BRUNA LEITE SUFIATE

ENZIMAS VEGETAIS E FÚNGICAS COM POTENCIAL NEMATICIDA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 02 de março de 2018.

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“A maior recompensa para o trabalho do homem não é o que ele ganha com isso, mas o que ele se torna com isso.”

John Ruskin

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BIOGRAFIA

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RESUMO

SUFIATE, Bruna Leite, D.Sc., Universidade Federal de Viçosa, março de 2018. **Enzimas vegetais e fúngicas com potencial nematicida.** Orientador: José Humberto de Queiroz. Coorientador: Filippe Elias de Freitas Soares.

Fungos e plantas produzem enzimas que possuem as mais diversas aplicações biotecnológicas. Uma possível e promissora aplicação dessas enzimas é o controle de nematoides, em substituição aos nematicidas químicos prejudiciais à saúde humana e animal, ao meio ambiente e à microbiota do solo. Outra possível aplicação das enzimas de fungos é o uso da dextranase na redução de dextrana na indústria sucroalcooleira. Dessa forma, o presente trabalho teve como objetivo investigar a produção de enzimas pelo fungo *Pleurotus eryngii* e pelas plantas *Euphorbia milii* e *E. trigona*, purificar e caracterizar as enzimas de *E. milii*, *E. trigona* e *Pochonia chlamydosporia*, e testar a atividade nematicida das enzimas das plantas *E. milii*, *E. trigona* e do fungo *P. eryngii*. O fungo *P. eryngii* e seu extrato reduziram significativamente ($p<0,01$) o número de larvas intactas de *Panagrellus* sp. após 24 horas de tratamento em 60 e 90%, respectivamente. Este efeito não está relacionado à atividade enzimática, mas sim à presença de outros metabólitos. Os ovos de *Meloidogyne javanica*, quando tratados com o extrato de *P. eryngii*, mostraram redução de 53% ($p<0,01$) no número de ovos intactos. A redução de ovos intactos de *M. javanica* é atribuída à atividade enzimática, uma vez que o extrato apresentou atividade quitinolítica e proteolítica de, respectivamente, 32,74 e 3,57 U/mL. A purificação do látex de *E. trigona* por cromatografia de exclusão molecular permitiu a purificação de três proteases distintas. O peso molecular das proteases foi estimado em aproximadamente: 36, 31 e 29 kDa, para a trigonina 1, 2 e 3, respectivamente. O pH e a temperatura que proporcionaram maior atividade de protease foram 4,0, 6,0 e 9,0, e temperatura de 70 ℃. As proteases foram inibidas por fluoreto de fenilmetilsulfonila e iodoacetamida. O pool das três proteases presentes no látex de *E. trigona* reduziram significativamente ($p<0,01$) o número de J_2 vivas de *M.*

incognita em 96% após 24 horas de tratamento. A purificação parcial das proteases presentes no látex de *E. milii* indicou que estas proteases correspondem à milina e miliina. Estas enzimas reduziram em 65,59% e 96,46% o número de larvas de *Panagrellus redivivus* após 24 e 48 h, respectivamente ($p<0,01$). A dextranase produzida pelo fungo *P. chlamydosporia* foi purificada à homogeneidade em duas etapas, com rendimento de 152%, fator de purificação de 6,84 e atividade específica de 358,63 U/mg. Seu peso molecular foi estimado por SDS-PAGE em 64 kDa. A enzima apresentou maior atividade a 50 ℃ e pH 5,0, usando tampão citrato-fosfato 100 mM, foi inibida por Ag^{1+}, Hg^{2+}, Cu^{2+}, Mg^{2+}, e apresentou K_M de 1,77 g/L. A dextranase madura é composta por 585 resíduos de aminoácidos, com peso molecular predito de 64,38 kDa e pI 5,96. Esta dextranase demonstrou forte semelhança filogenética em comparação à dextranase de *Trichoderma harzianum*. Sua estrutura consiste de dois domínios: o primeiro composto por 15 cadeias beta, e o segundo composto por uma β-hélice paralela. Esses resultados evidenciam o potencial biotecnológico desses fungos, plantas e suas enzimas extracelulares no controle de nematoides, e fornecem informações acerca da dextranase produzida pelo fungo *P. chlamydosporia*, que podem ser utilizadas para futuras aplicações industriais desta enzima.

ABSTRACT

SUFIATE, Bruna Leite, D.Sc., Universidade Federal de Viçosa, March, 2018. **Vegetable and fungic enzymes with nematicidal potential.** Adviser: José Humberto de Queiroz. Co-adviser: Filippe Elias de Freitas Soares.

Fungi and plants produce enzymes that have several biotechnological applications. A possible and promising application of these enzymes is the control of nematodes, replacing chemical nematicides harmful to human and animal health, to the environment and to the soil microbiota. Another possible application of fungi enzymes is the use of dextranase to reduce dextran content in sugar industry. The objective of this work was to investigate the production of enzymes by *Pleurotus eryngii*, *Euphorbia milii* and *E. trigona*, to purify and characterize the enzymes from *E. milii*, *E. trigona* and *Pochonia chlamydosporia*, and to test the nematicidal activity of *E. milii*, *E. trigona* and *P. eryngii. P. eryngii* fungus and its extract significantly reduced ($p<0.01$) the number of intact *Panagrellus* sp. larvae after 24 hours treatment in 60 and 90%, respectively. This effect is not related to enzymatic activity, but to the presence of other metabolites. *M. javanica* eggs, when treated with *P. eryngii* extract, showed a 53% reduction ($p<0.01$) in the number of intact eggs. The *M. javanica* intact eggs reduction is attributed to enzymatic activity, once the extract showed proteolytic and chitinolytic activities of, respectively, 32.74 and 3.57 U/mL. The purification of *E. trigona* latex with size-exclusion chromatography allowed partially purification of three distinct proteases. The molecular weight of proteases was estimated at approximately: 36, 31 and 29 kDa, for trigonin 1, 2 and 3, respectively. The pH and temperature that provided highest protease activity were 4.0, 6.0 and 9.0, and temperature of 70 ℃. The proteases were inhibited by phenylmethylsulfonyl fluoride and iodoacetamide. The pool of three proteases present in *E. trigona* latex reduced significantly ($p<0.01$) the number of live *M. incognita* J_2 in 96% after 24 hours treatment. The partial purification of the proteases present in *E. milii* latex indicated that these proteases are milin and miliin. These enzymes reduced the *Panagrallus redivivus* larvae number by 65.59 %

and 96.46 % after 24 and 48 hours, respectively ($p<0.01$). Dextranase produced by the fungus *P. chlamydosporia* was purified to homogeneity in two steps, with a yield of 152 %, purification factor of 6.84 and specific activity of 358.63 U/mg. Its molecular weight was estimated by SDS-PAGE at 64 kDa. The enzyme presented higher activity at 50 ℃ and pH 5.0, using 100 mM citrate-phosphate buffer, was inhibited by Ag^{1+}, Hg^{2+}, Cu^{2+}, Mg^{2+}, and presented K_M of 1.77 g/L. Mature dextranase is composed of 585 amino acids residues, with a predicted molecular weight of 64.38 kDa and pI 5.96. This dextranase showed a strong phylogenetic similarity when compared to *Trichoderma harzianum* dextranase. Its structure consists of two domains: the first composed by 15 β strands, and the second composed by a right-handed parallel β-helix. These results evidence the biotechnological potential of these fungi, plants and their extracellular enzymes to nematodes control, and provide information about the dextranase produced by *P. chlamydosporia* fungus, which can be used for future industrial applications of this enzyme.

1. INTRODUÇÃO GERAL

Esta tese está dividida em quatro capítulos. Nos dois primeiros capítulos foi investigada a atividade nematicida de enzimas encontradas no látex de plantas do gênero *Euphorbia*, e no terceiro capítulo foi investigada a atividade nematicida do fungo *Pleurotus eryngii* e de suas enzimas.

Os nematoides causam perdas nas principais culturas de importância econômica ou de subsistência em todos os continentes. Em escala global, estima-se que os prejuízos decorrentes da ação de fitonematoides sejam calculados entre 78 e 125 bilhões de dólares (Lopes e Ferraz, 2016).

O uso de nematicidas químicos corresponde a um dos métodos mais utilizados no controle de fitonematoides, no entanto estes compostos podem ser altamente prejudiciais à saúde humana e animal, ao meio ambiente e à microbiota do solo, e por isso, seu uso tem sido evitado em um número cada vez maior de países. Além disso, o uso constante desses nematicidas tem como possível consequência o desenvolvimento de resistência pelos nematoides. Diante do exposto, surge a necessidade de desenvolver novas estratégias de combate aos fitonematoides (Brand et al., 2010; Khalil, 2013).

Extratos de plantas têm sido usados há séculos, principalmente em países em desenvolvimento, no tratamento de nematoides gastrointestinais que parasitam seres humanos e animais. Entre os extratos de plantas mais estudados para esta finalidade, estão os extratos do látex e dos frutos de *Carica papaya*, *Ficus* spp., *Ananas comosus* e *Actinidia chinensis*. A atividade nematicida relacionada a esses extratos é, muitas vezes, atribuída à atividade das cisteíno proteases que os compõem (Stepek et al., 2007).

O látex, encontrado nos laticíferos de cerca de 40 famílias de plantas, é uma emulsão aquosa ou suspensão, viscosa, opaca e branca. Sua composição inclui, além de lipídios, borrachas, resinas e açúcares, uma grande variedade de proteínas, tais como proteases, oxidases,

lectinas, proteínas de ligação à quitina, quinases, lipases, glucosidases e fosfatases (Flemmig et al., 2017; Sufiate et al. 2017).

Fungos filamentosos são uma das principais fontes de enzimas industriais devido à sua alta capacidade de produção de proteínas extracelulares. Dentre as muitas enzimas comercialmente disponíveis produzidas por estes fungos, podemos citar: glicoamilases, celulases, proteases, lipases, pectinases, quitinases, lacases, catalases, fitases e dextranases. Apesar do grande número de espécies de fungos filamentosos existente na natureza, apenas poucas dezenas destas espécies são comercialmente exploradas como produtoras de enzimas (Corrêa et al., 2014).

Alguns fungos filamentosos são classificados como fungos nematófagos devido à sua capacidade de capturar e digerir nematódeos, que é atribuída, muitas vezes, à ação de enzimas, principalmente proteases e quitinases. Desta forma, estas enzimas podem ser usadas no controle de nematoides, com destaque para o fato de ser um método ecologicamente correto, uma vez que não gera resíduos químicos prejudicais ao meio ambiente (Soares et al., 2013b, 2015a, 2015b).

Nos capítulos 1 e 3, a atividade nematicida foi testada sobre larvas do gênero *Panagrellus*. Este gênero engloba nematoides de vida livre, sendo encontrado na maioria dos continentes, com exceção da Antártica e da Austrália (Stock e Nadler, 2006). Estes nematoides são altamente ativos em água, ao contrário da maioria dos nematoides parasitas de plantas, que se movem lentamente. Portanto, é mais fácil observar os efeitos tóxicos subletais dos compostos com atividade nematicida em larvas de *Panagrellus* sp. do que em fitonematoides (Kwok et al., 1992). Os nematoides do gênero *Panagrellus* são grandes, o que facilita sua manipulação, são de fácil cultivo em laboratório e possuem ciclo de vida curto, tornando possível a obtenção de milhares de indivíduos em poucos dias de cultivo. Por estes motivos, os nematoides do gênero *Panagrellus* são amplamente utilizados como modelo de experimentação em pesquisas envolvendo fungos nematófagos e compostos com atividade nematicida (Tarjan, 1995; Soares et al., 2013a, 2015a).

Nos capítulos 2 e 3, foram usadas larvas e ovos de nematoides do gênero *Meloidogyne*, popularmente conhecidos como nematoides das galhas. Este gênero é constituído por um grande grupo de cerca de 100 espécies descritas (Hunt e Handoo, 2009) sendo que destas, as espécies mais frequentemente encontradas são *Meloidogyne incognita*, *M.* arenaria, *M. javanica* e *M. hapla* (Agrios, 2005; Jones et al., 2013).

Estes nematoides causam muitas perdas, pois afetam várias plantas de interesse econômico. Estima-se que aproximadamente 5% da produção agrícola mundial seja destruída pelo gênero *Meloidogyne* anualmente (Karajeh et al., 2008), com um prejuízo anual estimado em bilhões de dólares (Agrios, 2005).

A casca dos ovos de *Meloidogyne* é formada por três camadas: uma camada lipídica interna, uma camada intermediária de quitina e uma camada vitelina externa. A camada vitelina é muito fina e composta por lipoproteínas, a camada de quitina consiste em complexos quitina-proteína e seu papel principal é proporcionar força estrutural, e a camada lipídica é composta por membranas de lipoproteínas, sendo responsável pela impermeabilidade dos ovos. Uma vez que a camada vitelina e de quitina sejam danificadas, a camada lipídica torna-se muito suscetível ao dano (Brand et al., 2010; Gortari e Hours, 2008; Khan et al., 2004).

A cutícula dos nematoides é uma estrutura extracelular complexa composta principalmente por proteínas, com vestígios de lipídeos e carboidratos, e podem variar amplamente entre as espécies e entre os estádios de desenvolvimento dentro de uma espécie (Fetterer e Rhoads, 1993).

Neste contexto, as enzimas provenientes de fungos e plantas são apontadas como uma alternativa em potencial no combate desses nematoides, especialmente as quitinases e proteases (Brand et al., 2010; Gortari e Hours, 2008; Khan et al., 2004).

Além das proteases e quitinases, os fungos podem produzir inúmeros outros tipos de enzimas, e dentre esta ampla variedade de enzimas, encontram-se as dextranases. No capítulo 4 é apresentada a caracterização *in vitro* e *in silico* da dextranase produzida pelo fungo nematófago *Pochonia chlamydosporia*.

Dextranases são enzimas que hidrolisam ligações α-(1,6), α-(1,2), α-(1,3) e α-(1,4) em polissacarídeos de dextrana (Jaiswal e Kumar, 2011). Dextranas são compostos altamente indesejáveis na indústria sucroalcooleira, produzidos a partir da sacarose por microrganismos que contaminam a cana-de-açúcar durante a colheita, transporte e armazenamento (Jiménez, 2005). A produção de dextrana resulta em diminuição da concentração de sacarose, menor taxa de recuperação da sacarose e aumento da viscosidade dos caldos de cana (Khalikova et al., 2005). Este aumento da viscosidade prejudica a qualidade do açúcar, pois não permite a correta retirada das impurezas dos méis, resultando na presença de resíduos nos cristais de açúcar, e na formação de cristais opacos irregulares, alongados e de cor caramelo (Batista, 2014; Boil e Wienese, 2002; Jiménez, 2009). A maior viscosidade como efeito da presença de dextrana resulta também em prejuízos nos equipamentos industriais, causando desestabilização do eixo das centrífugas e entupimento de filtros e bombas (Bukhari et al., 2015; Batista, 2014; Singleton et al., 2002).

Métodos físicos de remoção da dextrana, como ultrafiltração, diálise e osmose reversa são úteis em pequena escala, no entanto, em escala comercial, estes métodos são economicamente inviáveis. Desta forma, o método mais frequentemente utilizado na remoção de dextrana dos caldos de cana é a hidrólise enzimática pelo uso da dextranase (Boil e Wienese, 2002; Zhang et al., 2017).

P. chlamydosporia é um fungo parasita facultativo amplamente distribuído capaz de atuar tanto como saprófita no solo (Siddiqui et al., 2009), quanto como parasita de ovos e fêmeas de nematoides (Dallemole-Giaretta et al., 2013). Além disso, este fungo também é capaz de colonizar as raízes de uma ampla variedade de plantas monocotiledôneas (Lopez-Llorca et al., 2002) e dicotiledôneas (Bordallo et al., 2002).

Embora existam na literatura inúmeros estudos voltados ao uso do fungo *P. chlamydosporia* como ovicida e larvicida, e para produção de proteases e quitinases (Araújo et al., 2009, 2008; Braga et al., 2008, 2010, 2011; Frassy et al., 2010; Silva et al., 2010; Soares et al., 2015a; Tobin et

al., 2008), apenas recentemente foi relatado produzindo dextranase (Sufiate et al., 2018).

O constante requerimento industrial de preparações de enzimas altamente ativas, com atividade específica apropriada e estabilidade a diferentes pHs, temperaturas, íons metálicos, surfactantes e solventes orgânicos impulsiona a busca por novas fontes de enzimas (Morya et al., 2012).

Assim, este trabalho teve como objetivo investigar a atividade nematicida de enzimas fúngicas e vegetais, utilizando ensaios *in vitro* sobre nematoides, bem como técnicas de purificação e caracterização enzimáticas, e analisar a dextranase produzida pelo fungo nematófago *Pochonia chlamydosporia*, a fim de obter informações úteis para futuras aplicações industriais (Anexo I).

2. OBJETIVOS

2.1. Objetivos Gerais

Investigar a atividade nematicida de enzimas fúngicas e vegetais. Além disso, analisar a dextranase de *Pochonia chlamydosporia*.

2.2. Objetivos Específicos

Investigar a produção de enzimas pelo fungo *Pleurotus eryngii*, e pelas plantas *Euphorbia milii* e *E. trigona*;

Purificar as enzimas produzidas por *E. milii*, *E. trigona* e *Pochonia chlamydosporia*;

Caracterizar as enzimas purificadas de *E. milii*, *E. trigona* e *P. chlamydosporia*;

Avaliar a atividade nematicida do fungo *P. eryngii* e das plantas *E. milii* e *E. trigona*.

CAPÍTULO 1

Nematicidal activity of proteases from *Euphorbia milii*

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Nematicidal activity of proteases from *Euphorbia milii*

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Abstract

The present work aimed to characterize the proteases present in *Euphorbia milii* latex and to evaluate the nematicidal potential of these enzymes. Results suggest that proteases present in *E. milii* latex concentrate samples are milin and miliin. Difference ($p < 0.01$) between larvae number in the different groups was found, with 65.59 % and 96.46 % *Panagrellus redivivus* larvae reduction after 24 and 48 hours, respectively.

Keywords: *Euphorbia milii*; protease; nematode

1. Introduction

Euphorbia milii, also known as crown of thorns, Christ plant and Christ thorn, is a plant rich in latex, a white milky liquid found in the vacuole of specific secretory cells called laticifers (Mazou et al., 2016). Latex is a blend mainly

composed by lipids, rubber, resins, sugars and a wide variety of proteins, including enzymes such as peroxidases, proteases, esterases and phosphatases (Domsalla and Melzig, 2008).

Among the existing proteases in the *E. milii* latex, three have already been characterized: milin, eumiliin and miliin (Yadav et al., 2006, Fonseca et al., 2010, Moro et al., 2008). *E. milii* latex showed molluscicidal effects, therefore is possible that these isolated proteases also have nematicidal effects, promoting rupture of nematodes outer protective cuticle (Yadav and Jagannadham, 2008, Oliveira-Filho et al., 1999).

The present study aimed to characterize the proteases present in *E. milii* plant latex and to evaluate the nematicidal potential of these enzymes.

2. Material and Methods

2.1. Latex samples

E. milii latex was sampled from native specimens in Viçosa, Minas Gerais, Brazil, in fertile and poorly irrigated soil. The stem was cut, and the latex was collected into a plastic bottle and stored at 4 °C.

2.2. Nematodes

Free-living nematode *Panagrellus* sp. was provided by the Veterinary Medicine Department of Universidade Federal de Viçosa – Viçosa, Minas Gerais, Brazil. Nematodes were cultured in petri dishes containing oat and water and, when needed, were washed with water in a decanter. They were kept in a dark environment at approximately 22 °C, in order to decrease *Panagrellus* sp. reproduction rate and to avoid culture fermentation.

2.3. Enzymatic activity

Enzymatic activity was measured by the caseinolytic procedure described by Soares et al. (2011). A standard tyrosine curve was constructed, relating absorbance and tyrosine concentration. One protease unit was defined as the amount of enzyme required to release 1 µg of tyrosine per minute under these experimental conditions.

2.4. Purification and characterization

The first step of extracting, concentrating and separating of proteases contained in the latex was through centrifugation at 10,000 g and 4 °C for 30 minutes in order to remove larger particles and concentrate the proteases. Subsequently enzyme activity assays were performed to determine in which of the phases the crude extract proteases were.

The second step consisted of an ammonium sulphate precipitation, which was slowly added from 10 to 90% saturation, until the proteolytic activity was not present in the supernatant, only in the precipitate.

Proteolytic activity of the precipitate was analyzed at different temperatures (30, 40, 50, 60, 70 and 80 °C) and different pHs (4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0). Enzyme activity assays were performed using pH and temperature conditions that resulted in increased activity, with addition of the inhibitors β-mercaptoethanol, EDTA (Ethylenediamine tetraacetic acid) and PMSF (Phenylmethylsulfonyl fluoride) to determine the inhibitors effect on protease activity.

Zymogram was performed using a SDS-free 12% polyacrylamide gel containing 0.1% copolymerized casein, according to the modified methodology of Laemmli (1970), described by Soares et al. (2013). The process was performed in a refrigerator cabinet at 4 °C and 90 V. Then, the gel was washed with distilled water and incubated with phosphate-citrate buffer 50 mM pH 7.0 for 1 hour at 60 °C. Enzymatic activity was revealed by staining with Coomassie Brilliant Blue R-250 0.1% dissolved in ethanol:acetic acid:water (30:7:63). Proteases were visualized as negative contrast bands on the gel.

2.5. Nematicidal activity

Nematicidal activity of the concentrated proteases was analyzed in vitro on *Panagrellus redivivus* larvae. Four groups were formed in sterile tubes, two treated groups and two control groups. One of the control and treated groups were evaluated at 24 and the others at 48 hours, with 6 replicates for each group. About 70 *P. redivivus* larvae were poured into each sterile tube containing *E. milii* proteases. The control group contained approximately the same number of *P. redivivus* larvae treated with protease denatured by boiling. The tubes were incubated at 28 °C in a dark environment for 24 and 48 hours. After these periods, total number of intact larvae present in each tube was counted by optical microscopy according to the modified methodology described by Soares et al. (2013). The data obtained were interpreted statistically by variance analysis at significance levels of 1 and 5% probability. The larvae destruction efficiency when compared to the control was done by Tukey test at the 1% probability level. Subsequently, the average reduction percentage of the larvae was calculated according to the following equation:

$$\%\text{Reduction} = \left(\frac{\overline{X}\text{ larvae recovered from control} - \overline{X}\text{ larvae recovered from treatment}}{\overline{X}\text{ larvae recovered from control}}\right) \times 100$$

3. Results and discussion

E. milii is a plant very rich in latex, thus the extraction resulted in a large volume of latex, with approximately 30 mL being obtained in 30 minutes. After the first ammonium sulfate addition, the latex presented a rubbery solid phase, in which the proteolytic activity was measured. Since no protease was detected, this phase was discarded. In the subsequently ammonium sulfate additions, the existent phases were only the supernatant and the precipitate.

Ammonium sulfate precipitation was able to separate the proteases from the contaminants at 60% saturation, and a pool was made between the 60% and 70% precipitates to ensure greater presence of latex proteases.

Cuticle constitution and nematodes mobility were visually analyzed. In both control groups, 24 and 48 hours, nematodes still moved normally, however in the treated tubes no nematode movement was observed. This indicates that the proteases may have killed all nematodes or have some paralyzing effect on them.

Analysis of variance shows that there was significant difference between treated and control groups, therefore the differences were real, not casual. The nematode reduction percentage evidences the high efficiency of *E. milii* latex proteases in nematodes control, with 65.59% reduction after 24 hours treatment and 96.46% reduction after 48 hours treatment.

The temperature of 60 °C resulted in higher enzymatic activity. This data agrees with the data obtained for the proteases milin (Yadav et al., 2006), miliin (Moro et al., 2008), and eumiliin (Fonseca et al., 2010). The pH values for the highest proteases activity were 7.0 and 11.0, but the activity also remained high at

pH 8.0, which agrees with the data obtained for eumiliin (pH 8.0), milin (pH 8.0) and miliin (pH 7.0 and 11.0) founded in *E. milii* (Fonseca et al., 2010, Yadav et al, 2006, Moro et al., 2008). This fact indicates that miliin is probably the most present protease in *E. milii* latex, once it had greater influence on pH value for proteolytic activity.

Assays with inhibitor were performed in order to identify metalloproteases, cysteine proteases and serine proteases. β-mercaptoethanol was used to identify eumiliin, since it is a cysteine protease, in which its disulfide bonds are essential for total activity (Fonseca et al., 2010). As β-mercaptoehtanol had no effect on proteolytic activity, it is suggested that eumillin is not present in the latex. PMSF completely inhibited the proteases activity, showing that all proteins present in the precipitate are serine proteases, which are milin and miliin (Yadav et al, 2006, Moro et al., 2008), reinforcing the hypothesis that eumiliin is not present in the analyzed samples. EDTA had no significant effect on protease activity.

As the tests with inhibitors suggested that there was no eumillin in the tested samples, a zymogram gel was carried out to identify sample existing proteases. The result is shown in Figure 1, where only two bands are evident, thus confirming that there are only two proteases present in *E. milii* latex. This gel also reveals that one of the proteases is present in higher quantity than the other due to the ticker band located higher up in the gel, indicating a greater molecular weight. This way, we can infer that proteases present in the concentrated samples are milin and miliin, and that miliin, which has the highest molecular mass (79 kDa), is present in higher quantity than milin (51 kDa). Thus, confirming the hypothesis

suggested by the pH test, in which the pH of the highest activity was miliin's pH (Table 1).

Table 1 – Proteases concentration present in *Euphorbia milii* latex.

Sample	Protein (mg/mL)	Enzymatic Activity (U/mL)	Specific Activity (U/mg)	Purification Factor	Recovery (%)
Crude extract	1.670	145.68	87.23	1.00	100.00
Precipitation	0.490	67.55	137.85	1.58	46.36

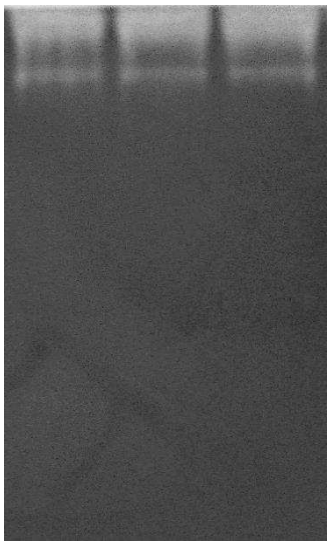

Figure 1 - Zymogram gel. The marked columns are replicates of the sample obtained precipitation with ammonium sulfate.

4. Conclusions

The results suggest that proteases present in *E. milii* latex concentrate samples from this experiment are milin and miliin, and that miliin is present in higher quantity than milin.

The treatment with partially purified proteases evidences the high efficiency of *E. milii* latex proteases in nematodes control, with 65.59 % reduction after 24 hours treatment and 96.46 % reduction after 48 hours treatment.

This is the first report of *E. milii* proteases on *Panagrellus redivivus* larvae, therefore, there is still much to be studied regarding action and mechanism of these nematicidal enzymes.

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CAPÍTULO 2

Three proteases from *Euphorbia trigona* latex with nematicidal activity on the root-knot nematode *Meloidogyne incognita*

Submetido ao periódico: Pesquisa Agropecuária Brasileira

Three proteases from *Euphorbia trigona* latex with nematicidal activity on the root-knot nematode *Meloidogyne incognita*

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Abstract – *Euphorbia trigona* is a succulent plant from Africa, known as African milk tree because of its high latex production, cultivated in various countries for ornamental purposes. The aim of this study was to partially purify and to characterize the proteases present in *E. trigona* latex, and to test its nematicidal activity on *Meloidogyne incognita* larvae. The purification of *E. trigona* latex with size-exclusion chromatography allowed partially purification of three distinct proteases, named trigonin 1, 2 and 3. The molecular weight of proteases was estimated at approximately: 36, 31 and 29 kDa, for trigonin 1, 2 and 3, respectively. The pH and temperature that provided highest protease activity were pH values of 4.0, 6.0 and 9.0, and temperature of 70 °C. The proteases were inhibited by phenylmethylsulfonyl fluoride and iodoacetamide. The three proteases present in *E. trigona* latex reduced significantly ($p < 0.01$) the number of live *M. incognita* J_2 in 96% after 24 hours treatment.

Index terms: Purification; nematicidal; enzymes; characterization; trigonin

Três proteases do látex de *Euphorbia trigona* com atividade nematicida sobre o nematoide das galhas *Meloidogyne incognita*

Resumo – *Euphorbia trigona* é uma planta suculenta originária da África, conhecida como árvore de leite africana devido à sua alta produção de látex, sendo cultivada em diversos países para fins ornamentais. O objetivo deste estudo foi purificar parcialmente e caracterizar as proteases presentes no látex de *E. trigona*, e testar sua atividade nematicida em larvas de *Meloidogyne incognita*. A purificação do látex de *E. trigona* por cromatografia de exclusão molecular permitiu a purificação parcial de três proteases distintas, denominadas trigonina 1, 2 e 3. O peso molecular das proteases foi estimado em aproximadamente 36, 31 e 29 kDa para a trigonina 1, 2 e 3, respectivamente. O pH e a temperatura que proporcionaram maior atividade de protease foram pH 4,0, 6,0 e 9,0, e temperatura de 70 °C. As proteases foram inibidas por fluoreto de fenilmetilsulfonila e iodoacetamida. As três proteases presentes no látex de *E. trigona* reduziram significativamente ($p < 0,01$) o número de J_2 vivas de *M. incognita* em 96% após 24 horas de tratamento.

Termos para indexação: Purificação; nematicida; enzimas; caracterização; trigonina

Introduction

Latex is found in laticifers of approximately 40 plant families, corresponding to about 9% of all angiosperms. It is an aqueous emulsion or suspension, generally viscous, opaque and white. The latex composition include lipids, rubber, resins, sugars and a wide variety of proteins, which are proteases, oxidases, lectins, chitin-binding proteins, chitinases, lipases, glucosidases, and phosphatases (Flemmig et al., 2017; Sufiate et al. 2017).

Plant latex is a rich source of several proteases, most belonging mainly to cysteine or serine super family (Domsalla et al., 2010; Patel et al., 2012). The property of being active over wide range of temperature and pH found in proteolytic enzymes from plant latex results in a crescent notoriety for these enzymes in pharmaceutical industry and biotechnology (Domsalla et al., 2010).

Euphorbia, belonging to Euphorbiaceae family, is a cosmopolitan genus of about 2000 species, which have several growth forms: herbs, shrubs, trees, geophytes, and multiple succulent forms. This genus shares in common the characteristics of having specialized, highly reduced, flowerlike inflorescences, and the presence of milky white latex (Fonseca et al., 2010, Gunawardana et al., 2015). *Euphorbia trigona* is a succulent plant from Africa, known as African milk tree because of its high latex production, cultivated in various countries for ornamental purposes (Villanueva et al., 2015). Many studies suggest that the latex of plants from *Euphorbia* genus is a rich source of proteases (Badgujar & Mahajan, 2013; Domsalla et al. 2010; Fibriana & Upaichit, 2015; Flemmig et al., 2017; Mahajan & Adsul, 2015; Patel et al., 2012; Rezanejad et al., 2015; Sufiate et al., 2017; Yadav et al., 2012). Recently, our research group reported for the first

time proteases from *E. milii* latex with nematicidal activity on *Panagrellus* sp. larvae (Sufiate et al., 2017).

Nematodes from *Meloidogyne* genus are known as root knot nematodes. They are responsible for production reduction of several plants with economic importance, causing many losses to agriculture. Although chemical nematicides are efficient in nematodes control, they are extremely toxic and non-specific (Adegbite, 2011). Thus, there is the need to develop new eco-friendly strategies to combat these nematodes.

The aim of this study was to partially purify and to characterize the proteases present in *E. trigona* latex, and to test its nematicidal activity on *M. incognita* larvae.

Material and Methods

Latex obtainment

Euphorbia trigona latex was collected through superficial cuts on the stem of native plants from Viçosa, Minas Gerais, Brazil. The latex was collected in microtubes and immediately stored at -20 °C. After five hours, the latex was thawed at room temperature, and then it was crushed. The clear supernatant was collected, which was denominated as crude extract.

Obtaining of *Meloidogyne incognita* juveniles

Pure population of *Meloidogyne incognita* collected in Lavras, Minas Gerais, identified by analysis of esterase phenotypes (Carneiro & Almeida, 2001), were maintained in soybean plant during 60 days. After this period, the root system of the plants was submitted to Baermann funnel for hatching eggs and

obtaining second stage juveniles (J_2), which were quantified in Peters' chamber. The nematode suspensions were calibrated to 50 J_2/mL.

Protease and protein assay

Enzymatic activity was measured through method described and modified by Soares et al. (2013). To calculate the protease unit (U), a tyrosine standard curve was constructed relating the absorbance obtained and the tyrosine concentration. One protease unit was defined as the amount of enzyme required to release 1.0 μg of tyrosine per minute under the assay conditions.

Protein content was determined according to Bradford (1976). A standard curve was constructed using bovine serum albumin relating the protein content and the absorbance.

Purification

For proteases purification, the crude extract was applied in a gel filtration column Sephacryl® S-300 previously equilibrated with citrate-phosphate buffer 25 mM (pH 6.0), connected to a vacuum pump and an automatic collector, at 4 °C. The flow was adjusted to 0.5 mL/min. Proteases elution was monitored by proteases activity, using glycine-sodium hydroxide buffer 100 mM (pH 9.0) at 70 °C, and by protein content. Fractions with high protease activity were pooled, constituting the proteases partially purified.

A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% was used to monitor the purification steps (Laemmli, 1970). The gel was stained with coomassie blue to allow visualization of the protein bands. A zymogram was prepared from a PAGE (10%) containing casein 0.1% co-

polymerized. The gel was incubated in citrate phosphate buffer 100 mM (pH 6.0) at 70 °C during 30 minutes. Then, it was stained with coomassie blue, and the protease activity was observed due to degradation halos.

Proteases characterization

Protease activity was determined in different pH values, ranging from 2.2 to 10.0, using citrate-phosphate buffer 100 mM (pH 2.2 to 8.0), and glycine-sodium hydroxide buffer 100 mM (pH 8.0 to 10.0). In this assay, the incubation temperature was 70 °C.

For temperature effect characterization in the protease activity, different temperature values ranging from 40 to 80 °C were utilized in the pH value that provided high protease activity during the previous assay.

Protease activity was measured in presence of the following inhibitors at 10 mM concentration: iodoacetamide, phenylmethylsulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA). This assay was conducted using citrate-phosphate buffer 100 mM (pH 3.0 and 6.0), and glycine-sodium hydroxide buffer 100 mM (pH 9.0), at 70 °C. All the protease activity assays were performed in triplicate.

Nematicidal assay

The effect of *E. trigona* proteases on *M. incognita* juveniles was tested. Two groups were formed in microtubes, a treated group containing enzymes and approximately 50 *M. incognita* J_2, and a control containing the same number of *M. incognita* J_2, without enzymes. This assay had seven replicates for each group. The microtubes were incubated at 28 °C, in a dark room, during 24 hours. After

this period, the number of live *M. incognita* J_2 was counted in each tube of both groups. For data analysis, analysis of variance was used at significance levels of 1 and 5%. The destruction efficiency of *M. incognita* larvae in relation to control was evaluated by Tukey test at 1% significance level (Ayres et al., 2003). Subsequently, the percent reduction of larvae number was calculated according to the following equation:

$$\%\text{Reduction} = \left(\frac{\overline{X}\text{ larvae from control} - \overline{X}\text{ larvae from treatment}}{\overline{X}\text{ larvae from control}}\right) \times 100$$

Results and Discussion

The purification methodology allowed partially purification of three distinct proteases, which were named trigonin 1, 2 and 3 (Table 1). In the SDS-PAGE, there are three protein bands present in the pool formed after the gel filtration chromatography (Figure 1a). In the zymogram, there are also three degradation halos (Figure 1b), confirming that the three proteins present in the pool are three proteases. However, the methodology used in this study did not allow that each of the enzymes were purified separately. The molecular weight of proteases was estimated at approximately: 36, 31 and 29 kDa for trigonin 1, 2 and 3, respectively (Figure 1). These molecular weights are similar to those related for hirtin from *E. hirta* (34 kDa) (Patel et al., 2012), eumiliin from *E. milii* (30 kDa) (Fonseca et al., 2010), and neriifolin from *E. neriifolia* (35 kDa) (Yadav et al., 2012).

Table 1. Purification steps of proteases from *Euphorbia trigona* latex.

Step	Total enzyme activity (U) (1)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	309.44	3.53	87.58	100%	1.00
Gel filtration	170.53	0.52	327.17	55%	3.74

(1)One protease unit (U) was defined as the amount of enzyme required to release 1.0 μg of tyrosine per minute under the assay conditions.

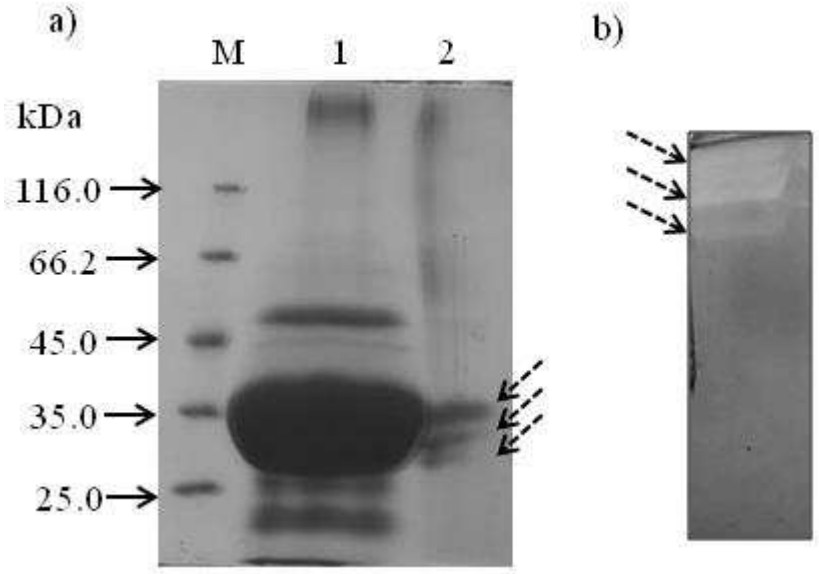

Figure 1. a) Purification analysis of proteases from *Euphorbia trigona* latex through SDS-PAGE 10%. Lane M: Protein molecular weight markers (Thermo Scientific); Lane 1: crude extract; Lane 2: proteases partially purified. Dashed arrows indicate the three proteases from E. trigona latex. **b)** Zymogram of the proteases partially purified from *E. trigona*. Dashed arrows indicate the clear bands formed by proteases activity.

The proteases showed highest activity at pH values of 4.0, 6.0 and 9.0 (Figure 2a). In relation to the temperature effect on protease activity, the 70 °C

temperature resulted in highest activity. After this temperature, there was an abrupt decline in protease activity (Figure 2b).

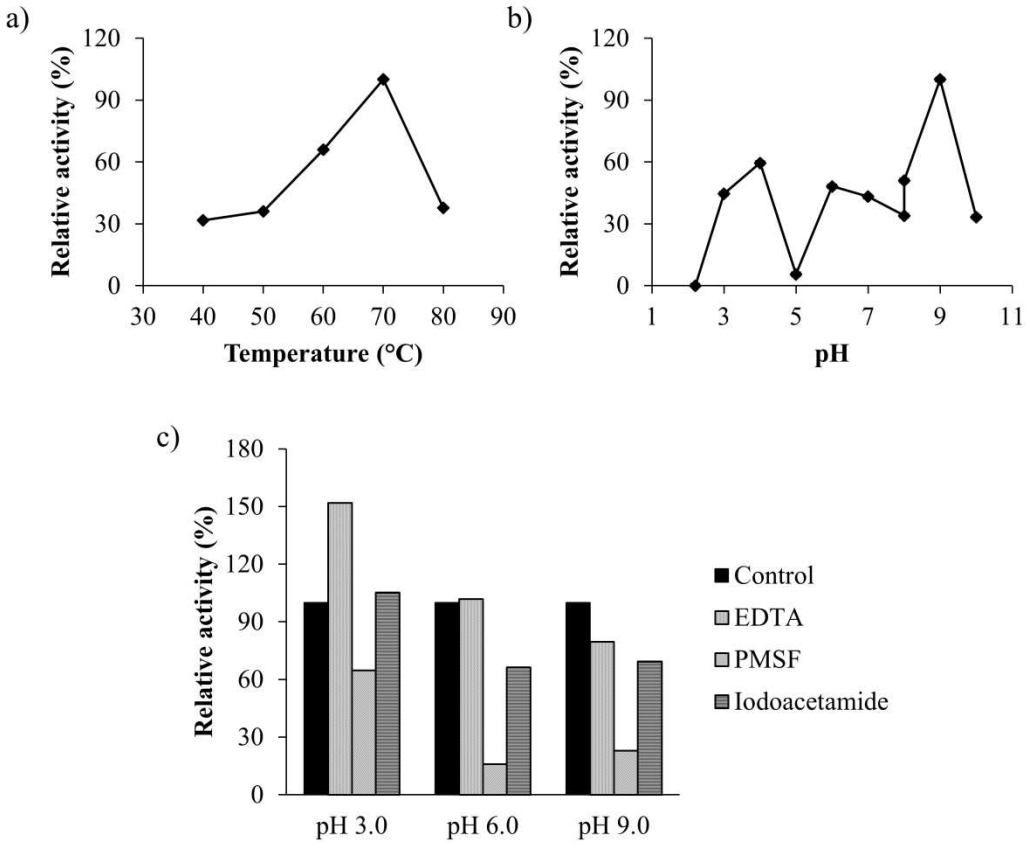

Figure 2. a) pH effect on enzymatic activity of proteases from *Euphorbia trigona* latex. **b)** Temperature effect on enzymatic activity of proteases from *E. trigona*. **c)** Effect of inhibitors on enzymatic activity of proteases partially purified from *Euphorbia trigona* latex.

These results differ from those observed by Fibriana & Upaichit (2015), which, evaluating the crude extract from *E. trigona*, determined that the pH 6.0 and temperature of 50 °C resulted in highest protease activity. The proteases from this present study showed the highest optimum temperature in comparison with different proteases from *Euphorbia* genus (Arima et al., 2000; Badgujar & Mahajan, 2012; Fonseca et al., 2010; Kumar et al., 2011; Lynn & Clevette-Radford, 1983, 1984; Moro et al., 2013; Patel et al., 2012; Rezanejad et al., 2015;

Yadav et al., 2012). The pHs optimum values observed in this study, 4.0, 6.0 and 9.0, were similar to described for microsciadin from *E. microsciadia* (pH 4.5) (Rezanejad et al., 2015), nivulian from *E. nivulia* (pH 6.6) (Badgujar & Mahajan, 2012), and miliin from *E. milii* (pH 9.0) (Moro et al., 2013).

Assays with proteases inhibitors were performed with EDTA, PMSF and iodoacetamide to identify metallo proteases, serine proteases and cysteine proteases, respectively. PMSF inhibited proteases activity in 35, 84 and 77% at pH values 3, 6 and 9, respectively. Iodoacetamide reduced proteases activity in 34 and 31% at pH 6 and 9, respectively, and it had no effect on proteases activity at pH 3. EDTA presence was responsible for increase activity in 52% at pH 3 (Figure 2c). These results indicate presence of serine and cysteine proteases in *E. trigona* latex. Serine proteases were described in *E. hirta* and *E. neriifolia* (Patel et al., 2012; Yadav et al., 2012), and cysteine proteases were observed in *E. microsciadia* and *E.nivulia* (Badgujar & Mahajan, 2012; Rezanejad et al., 2015).

The three proteases present in *E. trigona* latex reduced significantly ($p < 0.01$) the number of live *M. incognita* J_2. The efficiency of *E. trigona* proteases in nematodes control is showed by the high reduction percentage observed, with 96% reduction in 24 hours of treatment (Figure 3). In a similar study, the proteases from *E. milii* reduced the number of *Panagrellus* sp. larvae in 66 and 96% after 24 and 48 hours treatment (Sufiate et al., 2017). The present study is the first report of *E. trigona* proteases with nematicidal activity, therefore more studies regarding these enzymes action on nematodes are needed.

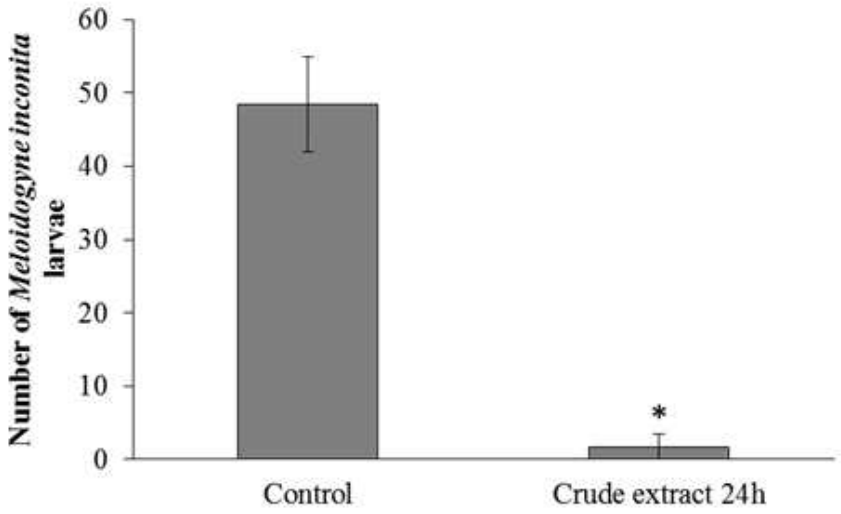

Figure 3. Average number of *Meloidogyne incognita* larvae after 24 hours treatment with the extract obtained from *Euphorbia trigona* latex. The control had no enzymes. Asterisk indicates significant difference ($p < 0.01$) between the treated group and the control by Tukey test at 1% significance level.

Conclusions

1. *E. trigona* latex contains three distinct proteases, named trigonin 1, 2 and 3, with an estimated molecular weight of 36, 31 and 29 kDa, respectively.
2. The pH and temperature that provide highest protease activity are pH values of 4.0, 6.0 and 9.0, and temperature of 70 °C.
3. There are serine and cysteine proteases present in *E. trigona* latex.
4. The three proteases present in *E. trigona* latex have nematicidal activity on *M. incognita* J_2 larvae.

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CAPÍTULO 3

Nematicidal action of *Pleurotus eryngii* metabolites

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Nematicidal action of *Pleurotus eryngii* metabolites

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Abstract

Pleurotus eryngii is one of the most cultivated and consumed mushroom species in North Africa, Europe and Asia. Fungi from *Pleurotus* genus have demonstrated nematophagous activities, however most of the literature reports are focused on *Pleurotus ostreatus*. The aim of this work was to evaluate *P. eryngii* action on *Panagrellus* sp. and to evaluate the effect of this fungus culture extract on *Meloidogyne javanica* eggs. *P. eryngii* fungus and its extract significantly reduced ($p<0.01$) the number of intact *Panagrellus* sp. larvae after 24 hours treatment in 60 and 90%, respectively. This effect is not related to enzymatic activity, but to the presence of other metabolites. *M. javanica* eggs, when treated with *P. eryngii* extract, showed a 53% reduction ($p<0.01$) in the number of intact eggs. The *M. javanica* intact eggs reduction is attributed to enzymatic activity, once the extract showed proteolytic and chitinolytic activities of, respectively, 32.74 and 3.57 U/mL. *P. eryngii* fungus has predatory activity against *Panagrellus* sp. larvae due to toxins production and negatively affects *M. javanica* eggs and juveniles development due to chitinases and proteases production, evidencing this fungus potential to be used in biological control.

Keywords: biological control; nematode; toxin; protease; chitinase

1. Introduction

Root-knot nematodes are the plant-parasitic nematodes from the genus *Meloidogyne*. They affect several plants of economic importance, causing many losses. These nematodes were traditionally combated through the use of chemical pesticides, among which, methyl bromide was the most used. Actually, this

pesticide was banned from many countries due to its negative environmental impact. In addition, nematodes have demonstrated resistance to a wide variety of known and used pesticides. Therefore, there is a need to develop new strategies to combat root-knot nematodes with ecofriendly weapons (Brand et al., 2010).

Panagrellus sp. is a free-living nematode highly active in water, unlike most plant parasitic nematodes, which move slowly. Thus, *Panagrellus* sp. is more suitable to be used as test organism in the search for new compounds with nematicidal action, once sublethal toxic effects can be easily observed by motility reduction (Kwok et al., 1992).

Pleurotus eryngii, also known as king oyster mushroom, is an edible mushroom with good flavor, high nutritional value and some medicinal properties. These characteristics made it one of the most cultivated and consumed mushroom species in North Africa, Europe and Asia (Yang et al., 2013). Fungi from *Pleurotus* genus have demonstrated nematicidal activities against larvae and adults of different nematodes species, in addition to proteases and chitinases production (Barron and Thorn, 1987; Genier et al., 2015; Gortari and Hours, 2008; Heydari et al., 2006; Lopes et al., 2015), however most of the reports are focused on *P. ostreatus*.

The aim of this work was to evaluate *P. eryngii* action on *Panagrellus* sp. and to evaluate this fungus culture extract effects on *M. javanica* eggs.

2. Material and methods

2.1. Organisms

The fungus *P. eryngii* was acquired from the collection of the Department of Microbiology, Universidade Federal de Viçosa. This fungus has been

maintained by continuous transfer to a 2% (w/v) solid Potato Dextrose Agar medium (2% PDA). To prepare the inoculum, the fungus was again replicated in Petri dishes containing PDA 2% and maintained at 28 °C during 7 days for inoculum removal as 2 cm disks.

The free-living nematodes from the *Panagrellus* genus were cultured in Petri dishes with a medium composed by distilled water and oat bran in a dark room at the Department of Biochemistry and Molecular Biology, Universidade Federal de Viçosa. Before test trials, the nematodes were extracted whit a Baerman apparatus and collected in hemolysis tubes after decanting.

M. javanica eggs were obtained from the Department of Phytopathology, Universidade Federal de Viçosa. They were collected from tomato roots kept in greenhouse according to methodology described by Hussey and Barker (1973), modified by Boneti and Ferraz (1981).

2.2. Extract production

The inoculum was transferred to a solid culture media in Erlenmeyer flasks. The culture medium was composed by wheat bran, which was washed with boiling water and then dried at 50 °C for 8 hours, 5 g, glucose 0.125 g and 7.5 mL of a solution containing K_2HPO_4 5g/L, $MgSO_4$ 0.1 g/L and yeast extract 5 g/L. After inoculation, the flasks were kept at 28 °C for 21 days. Then, the conidia were sampled and macerated with citrate phosphate buffer 100 mM pH 6,0, and subsequently centrifuged at 10,000 x g, 4 °C for 10 minutes. The supernatants, once recovered, constituted the extract (Gouveia et al., 2017).

2.3. Experimental Assays

Three in vitro experimental assays, named A, B and C were performed. In the test trial A, the interaction between *P. eryngii* conidia and *Panagrellus* sp. larvae was evaluated in laboratorial conditions. In the assays B and C, the effect of the extract obtained from *P. eryngii* cultivation was tested on *Panagrellus* sp. larvae and on *M. javanica* eggs, respectively.

2.3.1. Assay A

This test was composed of two groups: one treated and one control, with six replicates for each group. For this purpose, Petri dishes of 4.5 cm diameter containing a 2% (w/v) solid water-agar medium (2% WA) were previously marked in fields with 4 mm diameter. Each Petri dish contained 1000 *Panagrellus* sp. larvae. In the treated group, the Petri dishes also contained a *P. eryngii* inoculum, and in the control group there were only the *Panagrellus* sp. larvae, without fungus. These two groups were incubated in BOD incubator, in the dark, at 28 °C. During three days, every 24 hours, 10 random fields of 4 mm diameter were analyzed, in each Petri dish, using an optical microscope at 10x objective, and the number of intact larvae in each plate was counted.

2.3.2. Assay B

The assay B was constituted of three groups, with six replicates for each group. One replicate consisted of one microtube containing about 40 *Panagrellus* sp. larvae. The first group was treated with 40 µL of the extract obtained from *P. eryngii* cultivation, the second group was treated with 40 µL of the extract previously boiled for 30 minutes, and the third group was the control, to which 40 µL of distilled water was added. These groups were incubated in BOD incubator,

in the dark, at 28 °C, during 24 h. After this period, the number of intact larvae in each microtube was counted with an optical microscope at 10x objective.

2.3.3. Assay C

In the assay C, the extract obtained from *P. eryngii* cultivation was tested on *M. javanica* eggs. This assay was composed of two groups: one treated and one control. For each group, there were six replicates. Approximately 15 eggs were incubated in microtubes with 50 µL of the extract, during 24 h. For the control group, the extract was previously boiled during 30 minutes. After 24 h, the number of intact eggs in each microtube was counted with the aid of an optical microscope.

2.4. Statistical analyses

Panagrellus sp. larvae and *M. javanica* eggs reduction percentages, for each one of the assays, were determined through the formula below:

$$\%\text{Reduction} = \frac{(\overline{X}\ \text{intact eggs or larvae from control} - \overline{X}\ \text{intact eggs or larvae from treatment})}{\overline{X}\ \text{intact eggs or larvae from control}} \times 100$$

The data obtained were interpreted by analysis of variance (ANOVA) with significance levels of 5 and 1%. Subsequently, data were analyzed by Tukey test, with 1% significance.

2.5. Protease and chitinase activities measurements

Proteases and chitinases activities were measured from the extract obtained from the culture medium, as described by Braga et al. (2011) and Soares et al. (2015), respectively. One protease unit (U) was defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under the assay

conditions. One chitinase unit (U) was defined as the amount of enzyme that catalyzes the release of 1 μmol of reducing sugar per minute under the assay conditions. All enzymatic activity measurements were performed in triplicate.

3. Results and Discussion

P. eryngii fungus significantly reduced ($p<0.01$) the number of intact *Panagrellus* sp. larvae *in vitro*, with reduction percentages of 60 ± 6% after 24 hours treatment and 90 ± 2% after 72 hours treatment (Assay A) (Figure 1). The fungus demonstrated high capture activity, which could be observed through fungus hyphae interaction with the *Panagrellus* sp. larvae (Figure 2a). Figure 2b shows the complete larvae destruction and colonization by *P. eryngii* fungus.

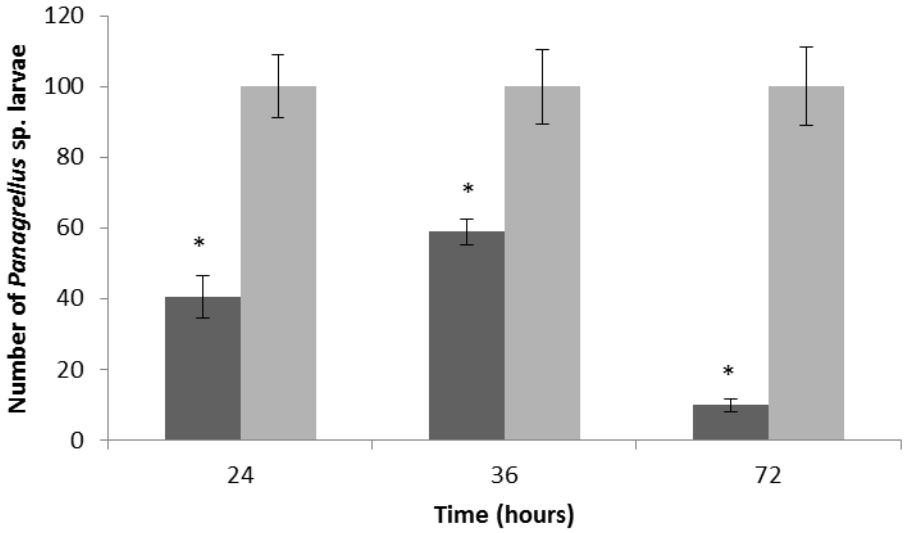

Figure 1. Daily average, standard deviation and percentage reduction of *Panagrellus* sp. larvae in water-agar (2% WA) during three days of treatment with *Pleurotus eryngii* fungus and the control group without fungus. Asterisk indicates significant difference ($p<0.01$) between the fungus-treated group and the control by Tukey test at 1% probability level.

The extract obtained from *P. eryngii* cultivation and the same extract previously boiled significantly reduced ($p<0.01$) the number of *Panagrellus* sp. larvae after 24 hours treatment in 90 ± 3 and 91 ± 3%, respectively, and there was no statistical difference ($p>0.01$) between these two treatments (Assay B) (Figure 3). Boiling the extract denatures the enzymes present in the medium, thus it can be inferred that this *Panagrellus* sp. larvae number reduction is due not to enzymes activities, but due to the presence of other metabolites.

These results are in agreement with that observed by Hibett and Thorn (1994). They described that fungi from *Pleurotus* genus produce a toxin derived from linoleic acid, constituting the main predatory activity mechanism of fungi from this genus on nematodes.

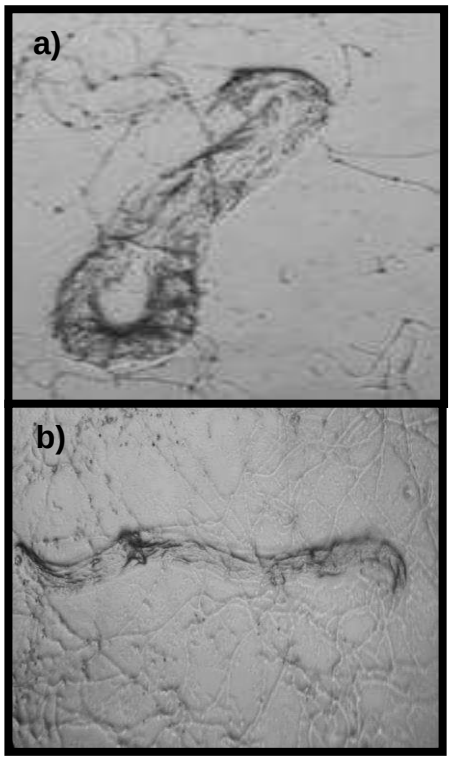

Figure 2. a) Initial destruction of larvae and colonization by *Pleurotus eryngii* hyphae. **b)** Complete destruction of *Panagrellus* sp. larvae by *Pleurotus eryngii*.

The fungus *P. ostreatus*, when tested directly over *Panagrellus* sp. larvae, showed a reduction percentage of 95% after 3 days treatment. The *P. ostreatus* enzymatic extract containing proteases was also tested over *Panagrellus* sp. larvae, resulting in a reduction percentage of 66% (Genier et al., 2015). Barron and Thorn (1987) reported that *P. ostreatus* can immobilize and attack nematodes through toxins production, which inactivates, but not kill, then.

Lopes et al. (2015) demonstrated the predatory activity of the fungus *P. eryngii* on *Ancylostoma caninum* larvae, with a reduction percentage of 48%. The filtrate obtained from *P. eryngii* cultivation showed predatory in vitro activity against *M. javanica* larvae, paralyzing 42% of second-stage juveniles after 24 hours treatment (Heydari et al., 2006).

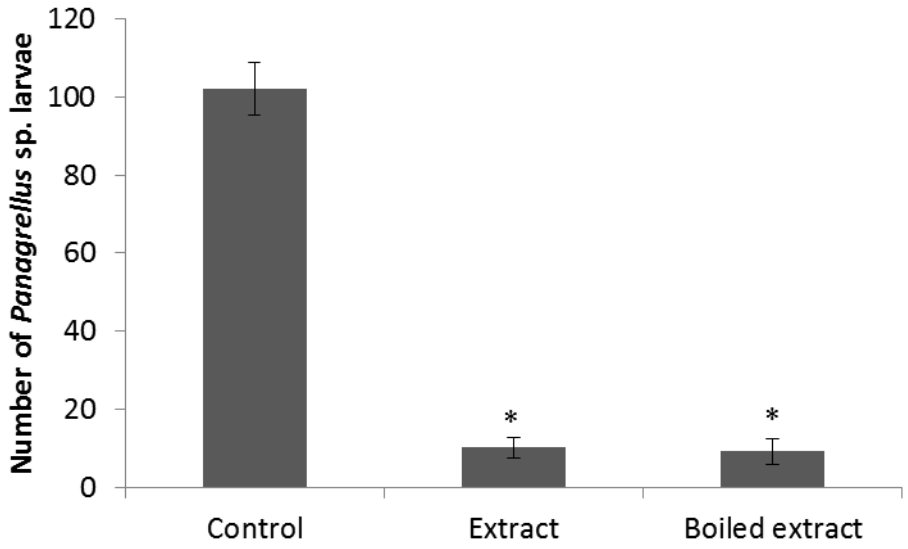

Figure 3. Average number of *Panagrellus* sp. larvae after 24 hours treatment with the extract obtained from *Pleurotus eryngii* cultivation and the same extract previously boiled. To control, distilled water was used. Asterisk indicates significant difference ($p<0.01$) between the treated group and the control by Tukey test at 1% probability level.

In this present work, *M. javanica* eggs treatment with *P. eryngii* extract reduced in 53 ± 11% ($p<0.01$) the number of intact eggs, when compared to the control treatment (Assay C) (Figure 4a). In this assay, the control was done using the extract previously boiled, thus the *M. javanica* intact eggs reduction is attributed to enzymes activities. The extract showed proteolytic and chitinolytic activities of, respectively, 32.74 and 3.57 U/mL.

Chitinases and proteases activities strongly affected the eggs structure and development causing eggshell ruptures, early hatching and vacuoles formation in eggs and juveniles. Figure 4b shows an intact egg, and Figure 4c shows a ruptured egg caused by proteases and chitinases action. In Figure 4d, is shown an early hatching of a vacuolated juvenile, suggesting eggshell and juvenile content hydrolysis. The early hatching is evidenced by incomplete ecdysis, indicated by an arrow in the Figure 4d.

Normally, the first moult occurs in the egg, when the first-stage juvenile (J1) moults to become the infective second-stage juvenile (J2). If the first moult does not occur completely before hatching, the development from J1 to J2 is impaired, negatively affecting the nematode infectivity and, consequently, its life cycle (Chitwood and Perry). Khan et al. (2004), evaluating the effects of *Paecilomyces lilacinus* protease and chitinase over *M. javanica* eggs, also observed vacuoles formation in eggs and juveniles, in addition to lower hatchability. Marino and Silva (2013) evaluated the fungus *P. ostreatus* efficiency in lettuce *M. incognita* control. These authors observed that the lettuce treated with this fungus reduced *M. incognita* root-knot number and egg mass.

Wang and Ng (2001) identified a protease produced from *P. eryngii* fruiting bodies. Palmieri et al. (2001) identified a protease produced from *P.*

ostreatus in a liquid culture medium, however this is the first report regarding *P. eryngii* chitinases production.

Wheat bran washing removes about 50 mg of carbohydrates per wheat bran gram, among which 45 mg correspond to sucrose (Gouveia et al., 2017). Thus, the mainly difference between this tested washed wheat bran culture medium and a non-washed wheat bran culture medium was that the first one is rich in glucose, once wheat bran was previously washed and glucose was added, and the second one is rich in sucrose.

There are no reports about *Meloidogyne* sp. eggs destruction by the fungus *P. eryngii*, nor regarding *P. eryngii* enzymes production with ovicidal activity. Most reports relate fungus nematicidal activity to toxins production, but to have effect on eggs, fungus need to promote eggshell lysis, which occurs due to mechanic and enzymes activities (Gortari and Hours, 2008; Morton et al., 2004).

Meloidogyne eggshells are formed by three layers: an inner lipid layer, a middle chitinous layer, and an outer vitelline layer. The vitelline layer is very thin and composed by lipoproteins, the chitin layer consists of chitin-protein complexes and its main role is to provide structural strength, and the lipid layer is composed by lipoproteins membranes, being responsible for egg impermeability. If the vitelline and chitinous layers are damaged, the lipid layer becomes very susceptible to damage. In this context, fungi have been shown as a potential alternative to combat these nematodes through enzymes production, especially chitinases and proteases (Brand et al., 2010; Gortari and Hours, 2008; Khan et al., 2004).

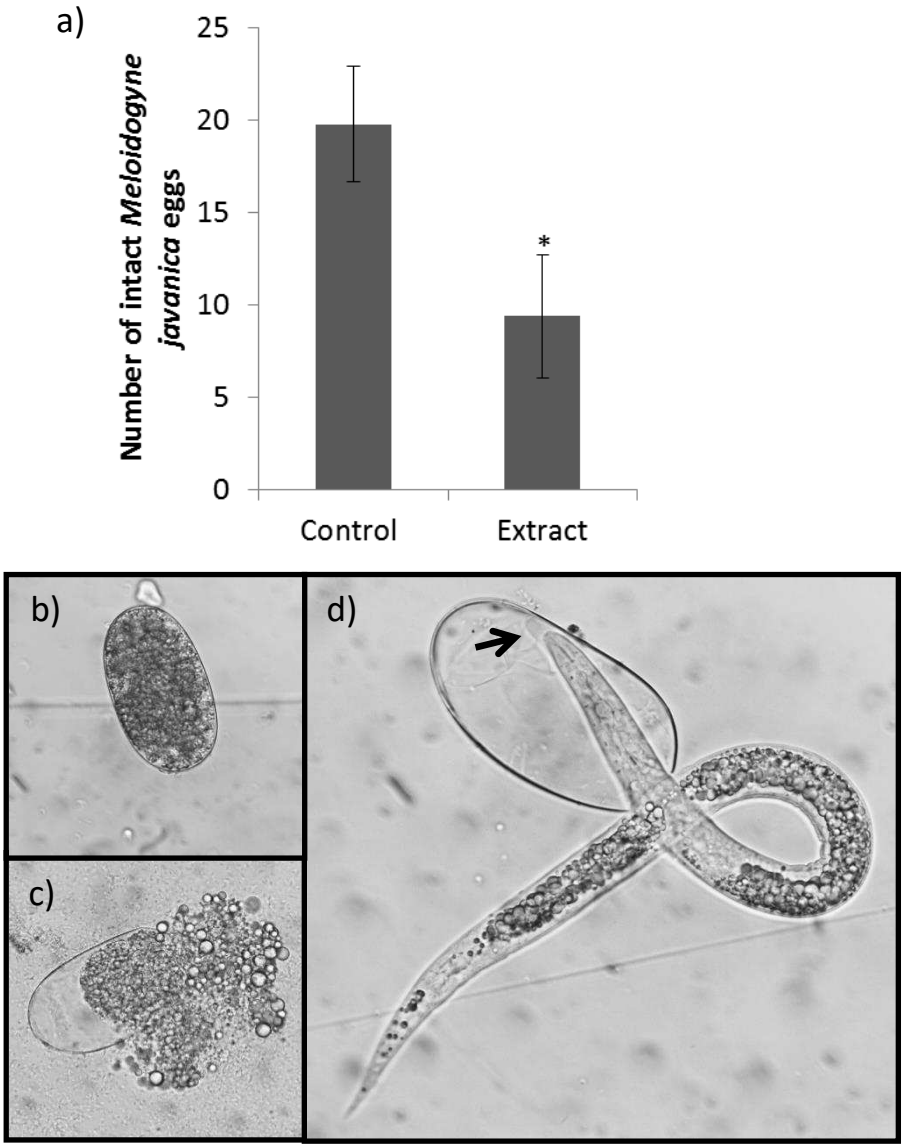

Figure 4. Activity effects of proteases and chitinases present in extract obtained from *Pleurotus eryngii* cultivation on *Meloidogyne javanica* eggs. **a)** Average number of *Meloidogyne javanica* eggs after 24 hours treatment with the extract obtained from *Pleurotus eryngii* cultivation. To control, the same extract previously boiled was used. Asterisk indicates significant difference ($p<0.01$) between the treated group and the control by Tukey test at 1% probability level. **b)** Intact *M. javanica* egg observed in the control. **c)** Ruptured *M. javanica egg* by proteases and chitinases action. **d)** Early hatching of a vacuolated juvenile. The early hatching is evidenced by incomplete ecdysis, indicated by the arrow.

4. Conclusions

Pleurotus genus has proven activity against nematodes (Barron and Thorn, 1987; Genier et al., 2015; Heydari et al., 2006; Lopes et al., 2015), but this is the first report regarding *P. eryngii* proteases and chitinases action on *M. javanica* eggs. The results demonstrate that *P. eryngii* fungus has predatory activity against *Panagrellus* sp. larvae through toxins production and negatively affects *M. javanica* eggs and juveniles development due to chitinases and proteases production, evidencing this fungus potential to be used as biological control.

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Carbohydr. Polym. 95, 615-620.

CAPÍTULO 4

In vitro and _in silico_ characterization of a novel dextranase from _Pochonia chlamydosporia_

Aceito para publicação no periódico: 3 Biotech

In vitro and *in silico* characterization of a novel dextranase from *Pochonia chlamydosporia*

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[e] Scholarship CNPq

Abstract

The objective of this study was to purify, characterize, and phylogenetically and structurally analyze the dextranase produced by the fungus *Pochonia chlamydosporia*. Dextranase produced by the fungus *P. chlamydosporia* was

purified to homogeneity in two steps, with a yield of 152 %, purification factor of 6.84 and specific activity of 358.63 U/mg. Its molecular weight was estimated by SDS-PAGE at 64 kDa. The enzyme presented higher activity at 50 °C and pH 5.0, using 100 mM citrate-phosphate buffer, was inhibited by Ag^{1+}, Hg^{2+}, Cu^{2+}, Mg^{2+}, and presented K_M of 23.60 µM. Mature dextranase is composed of 585 amino acids residues, with a predicted molecular weight of 64.38 kDa and pI 5.96. This dextranase showed a strong phylogenetic similarity when compared to *Trichoderma harzianum* dextranase. Its structure consists of two domains: the first composed by 15 β strands, and the second composed by a right-handed parallel β-helix.

Keywords: enzyme; *Verticillium chlamydosporium*; purification; 3D structure

Introduction

Dextranases (EC 3.2.1) are enzymes that hydrolyze α-(1,6), α-(1,2), α-(1,3) and α-(1,4) linkages in dextran polysaccharides. The main products from dextran hydrolysis by dextranase activity are glucose, isomaltose and isomalto-oligosaccharides and, thus, dextranases are commonly known as glucanases. Dextranases comprise a large and diverse group of enzymes which differ from one another due to the mode of action and resulted dextran hydrolysis products (Jaiswal and Kumar 2011; Picozzi et al. 2015).

Sugarcane, in the field, during transportation and in industry, is subject to microbial infections that lead to dextran production from sucrose. According to Boil and Wienese (2002), dextran presence in sugar processing results mainly from post-harvest delay and lack of hygiene in the factories.

Among the problems caused by dextran in the sugar industry we can mention sucrose concentration reduction as a result of its use in dextran production, lower sucrose recovery as a consequence of crystallization inhibition, and increase in the sugarcane juice viscosity (Khalikova et al. 2005). Moreover, dextran affects sugar quality, once syrup removal does not occur properly, forming residues in the sugar crystals (Batista 2014; Boil and Wienese 2002), as well as the formation of opaque, irregular, elongated and caramel-like crystals (Jiménez 2009). In addition to sucrose loss and worsening final product quality, the presence of dextran in the sugarcane juice also results in a higher equipment wear and compromise sucrose content quantification, which is the basis for the sugarcane payment (Singleton et al. 2002).

Physical separation methods, such as ultrafiltration, dialysis and reverse osmosis are useful methods for polysaccharide removal on a reduced scale, however, on a commercial scale, their use is not yet economically viable (Boil and Wienese 2002).

Therefore, in most cases, the methods used to remove dextran present in sugar solutions utilize enzymatic hydrolysis through dextranase use. However, for dextranase application to be economical, the availability of a suitable enzyme for the degree of dextran removal is required, which depends on time, temperature and dextran concentration (Boil and Wienese 2002; Picozzi et al. 2015).

Dextranase production was verified in some bacterial species, filamentous fungi and a small number of yeasts (Bhatia et al. 2010; Jiao et al. 2014; Zhang et al. 2016). Recently, our research group described the first report of dextranase production by the fungus *Pochonia chlamydosoporia*, a facultative parasite of eggs and female nematodes found in the soil in various regions of the world. This

enzyme reduced the dextran content of sugarcane juice by 75 %, with 12-hours treatment (Sufiate et al. 2017).

Thus, the objective of this study was to purify, characterize, and phylogenetically and structurally analyze the dextranase produced by *P. chlamydosporia.*

Materials and methods

Dextranase production by *P. chlamydosporia*

P. chlamydosporia VC4 (syn. *Verticillium chlamydosporium*) was obtained from the soil of Viçosa city, Minas Gerais, southeast of Brazil (latitude 20°4502000S, longitude 42°5204000W), and was maintained on solid 2 % Potato Dextrose Agar medium (2 % PDA (w/v)) under refrigeration, at 4 °C. For inoculation in liquid culture medium, four equally sized disks were removed from petri dishes edges. These disks were inoculated into 250 mL flasks containing 100 mL of liquid medium previously autoclaved at 121 °C for 15 minutes. The liquid medium contained 10 g/L dextran, 5 g/L $NaNO_3$, 4 g/L KH_2PO_4, 0.5 g/L $MgSO_4.H_2O$, 0.5 g/L KCl, 0.178 g/L $ZnSO_4.7H_2O$, 0.18 g/L $FeSO_4.7H_2O$ and pH was adjusted to 5.5. After inoculation, the flasks were kept at 28 °C and 180 rpm for 7 days. After this period, the flasks content was filtered and centrifuged at 10,000 X g for 20 minutes. The supernatant constituted the crude extract.

Enzyme activity and protein quantification

Dextranase activity was measured by evaluating the amount of reducing sugars through 3.5-dinitrosalicylic acid (DNS) method (Miller 1959). The enzyme

assay was performed using 370 µL of 100 mM citrate-phosphate buffer pH 6.0, 100 µL of 1% dextran solution and 30 µL of enzymatic sample. Reagents were incubated at 50 °C for 10 minutes, and the reaction was stopped by adding 500 µL of the DNS reagent. Samples were heated for 5 minutes in a boiling water bath, and then 1 mL of water was added to each sample. Absorbance readings were performed at 540 nm. In order to obtain the amount of reducing sugars, a standard curve was constructed with varying glucose concentrations. One dextranase unit (U) was defined as the amount of the enzyme that catalyzes the release of 1 µmol of reducing sugar per minute under the assay conditions. All enzymatic activity measurements were performed in triplicate.

Protein concentration was estimated at each fraction from the chromatographic columns by absorbance measured at wavelength of 280 nm. To determine the protein concentration in the crude extract and in the pools obtained from the chromatographic columns, Bradford method (Bradford 1976) was used.

Ion-exchange chromatography on DEAE-sepharose and CM-sepharose

A 5 mL crude extract volume was subjected to DEAE-Sepharose™ Fast Flow (Amershan Biosciences®) ion-exchange column previously equilibrated in 25 mM citrate-phosphate buffer pH 6.0, connected to an automatic collector and a peristaltic pump. Flow rate was kept constant at 0.5 mL/min. After sample application, 25 mL of 25 mM citrate-phosphate buffer pH 6.0 was applied. Resin-bound proteins were eluted with 25 mL of the same buffer solution with increasing linear NaCl concentrations up to 1 M. The collected fractions contained 3 mL each and those containing high dextranase activity were selected and assembled to form a pool.

This pool was subjected to a CM-Sepharose ™ Fast Flow (Amershan Biosciences®) ion exchange column previously equilibrated in 25 mM citrate-phosphate buffer pH 6.0, connected to an automatic collector and a peristaltic pump. The flow rate was kept constant at 0.5 mL/min. After sample application, 18 mL of 25 mM citrate-phosphate buffer pH 6.0 was applied. Resin-bound proteins were eluted with 18 mL of the same buffer solution with increasing linear NaCl concentrations up to 1 M. The collected fractions contained 1.5 mL each and those containing high dextranase activity were selected and assemble to form a pool, which consisted of the purified enzyme.

PAGE

Samples were subjected to 10 % (w/v) polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Electrophoresis was performed at 80 V, and the gel was stained with silver nitrate to allow protein visualization.

An in-gel activity assay was carried out in order to confirm the dextranase activity of the purified dextranase from *P. chlamydosporia*. Samples were applied to 10 % native PAGE gel containing 1 % blue dextran, and a voltage of 80 V was used. The gel was incubated in 100 mM citrate-phosphate buffer pH 6.0 for 1 hour at 50 °C. Dextranase activity was detected as a clear band on blue background. Subsequently, the gel was stained with silver nitrate to confirm the enzyme presence.

Enzymatic characterization

pH and temperature effect on enzyme activity

An assay was conducted using 100 mM citrate-phosphate buffer with different pH values: 2.2; 3.0; 4.0; 5.0; 6.0; 7.0 and 8.0. Assays were performed under the conditions described above, using the buffer solution in such pH values. Dextranase activity was measured at different reaction incubation temperatures: 30, 40, 50 and 60 °C, keeping all the previously described conditions.

Thermal stability of the purified dextranase was evaluated by incubating enzymatic samples at 40 and 50 °C for 0 to 300 minutes, before being subjected to the enzymatic activity measurement.

Effect of metal ions and other reagents on enzymatic activity

The presence effect of the following ions, initially in saline form, on dextranase enzymatic activity was evaluated: $ZnCl_2$, $CaCl_2$, $MgSO_4$, $MnSO_4$, $CuSO_4$, $FeSO_4$, $AgNO_3$, $HgSO_4$, and EDTA (Ethylenediamine tetraacetic acid) and SDS (sodium dodecyl sulfate) reagents. Compounds were prepared at 10 mM concentration in 100 mM citrate-phosphate buffer pH 6.0. Enzymatic activity in the absence of any reagent was considered to be 100 %. The other reaction conditions followed as described in section 2.2.

Kinetic constants

Kinetic parameters K_M (Michaelis-Menten constant) and V_{max} (maximum velocity) were calculated by means of the velocity curve as a function of the substrate concentration, Michaelis-Menten model. For this, Curve Expert program, version 1.4 for Windows, was used.

In silico predictions, molecular modeling and phylogeny

FASTA sequence of dextranase precursor from *P. chlamydosporia* was retrieved from UNIPROTKB (ID A0A179FQV9). The target sequence was searched for similar sequences using BLASTp (protein-protein Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST/). Signal dextranase sequence was predicted using SignalP 4.0 server. Theoretical protein isoelectric point (pI) and molecular weight were calculated using the pI/MW tool (http://web.expasy.org/compute_pi/).

Dextranase sequences were selected from CAZy (http://www.cazy.org/GH49_characterized.html) and from National Center for Biotechnology Information (NCBI). The selected sequences were: *P. chlamydosporia* (XP_018144597.1), *Aspergillus niger* (BAA18971.1), *Lipomyces starkeyi* (AAS90631.1), *Talaromyces minioluteus* (AAB47720.1), *Trichoderma harzianum* (KKO97501.1), *Talaromyces cellulolyticus* (GAM43713.1), *Sporothrix schenckii* (ERS97553.1), *Sporothrix brasiliensis* (KIH89944.1) and *Penicillium subrubescens* (OKP15192.1). Dextranase amino acid sequences were aligned with ClustalX, and MEGA 7 was used to construct a neighbor joining (NJ) tree. The 3-dimensional (3D) structure for dextranase from *P. chlamydosporia* was predicted by protein modeling with the SWISS-MODEL server (http://swissmodel.expasy.org/), using a *Talaromyces minioluteus* (syn. *Penicillium minioluteum*) (UniProt Acession Nº P48845) dextranase template.

Results and discussion

Purification of dextranase produced by *P. chlamydosporia*

Dextranase produced by *P. chlamydosporia* was purified to homogeneity in two steps, the first step being anion-exchange resin chromatography (DEAE-Sepharose), and the second step a cation-exchange resin chromatography (CM-Sepharose). The enzyme was purified with a yield of 152 %, purification factor of 6.84 and specific activity of 358.63 U/mg (Table 1). In the first purification stage, increased total dextranase activity was observed, which suggests inhibitor elimination during this step. This, consequently, can explain the high yield obtained for the process.

Table 1 Purification steps of dextranase from *Pochonia chlamydosporia*.

Step	Total enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	1397.50	26.65	52.45	100%	1
DEAE Sepharose	2224.09	16.02	138.81	159%	2.65
CM Sepharose	2120.49	5.91	358.63	152%	6.84

One dextranase unit (U) was defined as the amount of the enzyme that catalyzes the release of 1 μmol of reducing sugar per minute under the assay conditions.

In SDS-PAGE (Fig. 1a), there is only a single band, corresponding to the purified dextranase molecular weight estimated at 64 kDa by mobility comparison with marker proteins. In the native PAGE prior to staining by silver nitrate (Fig. 1b) dextranase activity was confirmed in samples from the crude extract, DEAE-Sepharose chromatography and CM-Sepharose chromatography. Dextranase activity was observed as clear bands in the gel. This same native PAGE was stained by silver nitrate (Fig. 1c) in order to confirm dextranase presence and to observe other proteins occurrence in the samples. After staining with silver nitrate of native PAGE (Fig. 1c), it was possible to observe the presence of other protein

in the crude extract besides dextranase, and that dextranase activity band corresponds to the only protein band in the sample from the second chromatography, confirming its purification to homogeneity.

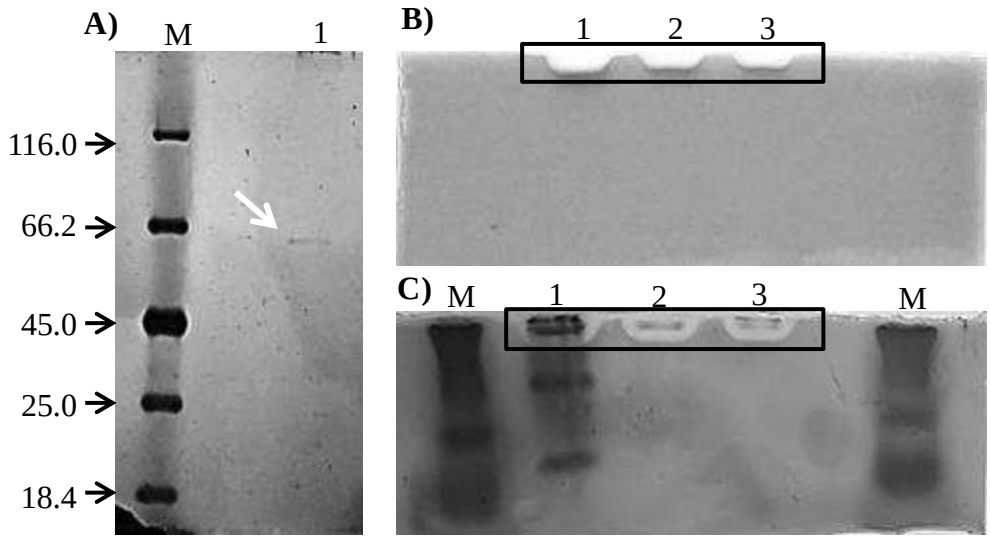

Fig. 1 Purified dextranase from *Pochonia chlamydosporia* on SDS-PAGE stained with silver nitrate. Lane M: Protein molecular weight markers (Thermo Scientific); Lane 1: purified dextranase (A). Clear bands, indicated by black rectangles, showing dextranase activity on a 10% native PAGE gel containing 1% (w/v) blue dextran, prior to staining with silver nitrate (B), and stained with silver nitrate (C). Lane M: Protein molecular weight markers (Thermo Scientific); Lane 1: crude extract; Lane 2: pool from DEAE-Sepharose; Lane 3: purified dextranase

This estimated molecular weight is close to the observed molecular weight values for purified dextranases from *Chaetomium erraticum*, *Paecilomyces marquandii*, *Penicilillium aculeatum*, *Penicillium funiculosum* and two dextranases from *Chaetomium gracile*, whose molecular weights were estimated at, respectively, 59, 73, 66.2, 67, 71 and 77 kDa (Abdel-Aziz et al. 2007; Hattori et al. 1981; Machado 2009; Mahmoud et al. 2014; Virgen-Ortíz et al. 2015).

Enzymatic characterization

pH and temperature effect on enzymatic activity

Purified *P. chlamydosporia* dextranase showed higher activity at pH 5.0 (Fig. 2a). In addition, it was observed that this dextranase has a high hydrolysis capacity also at pH 6.0, with relative activity above 90 %, and at pH 4.0, with a 74 % relative activity. Similarly, Machado (2009) determined that pH 6.0 provides higher activity of dextranase produced by *P. marquandii*, and that at pH 4.0, it has 90 % relative activity. Virgen-Ortiz et al. (2015) reported that pH 5.2 results in increased activity of the dextranase produced by *C. erraticum*, and observed relative activity above 85% in the pH ranging from 4.0 to 6.0. However, when studying *P. aculeatum* dextranase, Mahmoud et al. (2014) observed that the highest activity pH was 4.5, and that from this pH value, dextranase activity decreased abruptly.

The highest activity temperature of purified *P. chlamydosporia* dextranase was 50 °C (Fig. 2b). At 60 °C the enzyme demonstrated only 48 % activity, indicating that at this temperature protein denaturation may occur. These results are close to the results found by Mahmoud et al. (2014), which determined that dextranase produced by *P. aculeatum* exhibited higher activity at 45 °C, with relative activity of approximately 75 % at 40 and at 50 °C. On the other hand, Virgen-Ortiz et al. (2015) observed that *C. erraticum* dextranase exhibited maximum activity at 60 °C and more than 85 % of its maximum activity in the temperatures ranging from 55 to 65 °C, and Machado (2009) reported that *P. marquandii* dextranase presented higher activity temperature at 55 °C, and at 60 °C it retained 80 % of its activity. These results demonstrate that the dextranase

produced by the fungus *P. chlamydosporia* presents similar properties when compared to those already described in the literature.

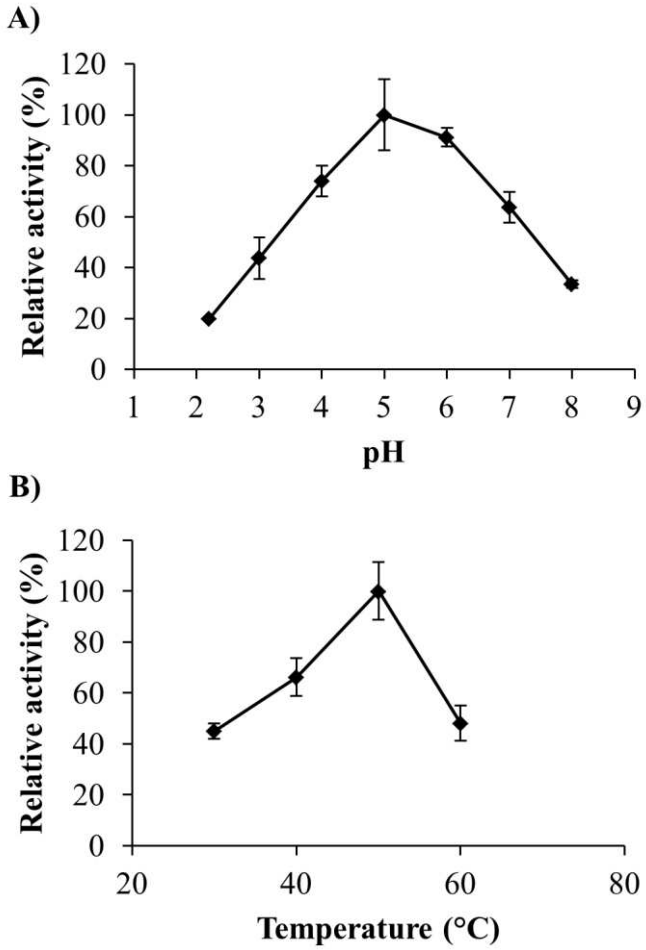

Fig. 2 pH (A) and temperature (B) effect on the enzymatic activity of purified dextranase from *Pochonia chlamydosporia*. 2.43 U/mL and 2.44 U/mL were defined as 100% relative activity for pH and temperature effect, respectively

P. chlamydosporia dextranase thermal stability was tested at 40 and at 50 °C (Fig. 3), which corresponds to the reaction temperature which resulted in higher enzymatic activity. Dextranase showed low thermal stability at 50 °C, exhibiting 35 % relative activity when previously incubated for 30 minutes prior enzymatic activity measurement. However, the enzyme was stable at 40 °C, with 48 % relative activity when previously incubated for 180 minutes. *Aspergillus penicillioides* dextranase showed little thermal stability, with relative activity

close to zero when incubated at 45 °C for 60 minutes (El-Shamy and Atalla 2014). Machado (2009) observed that dextranase produced by *P. marquandii* was much more stable at 37 °C, maintaining 85 % of its initial activity after has been incubated for 24 hours. On the other hand, when evaluating thermal stability at 55 °C, reaction temperature that resulted in higher activity, Machado (2009) also observed low stability, with relative activity, after 30 minutes of incubation, of approximately 20 %. Although there are dextranases produced by bacteria much more stable at higher temperatures, the industrial application of these enzymes becomes impracticable due to the extremely low amount of dextranase produced (Wynter et al. 1995). Thus, despite having intermediate stability, *P. chlamydosporia* dextranase has the advantage of being produced in higher quantity, with 4.82 U/mL activity in the crude extract.

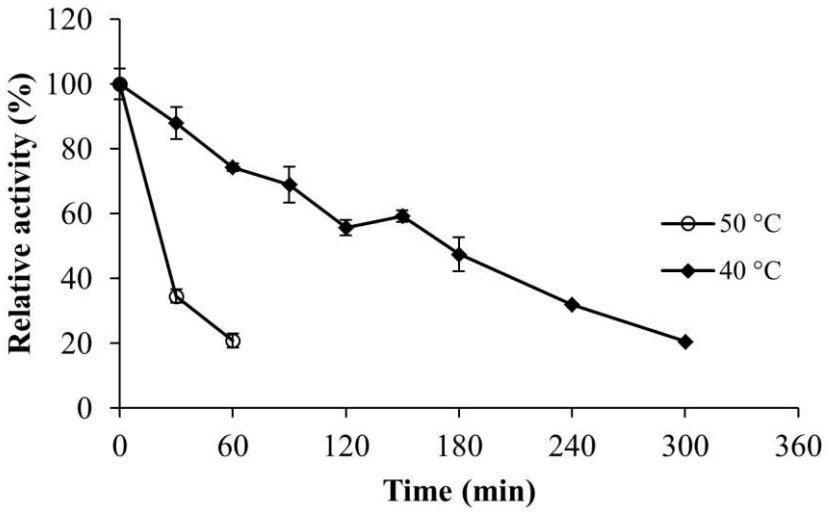

Fig. 3 Thermal stability of purified dextranase from *Pochonia chlamydosporia* at 40 and 50 °C. 2.44 U/mL was defined as 100% relative activity

Effect of metal ions and other reagents on enzymatic activity

Ag^{1+} and Hg^{2+} ions strongly inhibited purified dextranase, both reducing dextranase activity to 17 %. Cu^{2+}, Mg^{2+}, Fe^{2+}, Ca^{2+} and Zn^{2+} ions reduced enzyme activity to, respectively, 51, 60, 71, 77 and 88 %. The SDS and EDTA compounds had little influence on dextranase activity, reducing it to 79 and 81 %, respectively (Table 2).

Table 2 Effect of metal ions and other reagents on the enzymatic activity of purified dextranase from *Pochonia chlamydosporia*.

Compound	Relative activity (%)
Control	100.00 ± 10.85
Zn^{2+}	87.81 ± 0.77
Ca^{2+}	77.00 ± 8.48
Mg^{2+}	59.67 ± 2.66
Cu^{2+}	51.05 ± 7.29
Mn^{2+}	88.96 ± 15.11
Fe^{2+}	70.76 ± 9.75
Ag^{1+}	17.22 ± 0.02
Hg^{2+}	17.40 ± 0.87
EDTA	80.52 ± 6.92
SDS	79.03 ± 6.68

P. marquandii dextranase activity increased by 20 and 15 % in the presence of, respectively, Fe^{2+} and Mn^{2+} at 10 mM concentration and decreased by approximately 50 % of its activity in the presence of Cu^{2+} and Pb^{2+} and 40 % in the presence of Ag^{+}, also at 10 mM concentration (Machado 2009). *C. erraticum* dextranase suffered total inhibition in the presence of 1 mM Ag^{+} and 77 % inhibition in the presence of 1 mM Cu^{2+} and had its activity increased by 18 and 25 % when incubated with, respectively, 1mM Ca^{2+} and Co^{2+} (Virgen-Ortíz et

al. 2015). Cu^{2+} and Hg^{2+} completely inhibited *C. gracile* dextranase, and Fe^{3+} inhibited its activity by 40 % at 1 mM concentration (Hattori et al. 1981). *P. funiculosum* dextranase was totally inhibited by Hg^{2+}, had its activity reduced by approximately 70 % when incubated with Ag^{+}, and was activated by 2 mM Co^{2+}, Mn^{2+} and Cu^{2+} in 42, 39 and 21%, respectively (Sugiura et al. 1973).

Kinetic constants

The maximum velocity values and the Michaelis-Menten constant were determined by non-linear data adjustment to the Michaelis-Menten equation, with correlation coefficient (r^2) of 0.9935. *P. chlamydosporia* dextranase presented K_M and V_{max} values of, respectively, 1.77 g/L and 2.09 mM glucose/min at 50 °C and pH 6.0 for dextran with molecular weight ranging from 60,000 to 90,000. Considering the average dextran molecular weight as 75,000, the K_M value resulted in 23.60 μM. This value has the same order of magnitude of observed K_M values for other dextranases. Abdel-Naby et al. (1999) found that *P. funiculosum* dextranase showed K_M of 25 μM for dextran with molecular weight 260,000, and Machado (2009) reported K_M of 7.86 μM for *P. marquandii* dextranase.

In silico **predictions, molecular modeling and phylogeny**

The *P. chlamydosporia* dextranase precursor sequence, deposited in UNIPROTKB (ID A0A179FQV9), has 601 amino acid residues, with a peptide signal of 16 residues. Mature protein is composed of 585 amino acid residues, exhibiting molecular weight and pI of, respectively, 64.38 kDa and 5.96. This predicted molecular weight value coincides with the molecular weight value of 64 kDa estimated empirically by SDS-PAGE in the present study.

In addition, the dextranase precursor sequence of *P. chlamydosporia* (XP_018144597.1) shares a high degree of identity with other fungi dextranases, with 69, 79, 76, 70, 70 and 67 % identity with the dextranases of *Lipomyces starkeyi* (AAS90631.1), *Trichoderma harzianum* (KKO97501.1), *Talaromyces cellulolyticus* (GAM43713.1), *Sporothrix schenckii* (ERS97553.1), *Sporothrix brasiliensis* (KIH89944.1) and *Penicillium subrubescens* (OKP15192.1), respectively.

The selected fungi dextranases multiple sequence alignment revealed the occurrence of several conserved regions, occurring mostly among amino acid residues at positions ranging from 82 to 134, 324 to 377 and 403 to 478 (Fig. 4). Although sequence and structural homology methods are tools to determine global similarities between compared proteins, the enzyme molecular role is related to its active site. Thus protein comparisons focusing on global sequence and structural similarities may neglect proteins with conserved active sites but divergent sequences and structures (Morya et al. 2012)..

The phylogenetic tree constructed based on protein sequences using Neighbor Joining method suggested that dextranases from fungi had one common ancestor (Fig. 5). Phylogenetic analysis showed that these dextranases cluster into two subclades. One subclade contains dextranases from Sordariomycetes, including *S. schenckii* and *S. brasiliensis*. Another subclade consisted of six dextranases, and contains three clusters. The first cluster is composed by *T. minoluteus* and *T. cellulolyticus*, from Eurotiomycetes, and *L. starkeyi* from Saccharomycetes. The dextranase from *P. chlamydosporia* clusters with the dextranase from *T. harzianum*, both species belong to Sordariomycetes, constituting the second cluster. *P. subrubescens*, from Eurotiomycetes, was

distinct from others and formed the third cluster. Thus, though the dextranase from *P. chlamydosporia* has a distant evolutionary relationship with dextranases from some fungi, it clusters with dextranases from others fungi belong to different orders.

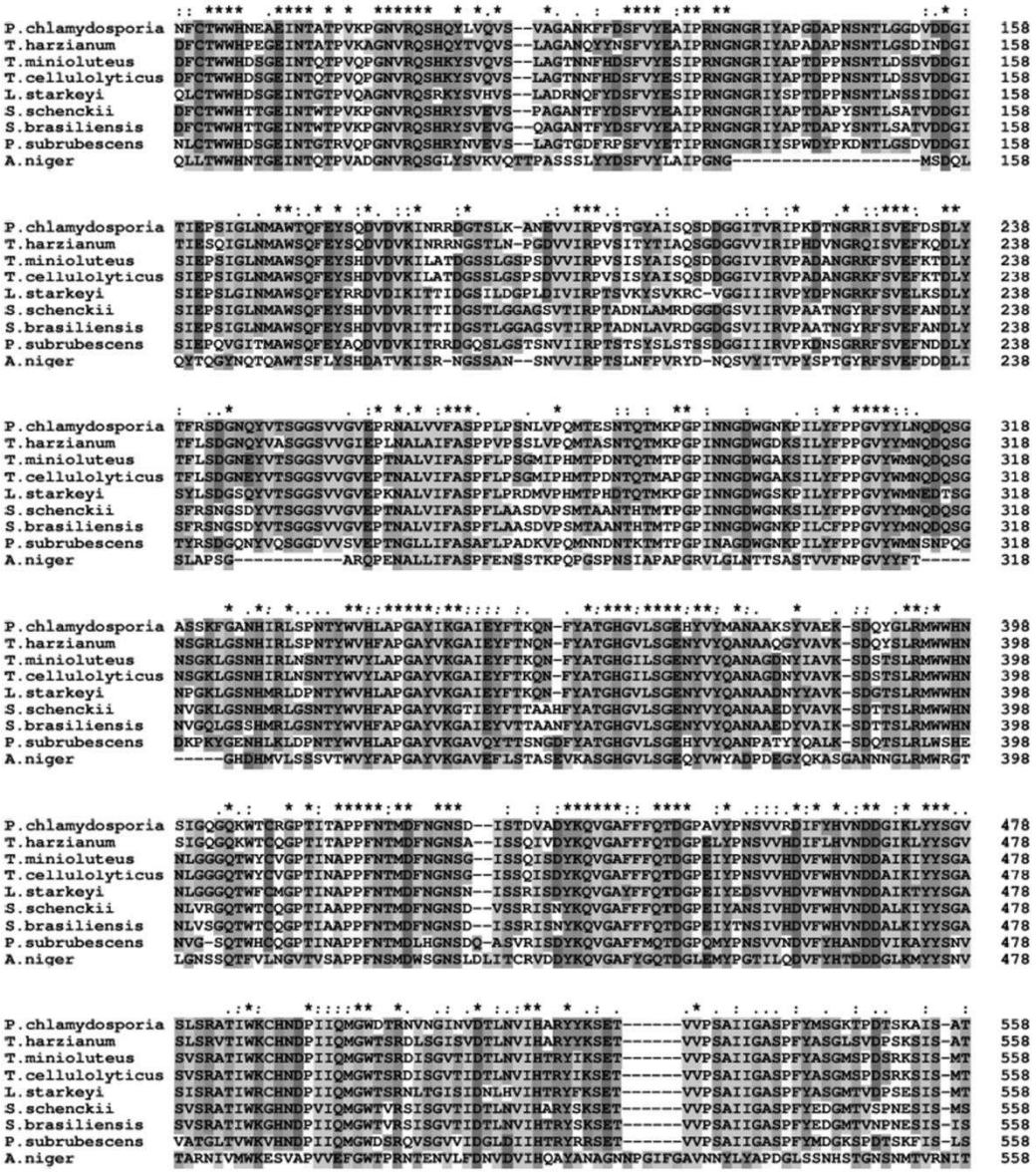

Fig. 4 Alignment of the dextranases from different fungi. Asterisk, colon and dot symbols indicate full conservation, and groups of strongly and weakly similar properties residues, respectively

The 3D structure (Fig. 6) of the dextranase from *P. chlamydosporia* was modeled using SWISS-MODEL employing dextranase structure from *Penicillium minioluteum* (anamorph: *T. minoluteum*) (PDB code: 1ogm.1) as template, sharing 75.44 % identity. Dextranase from *P. chlamydosporia* consists of two domains. The first domain is composed by 211 residues forming 15 β strands. The other domain consists in a right-handed parallel β-helix. The two domains are connected by several residues from the N-terminus of the β -helix. Asp395 is the catalytic amino acid residue from *P. minioluteum* dextranase, template of the dextranase structure of this present study (Larsson et al. 2003). This residue is shown conserved in the dextranases submitted to alignment, corresponding to the Asp422 residue in the *P. chlamydosporia* dextranase with the signal peptide (Fig. 4). This indicates that this residue may play catalytic role in the dextranase target of this study, since enzymes Asp residues are essential to enzyme activity involving glucans hydrolysis (Wang et al. 2014).

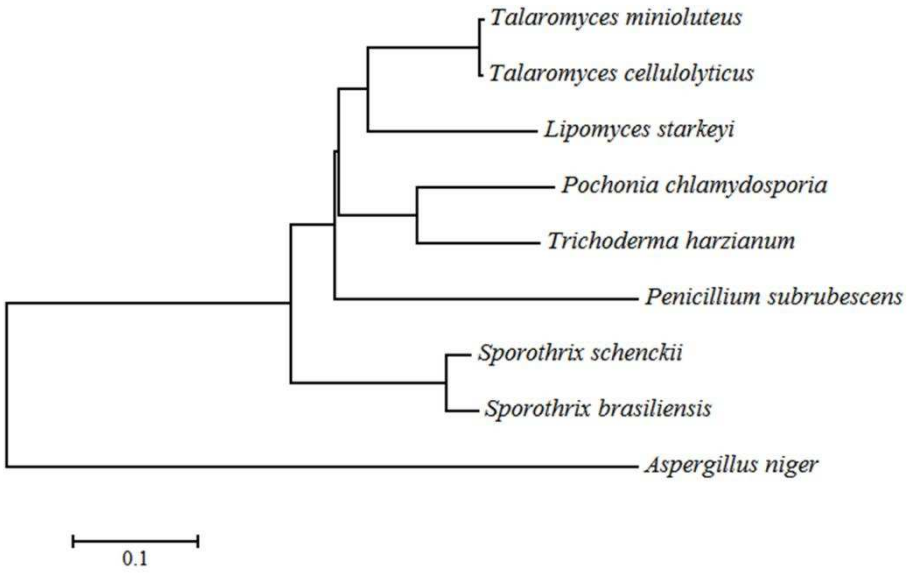

Fig. 5 Phylogenetic tree constructed by Neighbor-joining tree method based on dextranases sequences from different fungi species. *Pochonia chlamydosporia* 170 [XP_018144597.1], *Aspergillus niger* ATCC 9642 [BAA18971.1],

Lipomyces starkeyi KSM22M [AAS90631.1], *Talaromyces minioluteus* MUCL 38929 [AAB47720.1], *Trichoderma harzianum* T6776 [KKO97501.1], *Talaromyces cellulolyticus* Y-94 [GAM43713.1], *Sporothrix schenckii* ATCC 58251 [ERS97553.1], *Sporothrix brasiliensis* 5110 [KIH89944.1] and *Penicillium subrubescens* CBS 132785 [OKP15192.1]

P. chlamydosporia dextranase predicted structure is similar to that found for dextranases of different species. The dextranase structure of *Arthrobacter oxydans* predicted by Wang et al. (2014) is also composed of two domains, the first domain consisting of 13 β-strands containing 200 residues and the second domain a right-handed β-helix. In addition, isopullulanase from *A. niger*, an enzyme belonging to GH 49, is also structurally similar to dextranases, having two domains: one composed of 13 β-strands and the other domain composed of a right-handed β-helix (Mizuno et al. 2008).

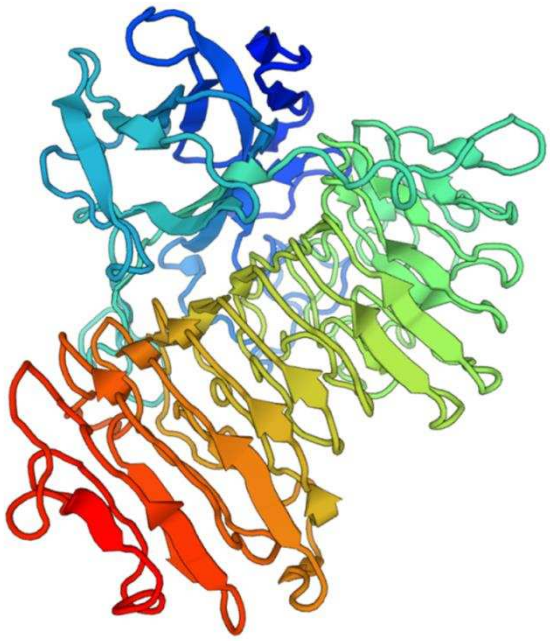

Fig. 6 3D structure of dextranase from *Pochonia chlamydosporia* predicted using the dextranase from *Talaromyces minioluteus* as template

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Compliance with Ethical Standards

Conflicts of interest

The authors declare that they have no conflict of interest.

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3. CONCLUSÕES GERAIS

- O fungo *Pleurotus eryngii* possui atividade nematicida predatória sobre larvas de *Panagrellus* sp.;

- O fungo *P. eryngii* e as plantas *Euphorbia milii* e *E. trigona* produziram enzimas extracelulares com atividade nematicida sobre diferentes nematoides;

- As enzimas produzidas por *P. eryngii*, *E. milii* e *E. trigona* possuem potencial para serem utilizadas no controle de nematoides;

- Foram identificadas duas proteases do látex de *E. milii* e três proteases do látex de *E. trigona*;

- A dextranase do fungo *Pochonia chlamydosporia* foi purificada e caracterizada, fornecendo informações úteis para futuras aplicações industriais (Anexo I).

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ANEXO I

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

16/11/2017 870170088278 10:12

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 024550 0

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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 16/11/2017 às 10:12, Petição 870170088278

Petição 870170088278, de 16/11/2017, pág. 1/28

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): Processo de produção de dextranase pelo fungo Pochonia chlamydosporia e seu uso na remoção de dextrana

Resumo: A presente invenção, destinada ao setor alimentício, pelas indústrias sucroalcooleiras, trata-se da otimização das condições de cultivo do fungo Pochonia chlamydosporia em fermentação submersa para a produção de dextranase e do uso dessa enzima na remoção de dextrana de extratos vegetais que contenham este polissacarídeo, mais especificamente de caldo de cana. As condições otimizadas permitiram aumento da produção de dextranase em 1,74 vezes, com pH e temperatura de maior atividade de, respectivamente, 5,0 e 50 °C, e massa molecular de 64 kDa. A adição de 15 U da dextranase do fungo P. chlamydosporia reduziu em 75% o teor de dextrana do caldo de cana, após 12 h de tratamento, quando comparado com o controle.

Figura a publicar: 1

PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 16/11/2017 às 10:12, Petição 870170088278

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Documentos anexados

Tipo Anexo	Nome
Comprovante de pagamento de GRU 200	comprovante de pagamento.pdf
Procuração	Procuração e DOU Dr. Afonso.pdf
Relatório Descritivo	Relatório descritivo .pdf
Reivindicação	Reivindicação .pdf
Desenho	Figura.pdf
Resumo	Resumo.pdf

Acesso ao Patrimônio Genético

☑ Declaração Negativa de Acesso - Declaro que o objeto do presente pedido de patente de invenção não foi obtido em decorrência de acesso à amostra de componente do Patrimônio Genético Brasileiro, o acesso foi realizado antes de 30 de junho de 2000, ou não se aplica.

Declaração de veracidade

☑ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.

PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 16/11/2017 às 10:12, Petição 870170088278