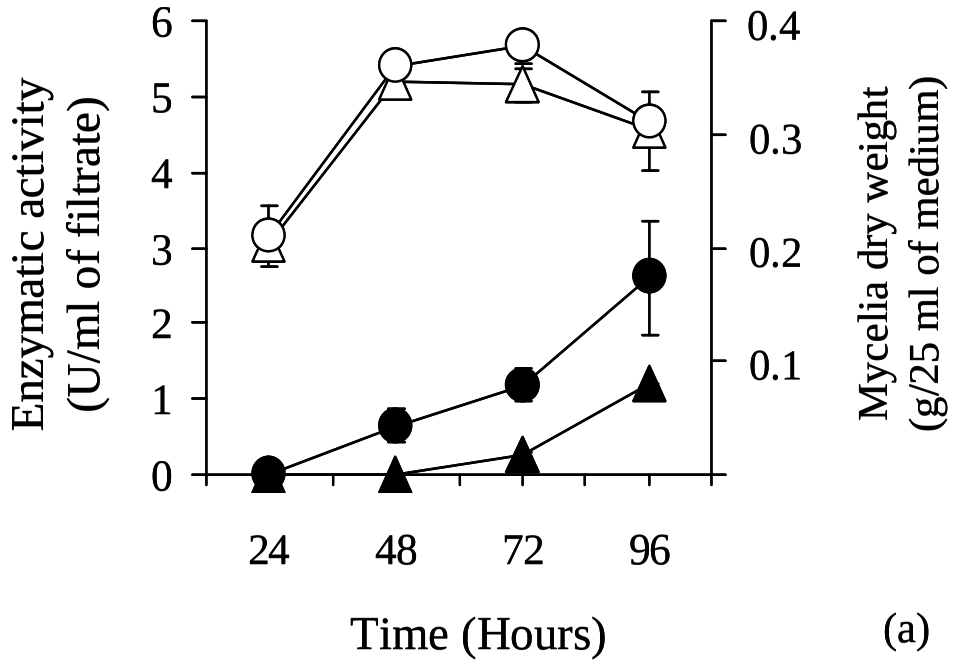


Figure 2


Enzymatic activity
(U/ml of filtrate)
Mycelia dry weight
(g/25 ml of medium)
0
1
2
3
4
5
6
0.1
0.2
0.3
0.4
24
48
72
96
Time (Hours)
(a)

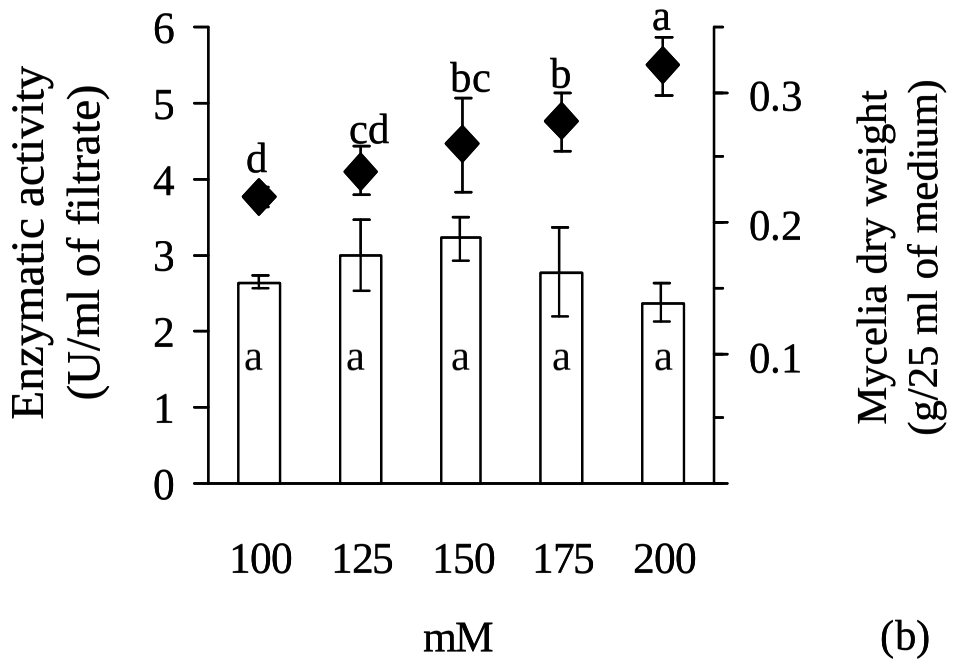
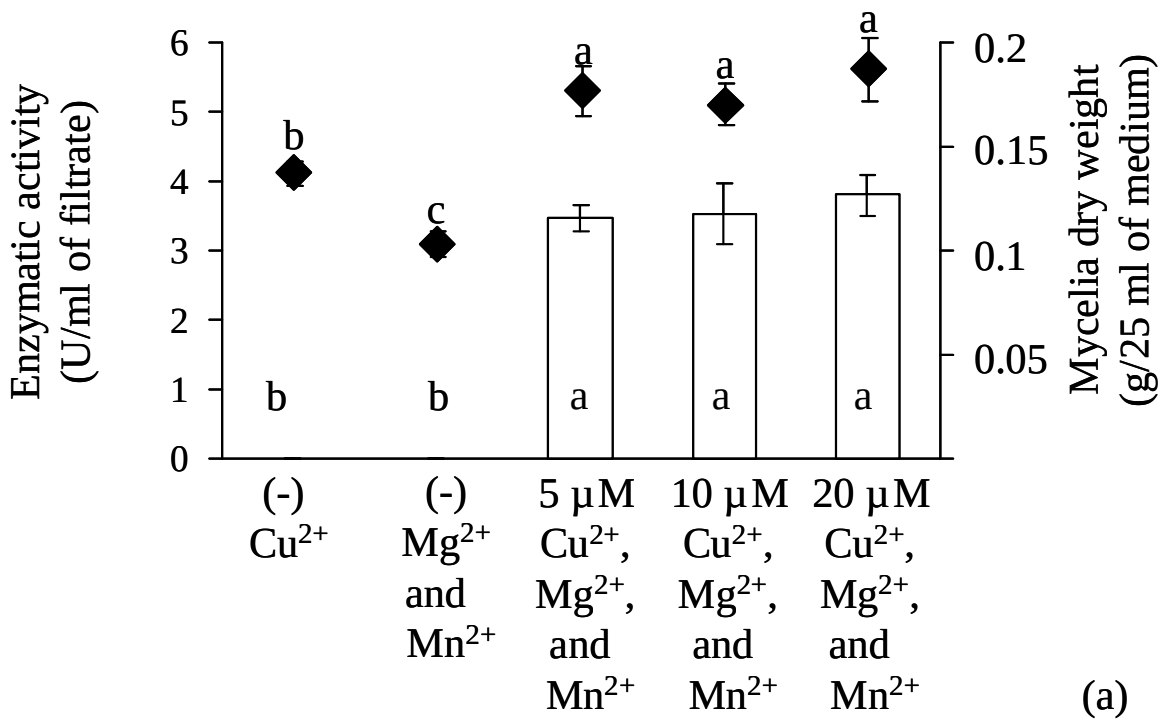
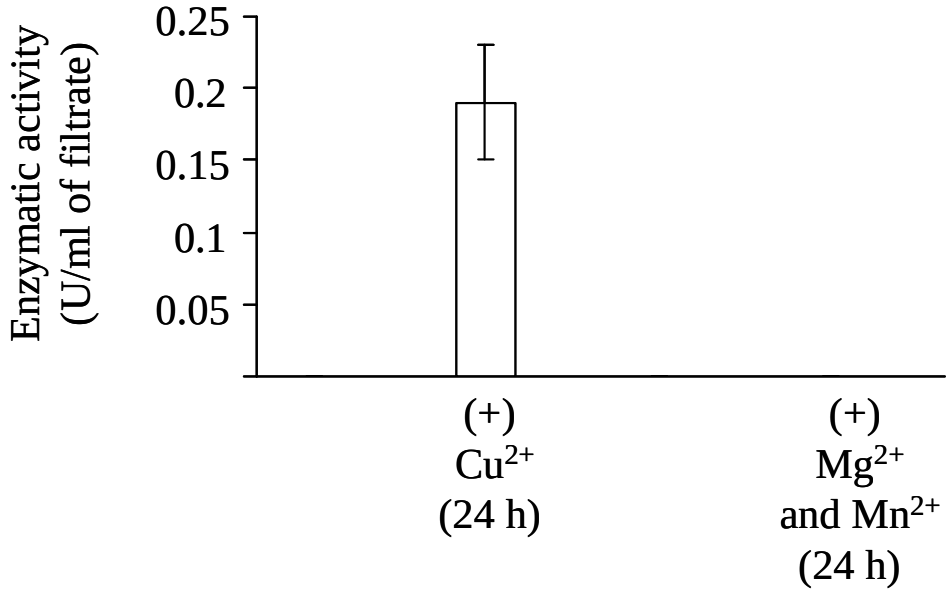

Enzymatic activity
(U/ml of filtrate)
Mycelia dry weight
(g/25 ml of medium)
0
1
2
3
4
5
6
0.1
0.2
0.3
d
cd
bc
b
a
a
a
a
a
a
100
125
150
175
200
mM
(b)

Figure 3

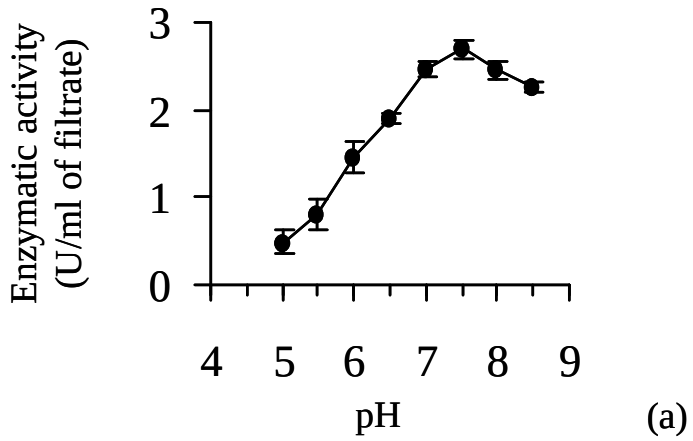

Enzymatic activity
(U/ml of filtrate)
Mycelia dry weight
(g/25 ml of medium)
0
1
2
3
4
5
6
0.05
0.1
0.15
0.2
b
c
a
a
a
b
b
a
a
a
(-) Cu2+
(-) Mg2+ and Mn2+
5 µM Cu2+, Mg2+, and Mn2+
10 µM Cu2+, Mg2+, and Mn2+
20 µM Cu2+, Mg2+, and Mn2+

(a)

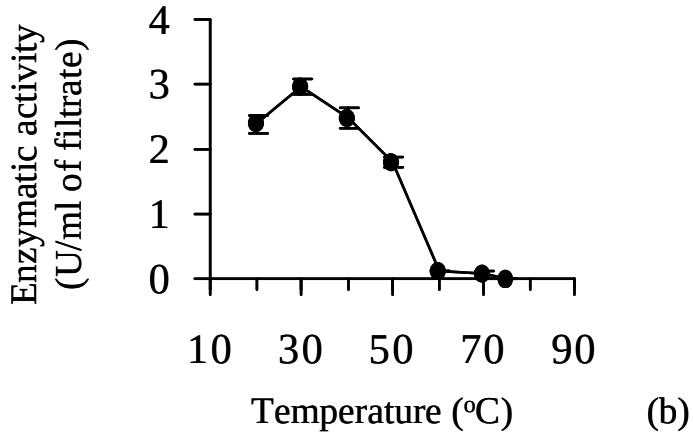

Enzymatic activity
(U/ml of filtrate)
0.05
0.1
0.15
0.2
0.25
(+) Cu2+ (24 h)
(+) Mg2+ and Mn2+ (24 h)

(b)

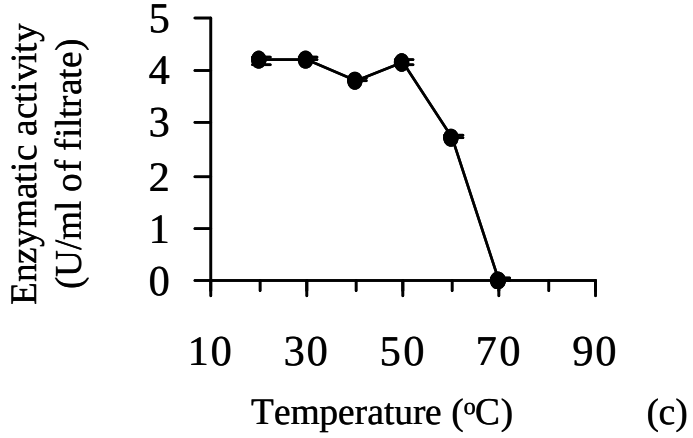
Figure 4


Enzymatic activity
(U/ml of filtrate)
0
1
2
3
4
5
6
7
8
9
pH

(a)


Enzymatic activity
(U/ml of filtrate)
0
1
2
3
4
10
30
50
70
90
Temperature (°C)

(b)


Enzymatic activity
(U/ml of filtrate)
0
1
2
3
4
5
10
30
50
70
90
Temperature (°C)

(c)

Figure 5

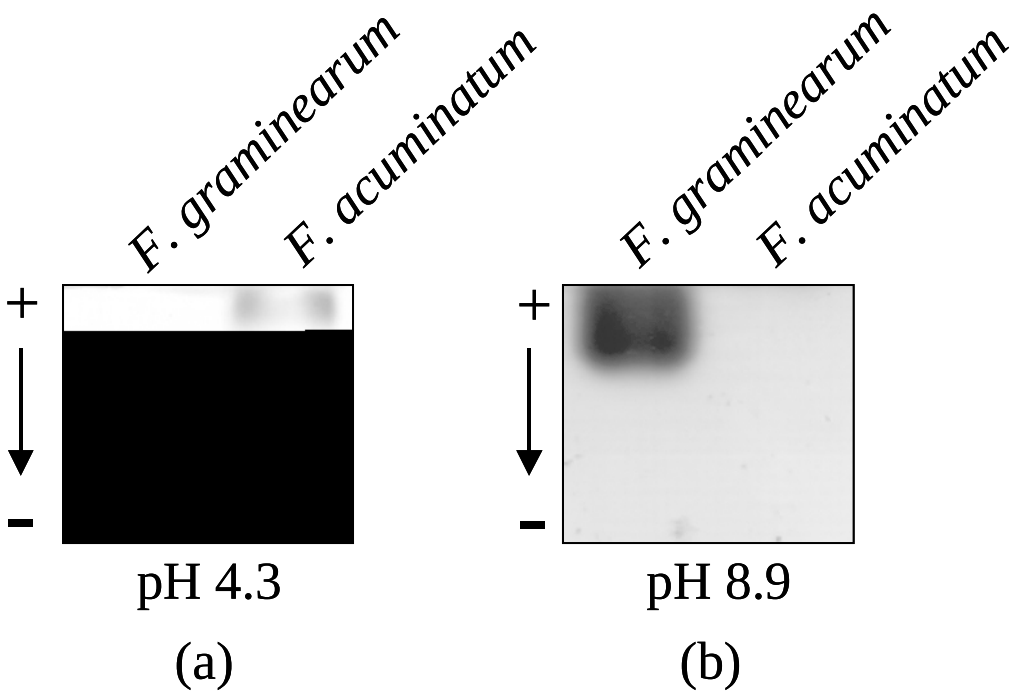
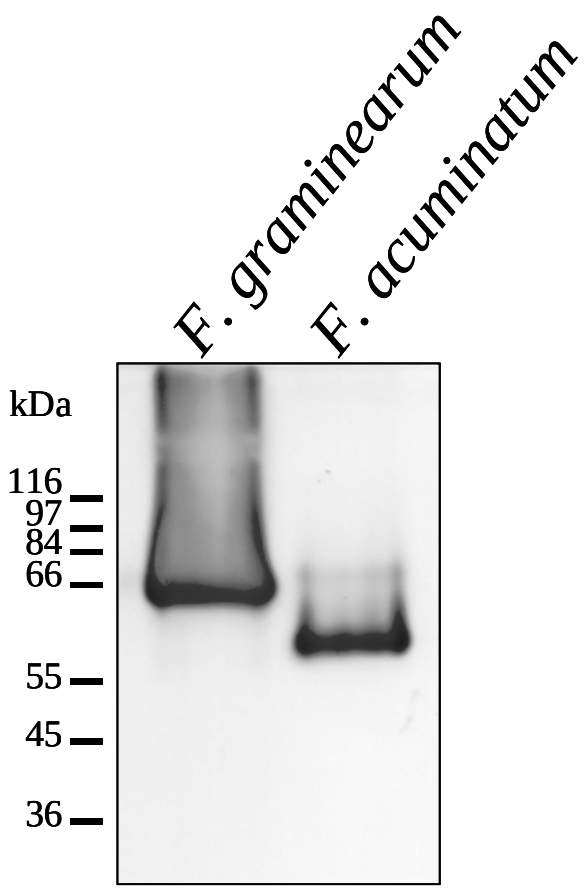

F. graminearum
F. acuminatum
+
-
pH 4.3
(a)
F. graminearum
F. acuminatum
+
-
pH 8.9
(b)

Figure 6



Livros Grátis

(http://www.livrosgratis.com.br)

Milhares de Livros para Download:

Baixar livros de Literatura
Baixar livros de Literatura de Cordel
Baixar livros de Literatura Infantil
Baixar livros de Matemática
Baixar livros de Medicina
Baixar livros de Medicina Veterinária
Baixar livros de Meio Ambiente
Baixar livros de Meteorologia
Baixar Monografias e TCC
Baixar livros Multidisciplinar
Baixar livros de Música
Baixar livros de Psicologia
Baixar livros de Química
Baixar livros de Saúde Coletiva
Baixar livros de Serviço Social
Baixar livros de Sociologia
Baixar livros de Teologia
Baixar livros de Trabalho
Baixar livros de Turismo