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Optimization of protease production by the fungus *Monacrosporium thaumasium* and its action against *Angiostrongylus vasorum* larvae

Otimização da produção de protease pelo fungo *Monacrosporium thaumasium* e sua ação contra larvas de *Angiostrongylus vasorum*

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Abstract

The objectives of this study were to optimize protease production from the nematophagous fungus *Monacrosporium thaumasium* (NF34a) and evaluate its larvicidal activity and biological stability. An isolate of the nematophagous fungus *Monacrosporium thaumasium* (NF34a) was used to produce the enzyme. The Plackett-Burman design was used in order to scan which components of the culture medium could have a significant influence on protease production by the fungus NF34a. An *in vitro* assay was also performed to evaluate the larvicidal activity of NF34a. It was observed that only one component of the culture medium (yeast extract), at the levels studied, had any significant effect ($p < 0.05$) on protease production. There was a reduction ($p < 0.01$) in the mean number of larvae recovered from the treated groups, compared with the control groups. The results confirm previous reports on the efficiency of nematophagous fungi for controlling nematode larvae that are potentially zoonotic. Thus, given the importance of biological control, we suggest that further studies should be conducted on the protease produced by the fungus *Monacrosporium thaumasium.*

Keywords: Nematophagous fungi, *Monacrosporium thaumasium*, protease, *Angiostrongylus vasorum.*

Resumo

O objetivo deste trabalho foi otimizar a produção de proteases do fungo nematófago *Monacrosporium thaumasium* (NF34a), avaliar sua atividade larvicida e sua estabilidade biológica. Foi utilizado um isolado do fungo nematófago *Monacrosporium thaumasium* (NF34a) para a produção de enzima. O delineamento Plackett–Burman foi utilizado com o objetivo de se escanear quais componentes do meio de cultura, poderiam ter influência significava na produção de protease pelo fungo NF34a. Foi realizado um ensaio *in vitro* para avaliar a atividade larvicida de NF34a. Observou-se que apenas um dos componentes do meio de cultura (extrato de levedura), nos níveis avaliados, apresentou um efeito significativo ($p < 0,05$) sobre a produção de protease. Houve redução ($p < 0,01$) entre as médias de larvas recuperadas dos grupos tratados em relação às dos grupos controle. Os resultados confirmam trabalhos anteriores da eficiência de fungos nematófagos no controle de larvas de nematóides potencialmente zoonóticos. Assim, devido à importância do controle biológico, os autores sugerem estudos mais aprofundados sobre a protease produzida pelo fungo *Monacrosporium thaumasium*.

Palavras-chave: Fungos nematófagos, *Monacrosporium thaumasium*, protease, *Angiostrongylus vasorum.*

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Nematophagous fungi have been shown to be potential sources of enzyme production (MEYER; WIEBE, 2003; ESTEVES et al., 2009). In laboratory practice, optimal culture media have been successfully used for producing proteases (BRAGA et al., 2011a). To achieve high levels of proteolytic activity, the culturing conditions need to be optimized. One strategy for optimization is to use the Plackett-Burman statistical experimental design (PLACKETT; BURMAN, 1946), in which the components of the culture medium are scanned in order to select the most significant ones for protease production.

There is increasing evidence showing that proteolytic enzymes may be involved in penetration of the cuticle and cellular digestion of the host nematode (LIANG et al., 2011). On the other hand, there are few reports about the molecular mechanism for the pathogenic action of fungi against nematodes, which means that this has not been fully elucidated (SOARES et al., 2012). The protease Mt1 of the nematophagous fungus *Monacrosporium thaumasium* has been used successfully to combat the first-stage larvae of *Angiostrongylus vasorum*, a potentially zoonotic nematode (SOARES et al., 2012). However, there are no reports on optimization of the production of this enzyme, or on its biological stability. Thus, there is a need for knowledge regarding some significant factors that could be used under natural conditions. Among these are (1) culture medium composition and (2) biological stability of these enzymes.

The objectives of this work were to optimize protease production from the nematophagous fungus *Monacrosporium thaumasium* (NF34a) and to evaluate its larvicidal activity and biological stability.

We used an isolate of the nematophagous fungus *Monacrosporium thaumasium* (NF34) for producing this enzyme. This isolate originated from Brazilian soil and has been maintained under laboratory conditions, through continual transfer to solid culture media in the dark for 10 days.

For the present study, fungal mycelia from this isolate were transferred to previously autoclaved flasks containing 50 mL of liquid medium. The mixtures were kept under continual stirring at 120 rpm for six days using the method described by Soares et al. (2012), with modifications.

Next, first-stage larvae (L_1) of *A. vasorum* were obtained from positive feces from dogs that had been kept in the Parasitology Department, Federal University of Minas Gerais, using the technique described by Lima et al. (1985).

The protease Mt1, produced by the nematophagous fungus *M. thaumasium* (NF34a), was purified in accordance with the protocol described by Soares et al. (2012). The proteolytic activity was measured by means of the caseinolytic method, as described by Braga et al. (2011a). A tyrosine standard curve was constructed to quantify enzyme activity. One unit of enzyme was defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under the assay conditions (60 °C and pH 8.0).

The Plackett-Burman design (PLACKETT; BURMAN, 1946) was used in order to scan the components of the culture medium and determine which of them might have significant influence on protease production by the fungus NF34a. The Minitab Release 15 software was used for this analysis. The variables studied were: glucose (g/L), yeast extract (g/L), K_2HPO_4 (g/L), $MgSO_4$ (g/L), $ZnSO_4$ (g/L), $FeSO_4$ (g/L), $CuSO_4$ (g/L) and pH. Based on the Plackett-Burman factorial design, each factor was analyzed on two levels: –1 to lower level and +1 to higher level. The analysed factors and respective levels were: glucose –1: 5 g/L, +1: 15 g/L; yeast extract –1 (5g/L), +1 (15 g/L); K_2HPO_4 –1 (2.5 g/L), +1 (7.5 g/L); $MgSO_4$ –1 (0.1 g/L), +1 (0.3 g/L); $ZnSO_4$ –1 (0.0025 g/L), +1 (0.0075 g/L); $FeSO_4$ –1 (0.0005 g/L), +1 (0.002 g/L); $CuSO_4$ –1 (0.000125 g/L), +1 (0.0005 g/L). The choice of levels to study was based on the work of Braga et al. (2011a). The study variables and their respective levels are shown in Table 1. The model is given by the following equation:

$$Y = \beta_o + \beta_i x_i + \beta_{ii} x_{ii} + \beta_{iii} x_{iii} + \ldots + \beta_n x_n \quad (1)$$

Where Y is the response (proteolytic activity in units), β_o is the intercept of the model, β_i is the linear coefficient and x_i is the level of the independent variable. The proteolytic activity was measured in triplicate and the mean of these values was used as the response Y (MUKHERJEE; RAI, 2011). The model selection was performed with the help of the Minitab Release 15 software.

Table 1. Matrix of Plackett-Burman experimental design.

Run	Glucose (g/L)	Yeast extract (g/L)	K_2HPO_4 (g/L)	$MgSO_4$ (g/L)	$ZnSO_4$ (g/L)	$FeSO_4$ (g/L)	$CuSO_4$ (g/L)	pH	Activity (U/mL)
1	15	5	7.5	0.1	2.5×10^{-3}	5×10^{-3}	5×10^{-3}	9	0
2	15	15	2.5	0.3	2.5×10^{-3}	5×10^{-3}	1.25×10^{-3}	9	0
3	5	15	7.5	0.1	7.5×10^{-3}	5×10^{-3}	1.25×10^{-3}	5	0
4	15	5	7.5	0.3	2.5×10^{-3}	2×10^{-3}	1.25×10^{-3}	5	31.89
5	15	15	2.5	0.3	7.5×10^{-3}	5×10^{-3}	5×10^{-3}	5	14.30
6	15	15	7.5	0.1	7.5×10^{-3}	2×10^{-3}	1.25×10^{-3}	9	3.616
7	5	15	7.5	0.3	2.5×10^{-3}	2×10^{-3}	5×10^{-3}	5	0
8	5	5	7.5	0.3	7.5×10^{-3}	5×10^{-3}	5×10^{-3}	9	17.26
9	5	5	2.5	0.3	7.5×10^{-3}	2×10^{-3}	1.25×10^{-3}	9	21.53
10	15	5	2.5	0.1	7.5×10^{-3}	2×10^{-3}	5×10^{-3}	5	38.96
11	5	15	2.5	0.1	2.5×10^{-3}	2×10^{-3}	5×10^{-3}	9	0
12	5	5	2.5	0.1	2.5×10^{-3}	5×10^{-3}	1.25×10^{-3}	5	24.82

Glucose –1: 5 g/L, +1: 15 g/L; Yeast extract –1 (5 g/L), +1 (15 g/L); K_2HPO_4 –1 (2.5 g/L), +1 (7.5 g/L); $MgSO_4$ –1 (0.1 g/L), +1 (0.3 g/L); $ZnSO_4$ –1 (0.0025 g/L), +1 (0.0075 g/L); $FeSO_4$ –1 (0.0005 g/L), +1 (0.002 g/L); $CuSO_4$ –1 (0.000125 g/L), +1(0.0005 g/L).

Table 2. Factors studied in the Plackett-Burman design.

	Effect	Coeff	SE Coeff	t-*test*	P>|*t*|
Intercept		12.699	2.064	6.15	0.009
Glucose (g/L)	4.192	2.096	2.064	1.02	0.385
Casein (g/L)	–19.425	–9.712	2.064	–4.71	0.018
K_2HPO_4 (g/L)	–7.808	–3.904	2.064	–1.89	0.155
$MgSO_4$ (g/L)	2.932	1.466	2.064	0.71	0.529
$ZnSO_4$ (g/L)	6.493	3.247	2.064	1.57	0.214
$FeSO_4$ (g/L)	6.603	3.301	2.064	1.60	0.208
$CuSO_4$ (g/L)	–1.890	–0.945	2.064	–0.46	0.678
pH	–11.260	–5.630	2.064	–2.73	0.072

Coeff: coefficient; SE: standard deviation; t-test: value of the variables determined by Student's t-test at the 5% probability level; P-value.

The *in vitro* assay was based on previous work by Braga et al. (2011b). The data obtained in this assay were interpreted by means of analysis of variance, with a significance levels of 1 and 5% probability. The efficiency of predatory action on L_1 in relation to the control was measured by means of the Tukey test at 1% probability (AYRES et al., 2003). Subsequently, the mean percentage reduction of larvae was calculated in accordance with the following equation:

$$\% = \frac{(\bar{x}L_1 \text{ recovered from control} - \bar{x}L_1 \text{ recovered from treatment})}{\bar{x}L_1 \text{recovered from control}} \times 100 \quad (2)$$

In order to determine the degree of stability of biological activity of the crude extract and the protease Mt1 (SOARES et al., 2012), these were incubated at 28 °C for 7 days. Every 24 hours, aliquots were removed and the enzyme activity was determined.

The Plackett-Burman design was used to investigate the significance of the components of the culture medium used for protease production from the fungus *M. thaumasium* (NF34a). In the present work, it was observed that one component of the culture medium (yeast extract), at the levels studied, had a significant effect ($p < 0.05$) on protease production (Table 2). The R^2 of the model was 93.01%, thus demonstrating that it is a reliable model. It was also observed that the variable of pH was significant ($p < 0.1$) with regard to protease production.

Regarding the enzymatic activity on *A. vasorum* L_1 (*in vitro* assay), over a 24-hour interval, the crude extract produced by NF34a showed a reduction of 77.4% in larval recovery rate. A difference was noted ($p < 0.01$) regarding the action of this extract from the isolate, in relation to the larvae present in the control group. On the other hand, it was demonstrated that both the crude extract and the protease Mt1 maintained intact biological activity after incubation at 28 °C for 7 days.

Soil contaminated with infective stages (eggs and/or larvae) has been shown to be one of the major sources of contamination by geohelminths (GORTARI et al., 2007). Fungal structures such as conidia have been shown to be effective in controlled trials against *A. vasorum* L_1 (BRAGA et al., 2009, 2011b). Moreover, use of crude extract is also an artifice, albeit recent, that can be used to combat the eggs and/or larvae of geohelminths (BRAGA et al., 2011a, b).

Proteases produced by nematophagous fungi have been studied because they are important in relation to degradation of the cuticle of nematodes (LIANG et al., 2011). Esteves et al. (2009) stated that there might be differences in the results from enzyme production between different isolates from nematophagous fungi. Furthermore, they stated that culturing conditions seemed to play an important role and should always be standardized so that significant comparisons can be made. Cayrol et al. (1989) argued that the culturing conditions might affect the toxic properties of a fungus. In a recent study, it was demonstrated that the nematophagous fungus *M. thaumasium* (NF34a) produces a serine protease (Mt1) with importance in relation to the infection process of first-stage larvae of *A. vasorum* (SOARES et al., 2012). However, the present study is the first to report on optimization of protease production from *M. thaumasium.*

The crude extract of *M. thaumasium* (NF34) was efficient in destroying *A. vasorum* L_1. Braga et al. (2009) showed that the isolate (NF34) was effective ($p > 0.05$) in capturing and destroying *A. vasorum* L_1 under laboratory conditions. Those authors reported that, at the end of the experiment (seven days), the percentage reduction was 74.5%. In that study, the efficiency of predatory action was measured in a solid culture medium, with the fungus already grown. In the present study, the observed percentage reduction was similar (77.4%). However, it needs to be noted that in the present study, this percentage reduction was achieved over a short interval of time (24 hours). These results may eventually contribute towards discovery of new methodologies that might assist in decontamination of geohelminth-infested environments.

In relation to time, the results showed that the enzymatic action of the nematophagous fungus NF34a was effective by the end of 24 hours, thus demonstrating that use of this protease is promising. Furthermore, even after seven days of exposure to conditions resembling natural conditions, the biological activity of this enzyme remained intact. This is an interesting point, because it justifies undertaking capture and destruction of infective larvae of nematodes under laboratory conditions and possibly in the field. This may in the future make a contribution as a tool for further research to combat the infectious forms of nematodes that are potentially zoonotic.

Regarding predatory nematophagous fungi, few reports on production of enzymes and their larvicidal activity have been published (BRAGA et al., 2011a, 2013). The results obtained confirmed that nematophagous fungi were efficient for controlling

nematode larvae that are potentially zoonotic. Thus, given the importance of biological control, we suggest that further studies should be conducted on the protease produced by the fungus *Monacrosporium thaumasium* and its larvicidal activity.

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