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Statistical screening for the chitinase production by nematophagous fungi from *Monacrosporium* genus

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Several chitinases with nematicidal action of these fungi have been identified in recent years, however, little is known about which nutritional factors from the culture medium are significant for its mass production. The aim of this study was to perform the statistical screening of the components of the culture medium for chitinase production by nematophagous fungi, *Monacrosporium sinense* (SF53) and *Monacrosporium thaumasium* (NF34) aiming to identify which of these components were significant and to evaluate the influence of pH on chitinase activity of these fungi. The effect of five variables (chitin, colloidal chitin, glucose, sodium nitrate and moisture) on chitinase production was analyzed using Plackett-Burman statistical design. Chitinase activity was determined at different pH (pH 3.5, 4.5 and 5.5) and Tris-HCl 50 mM (pH 7.0 and 8.0). In relation to the isolate NF34, at the levels evaluated, two variables were significant ($p < 0.05$) for chitinase production: chitin and $NaNO_3$. On the other hand, for SF53, at the levels evaluated, only glucose was significant ($p < 0.05$). Regarding the influence of pH on enzyme activity for *M. thaumasium* the pH value with the highest activity obtained was 5.5. However, for *M. sinense*, the highest activity was observed at pH 8. These data demonstrate that different species of nematophagous fungi may exhibit better enzymatic activities and consequently, better predatory activities at different environmental conditions.

Key words: Nematophagous fungi, chitinase, Plackett-Burman, nematicidal action, *Monacrosporium thaumasium, Monacrosporium sinense*.

INTRODUCTION

According to Li (2006), the second most abundant polymer in the planet Earth is chitin, a homopolymer composed of units of N-acetyl-β (1-4)-D-glucosamine (GlcNAc). In general biological terms, this polymer is

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essential for body constitution of a number of organisms and, among these are: cuticles and membranes of insects, cell walls of fungi and helminth eggshells (Gortari and Hours, 2008). Moreover, it has been demonstrated that the destruction of the exoskeleton of arthropods can be done in a biological way by using chitinases that certain fungi can produce (St et al., 1996; Bogus et al., 2005; Braga et al., 2013). Purified proteases and chitinases demonstrate nematicidal activity against gastrointestinal nematodes of domestic animals (Tikhonov et al., 2002; Braga et al., 2014). In this sense, Braga et al. (2010) demonstrated that the enzymatic crude extract of a nematophagous fungus showed effective ovicidal activity against larvae and nematode eggs.

Chitinases (EC 3.2.1.14) are glycosidases that hydrolyze β (1-4) linkages of N-acetylglucosamine residues of chitin. As mentioned earlier, these enzymes can be produced by nematophagous fungi as an important virulence factor in the infection process of "target organisms" (Braga and Araújo, 2014). Several chitinases with nematicidal action of these fungi have been identified in recent years, however, little is known about which nutritional factors from the culture medium are significant for its mass production (Tikhonov et al., 2002; Khan et al., 2004; Gan et al., 2007; Yang et al., 2010, 2013). However, little is known about other chitinases from nematophagous fungi and their detailed molecular mechanism of action of these pathogenic fungi against nematodes still needs further studies (Braga and Araújo, 2014; Yang et al., 2013). In addition, several studies have shown that the nutritional aspect of the culture medium used in the fungal growth may favor the predatory activity (Araújo et al., 2004; Braga et al., 2010), this way, shows the best components to be introduced into routine laboratory cultivation and subsequent biological activity assays with nematophagous fungi which is an important point to be studied.

In order to quickly clarify the influence of certain nutritional factors in the culture medium for the production of chitinases, it is necessary to use a mathematical technique to design experiments, reducing the amount of assays and also time saving (Ma et al., 2012). In this sense, Plackett-Burman design (Plackett and Burman, 1946) is a useful and efficient statistical approach to evaluate the effect of various factors on a specific response, and it has proved effective in studies involving the production of enzymes from nematophagous fungi (Soares et al., 2013a, b).

Thus, the aim of this study was to perform the statistical screening of the components of the culture medium for the production of chitinase by two isolates of nematophagous fungi from the *Monacrosporium* genus aiming to identify which of these components were significant and to evaluate the influence of pH on chitinase activity of these fungi.

MATERIALS AND METHODS

Organisms

Two isolates of nematophagous fungi from the *Monacrosporium* genus (*Monacrosporium sinense* (SF53) and *Monacrosporium thaumasium* (NF34)) derived from mycology collection of the Laboratory of Parasitology of the Veterinary Department of the Federal University of Viçosa, Minas Gerais, Brazil were used. These isolates were kept in test tubes at 4°C containing 2% corn-meal-agar (2% CMA) in the dark. From these test tubes, culture dishes were extracted and transferred to Petri dishes containing 2% potato dextrose agar (2% BDA), maintained at 26°C in the dark for 10 days (Braga et al., 2010).

Solid state fermentation

Conidia of *M. thaumasium* (NF34) and *M. sinense* (SF53) were placed in sterile saline to prepare the inoculum for the fermentation. Wheat bran (5 g) (commercially obtained in Viçosa, Minas Gerais, Brazil) supplemented with 1% chitin was used as substrate for the production of chitinase. Erlenmeyer flasks (125 mL) were used as experimental units. The moisture was adjusted to 50% (v/w) using saline solution containing K_2HPO_4 (5.0 g/l) and $MgSO_4$ (0.10 g/l). Each vial was covered with hydrophobic cotton and autoclaved at 121°C for 15 min. After cooling, each flask was inoculated with the conidial suspension previously prepared and incubated in a BOD incubator at 28°C for five days (Braga et al., 2013). After this period, chitinase produced was extracted according to the method of Soares et al (2010). The clear supernatant was used for the assays.

Enzymatic activity

Chitinase activity was measured through the measure of the reducing sugars using DNS (3,5-dinitrosalicylic acid) method (Miller, 1959). The assay was composed of: 10 µL of enzyme, 40 µL of sodium acetate buffer 50 mM pH 5.5 and 50 µL of 1% colloidal chitin previously prepared according to the method of Braga et al. (2013), incubated at 50°C. The reaction was stopped by addition of 100 µL of DNS reagent. The amount of reducing sugars released was determined by recording the absorbance at 540 nm. A control was performed by adding denatured enzyme to the reaction mixture. A standard N-acetylglucosamine curve was constructed by varying its concentration. One unit of chitinase was defined as the amount of enzyme required to liberate 1.0 µmol of N-acetylglucosamine per minute under the assay conditions.

Influence of pH on the chitinase activity

Chitinase activity was determined at different pH using the following buffers for the respective values of pH: sodium acetate 50 mM (pH 3.5, 4.5 and 5.5) and Tris-HCl 50 mM (pH 7.0 and 8.0). The test temperature was 50°C.

Statistical screening

The Plackett-Burmann factorial design was conducted to find which variables were significant for the production of chitinase by nematophagous fungi *M. thaumasium* (NF34) and *M. sinense* (SF53). The experiment was performed in duplicate. The values of activity correspond to the average of triplicates. Plackett-Burman

Table 1. High (+1) and low (-1) levels of the five variables (chitin, colloidal chitin, carbon source (glucose), nitrogen source ($NaNO_3$), moisture) in Plackett-Burman statistical design.

Variable	High level (+1)	Low level (-1)
Chitin	1.5	0.5
Colloidal chitin	1	0
Glucose	1	0
$NaNO_3$	1	0
Moisture	350	150

Table 2. Matrix of the Plackett-Burman experimental design of chitinase production (U/ml) by the nematophagous fungus *Monacrosporium thaumasium* (NF34).

Runs	Chitin	Colloidal chitin	Glucose	$NaNO_3$	Moisture	Activity (U/ml)
1	1.0	0	1	0	150	0.405
2	1.0	1	0	1	150	0.138
3	0.5	1	1	0	300	1.24
4	1.0	0	1	1	150	0
5	1.0	1	0	1	300	0
6	1.0	1	1	0	300	0.319
7	0.5	1	1	1	150	0.233
8	0.5	0	1	1	300	0.324
9	0.5	0	0	1	300	0.144
10	1.0	0	0	0	300	0.152
11	0.5	1	0	0	150	1.04
12	0.5	0	0	0	150	0.432

design can be used for rapid multifactorial screening in order to find the physical and chemical significant factors necessary for high enzyme production (Plackett and Burman, 1946). Each factor was assessed at two levels: high (+1) and low (-1), with five variables (chitin, colloidal chitin, carbon source (glucose), nitrogen source (sodium nitrate - $NaNO_3$), moisture) (Table 1). In this design, it is assumed that the main factors have no interactions. Thus, the multiple regression model of the first order is given by the following equation:

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

Where, Y is the response (chitinase activity in U/mL), βo is the intercept of the model and β_i to β_n are the coefficients of the response values, and x_i is the level of the independent variable.

Statistical analysis

Minitab 15 software was used for analysis of Plackett-Burman statistical design. Data were analyzed by analysis of variance (ANOVA) with statistical significance level of $p < 0.05$.

RESULTS

The effect of five variables (chitin, colloidal chitin, glucose, sodium nitrate, moisture) on chitinase production by *M. thaumasium* (NF34) and *M. sinense* (SF53) was analyzed using Plackett-Burman statistical design (Tables 2 and 3). In this study, the highest chitinase activity values were 1.24 U / mL (NF34) and 0487 U / mL (SF53), respectively.

In relation to the isolate NF34, it could be seen that, at the levels evaluated, two variables were significant ($p < 0.05$) (Figure 1A) for chitinase production: chitin and $NaNO_3$ (Table 4). The effects of these variables on the production of chitinase were negative. Thus, the higher the amount of chitin and $NaNO_3$ in the culture medium, the lower the enzyme production by *M. thaumasium*.

On the other hand, for SF53, could be seen that, at the levels evaluated, only the variable glucose was significant ($p < 0.05$) (Table 5 and Figure 1B). Likewise, the effect of glucose on chitinase production was negative. Thus, the higher the amount of glucose in the culture medium, the lower the production of chitinase by *M. sinense*.

The results of the influence of pH on chitinase activity of fungi are shown in Figure 2. For *M. thaumasium*, the pH value with higher activity obtained was 5.5. However, for *M. sinense*, the highest activity was observed at pH 8.

Table 3. Matrix of the Plackett-Burman experimental design of chitinase production (U/ml) by the nematophagous fungus *Monacrosporium sinense* (SF53).

Run	Chitin	Colloidal chitin	Glucose	$NaNO_3$	Moisture	Activity (U/ml)
1	1.0	0	1	0	150	0.265
2	1.0	1	0	1	150	0.335
3	0.5	1	1	0	300	0.306
4	1.0	0	1	1	150	0.257
5	1.0	1	0	1	300	0.246
6	1.0	1	1	0	300	0.133
7	0.5	1	1	1	150	0.208
8	0.5	0	1	1	300	0.198
9	0.5	0	0	1	300	0.292
10	1.0	0	0	0	300	0.403
11	0.5	1	0	0	150	0.303
12	0.5	0	0	0	150	0.487

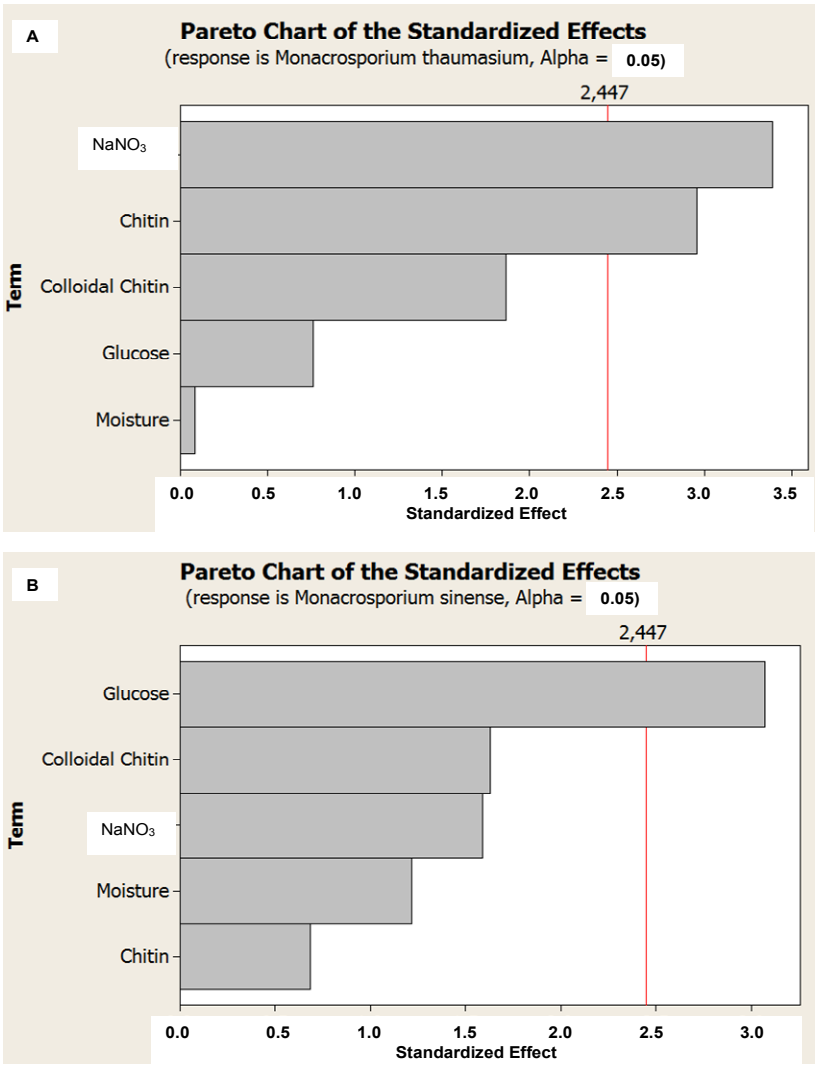

Figure 1. Pareto charts of the studied variables in the Plackett–Burman statistical design.

Table 4. Analysis of the factors studied in the Plackett-Burman statistical design of chitinase production by nematophagous fungus *Monacrosporium thaumasium* (NF34).

Parameter	Effect	P>|*t*|
Constant	-	0.002
Chitin	-0.3998	0.025
Colloidal chitin	0.2520	0.111
Glucose	0.1017	0.480
$NaNO_3$	-0.4583	0.015
Moisture	-0.0126	0.929

Table 5. Analysis of the factors studied in the Plackett-Burman statistical design of chitinase production by nematophagous fungus *Monacrosporium sinense* (SF53).

Parameter	Effect	P>|*t*|
Constant	-	0.000
Chitin	-0.0256	0.524
Colloidal chitin	-0.0616	0.155
Glucose	-0.1165	0.022
$NaNO_3$	-0.0598	0.165
Moisture	0.0462	0.267

DISCUSSION

The *Monacrosporium* genus has shown action on gastrointestinal nematode parasites of domestic animals and humans, which makes it a promising candidate for use in biological control of these organisms. In addition, it produces extracellular enzymes (chitinases and proteases) that actively participate in the process of infection of these organisms (Braga and Araújo, 2014). In this sense, the species *M. thaumasium* (NF34) and *M. sinense* (SF53) produce such enzymes, which must be well studied in this process. However, production of these enzymes involves a number of components that should be better studied for their obtaining.

Accordingly, the species *M. thaumasium (*NF34) produces chitinases that act on experimental model of free-living nematodes, which represents a new step in understanding the interaction of extracellular enzymes with the cuticle of the nematode (Soares et al., 2014). The species *M. sinense* produced extracellular proteases that are important virulence factors on nematodes (Soares et al., 2013b).

In the present study, it was demonstrated that both nematophagous fungi *M. thaumasium* and *M. sinense* produced chitinase when grown in rich chitin-based medium. To analyze the production of chitinases from nematophagous fungi *M. thaumasium* (NF34) and *M. sinense* (SF53), the Plackett-Burmann factorial design was performed in order to find out which culture variables were significant. It was observed that chitin and $NaNO_3$ are significant for chitinase production by isolate NF34. However, the effect of these variables on the production of chitinase is negative, that is, the lower the level, the higher the production of enzyme. Thus, the optimization of the production of chitinase using response surface methodology was not a step to be performed, since the presence of variables negatively affect enzyme production. Moreover, it was observed that the variable glucose was significant for the production of chitinase by the isolate SF53. However, the effect of the variable were also negative, again, the lower the level, the higher the production of enzyme. Also in this case, there was no need for the use of response surface methodology, once the presence of variable negatively affects enzyme production.

Soares et al. (2013a) used the Plackett-Burman statistical design to optimize the production of protease by *M. thaumasium* (NF34). In that study, the authors reported that the nitrogen source was the significant variable on enzyme production, a fact that is consistent with the results of this study, since the nitrogen source ($NaNO_3$) was also significant for the production of chitinase by *M. thaumasium*.

The evaluation of the ability to produce extracellular enzymes that are important in the process infection of nematophagous fungi *versus* nematodes has shown promising results under *in vitro* conditions (Araújo et al., 2004; Soares et al., 2010, 2012). In this sense, Braga et al. (2013) have suggested that nematophagous fungi produce other important enzymes, such as chitinases, in the infection process. Regarding this fact, Soares et al. (2012) demonstrated that the protease Mt1 produced by *M. thaumasium* was able to perform nematicidal activity. At that time, these authors reported that the Mt1 was able to destroy the potentially zoonotic nematode *Angiostrongylus vasorum* in dogs.

Moreover, Soares et al. (2012, 2013a, b) reported that fungi from *Monacrosporium* genus are capable of producing protease with nematicidal activity. However, to date, there are no reports of chitinase production by *M. thaumasium* and *M. sinense*, and this is the first report. Thus, the challenge today is to carry out further studies to generate knowledge on the large-scale production of these enzymes from culture media optimized for the growth of nematophagous fungi.

In the present study, the highest chitinase activity was observed at pH 5.5 for *M. thaumasium* and pH 8.0 for *M. sinense*. Soares et al. (2014) demonstrated that the fungus M. thaumasium (NF34) produced two chitinases

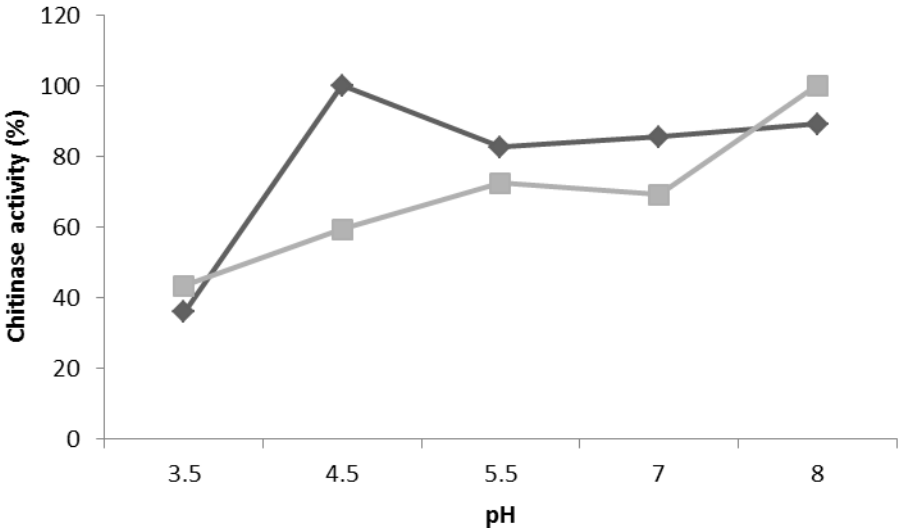

Figure 2. Effect of pH on chitinase activity of the nematophagous fungi *Monacrosporium thaumasium* (NF34) (black) and *M. sinense* (SF53) (gray).

with different biological characteristics, and one of these with maximum activity at basic pH. This is in agreement with the results shown in this study to NF34, which demonstrated that the higher enzyme activity was observed in basic pH. Thus, it is suggested that the more alkaline chitinase was the one produced in the evaluated culture medium.

Esteves et al. (2009) mentioned that the nematophagous fungi produce hydrolytic enzymes involved in the process of infection on nematodes, However, supplementation of culture media with rich substrates in chitin has been a strategy to stimulate the production of chitinases by these organisms, since the objective is to better exploit their predatory activities (Silva et al., 2010; Soares et al., 2012, Braga and Araújo, 2014).

These data demonstrate that different species of nematophagous fungi may present better enzymatic activity and consequently better predatory activities at different environmental conditions. Thus, further studies are required so that applicability of nematophagous fungi can be feasible and scientifically supported.

Conflict of interest

The authors declare that there is no conflict of interests.

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