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Full Length Research Paper

Anti-mycobacterium activity from culture filtrates obtained from the dematiaceous fungus C10

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The aim of this work was to increase the concentration of substances with anti-mycobacterium activity in culture filtrates obtained from the dematiaceous fungus C10. An experimental design was employed to study the effect of glucose, potato infusion and *Senna reticulata* infusion. The anti-mycobacterium activity was determined by evaluating the growth of bacteria in culture medium containing "culture filtrate" (the products of the submerged fermentation of the fungus). It was observed that the concentrations of glucose 30 g/L, potato infusion 50% v/v and *S. reticulata* infusion 0% v/v (a better result was obtained not using *S. reticulata* infusion) were the best conditions for metabolites production. The influence of each variable was determined and it was possible to produce a mathematical model and a surface response to demonstrate the influence of the studied variables. In conclusion, we note that the culture medium had a great importance in the production of culture filtrate with anti-mycobacterial activity and that the experimental design showed to be a functional statistical tool for studying the influence of culture medium composition.

Key words: Culture medium, *Senna reticulata*, endophytic fungi, experimental design.

INTRODUCTION

Bioprospecting is a term currently used to refer to the search for novel products or organisms of economic importance from the world's biota. The Amazon rain forest is one of the most species-rich containing the greatest microbial diversity in the word (Stinson et al., 2003; Souza et al., 2004). It has the potential for supplying materials and metabolites for an infinite number of researches. The Rain Forest is well known internationally for it´s vegetal diversity however inside trees, the microbiological diversity is even more impressive (Lodge et al., 1992; Azevedo et al., 2000; Araújo et al., 2002).

Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants. They cause no discernible manifestation of their presence and have typically gone unnoticed (Lu et al., 2000). These microorganisms constitute a valuable source of secondary bioactive metabolites that are produced to protect the plant in ways such as: plant growth regulation, antibacterial, antifungal, antiviral and insecticidal. These substances have been systematically for their potential therapeutic use (Lingham et al., 1993; Dreyfussn and Chapela, 1994; Bills et al., 1994; Pelaez et al., 1998).

Recently, endophytic fungi that were isolated from species of tropical Amazon rainforest were identified for producing substances with biological activity (Carvalho, 2005; Lima, 2007). More specifically, a strain known as C10, still not identified, produced substances that inhibit the growth of *Mycobacterium tuberculosis* (Lima, 2007).

These anti-mycobacterium substances should be characterized and identified. However, it is necessary to increase the concentration of them, to do a more adequate analytic study. An alternative, to increase the concentration of these substances, is to optimize culture composition. The aim of this work was to increase the concentration of substances with anti-mycobacterium

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Table 1. Level and factors used in the experimental design.

Level	-1	0	+1
Glucose g/L	0	15	30
Potato infusion (% v/v)	0	25	50
S. reticulata infusion (% v/v)	0	25	50

activity in culture filtrates obtained from the dematiaceous fungus C10.

MATERIALS AND METHODS

Microorganism

The strain C10 was isolated from *Senna reticulata* leaves as described by Pereira and Azevedo (1993). The tree was growing wild in National Institute for Research in Amazonia (INPA) land reserve, Latitude-south 3°10´43´´ and Longitude-east 59°99´99´´). The plant leaves were washed with sterile water and decontaminated with 70% ethanol for 1 min, 3.0% sodium hypochlorite for 4 min, 70% ethanol for 1 min and then rinsed with sterile water for three times. Discs of 7 mm in diameter were cut from the leaves using a sterile hole-punch and transferred to Petri dishes containing the culture media Potato Dextrose Agar. The developed colonies were transferred to PDA medium plates.

All the 62 isolates, including C10, were deposited in the "Microorganisms Collection of Medical Interest" from the National Institute for Research in Amazonia (INPA) and were investigated for the production of culture filtrates with anti-mycobacterium activity (Carvalho, 2005; Lima, 2007). The dematiaceous fungus C10 was one of the best producers of bioactive compounds.

Submerged fermentation bioprocess for producing the "culture filtrate"

The fungal inoculum was obtained by growing the C10 culture on potato dextrose agar (PDA) at room temperature for 7 days. The bioprocess was carried out in 125 ml Erlenmeyer flasks containing 50 ml of culture medium and $1x10^5$ fungal cells. The concentration of glucose, potato infusion and *S. reticulata* infusion was defined according to an experimental design. The bioprocess incubation was carried out in a rotary shaker under orbital agitation of 100 rpm at room temperature for 2 weeks. After this period, the culture medium was filtered and the filtrate (the culture filtrate) was submitted to an anti-mycobacterium activity assay.

Experimental design for the optimization of the bioprocess

In order to verify the influence that the content of glucose (g/L), potato infusion (% v/v) and *S. reticulata* infusion (% v/v) had on the production of culture filtrates that inhibit *M. tuberculosis* growth, a 2^3 experimental design, with three repetitions on the central point, was employed (Barros et al., 1995). The infusions were obtained by using potato pieces (100 g/L, 10 cm^2 surface per piece), or sterns of *S. reticulata* (100 g/L, pulverized). Both types were boiled in water for 10 min and then filtered with gauze. Table 1 shows the level and the factors used in the design. All eight experiments were performed in the same conditions as the three assays representing the central point (coded value 0). A statistical model to determine the inhibitive properties to *M. tuberculosis* growth was determined by the response regression procedure.

The statistical analysis was performed by using the STATGRAPHICS statistical software version 6.0 and the STATISTICA program version 5.0.

Anti-mycobacterium activity assay

This bioassay quantified the influence of culture filtrates in the growth of *M. tuberculosis* H37Rv by turbidimetry (660 nm), using a GeneQuant spectrophotometer (Amersham Pharmacia Biotech). The bioassay consisted in 0.5 ml of *M. tuberculosis* inoculum (3x105 cell/ml), 0.5 ml of culture medium (Middlebrook 7H9GC culture x 2) and 1 ml of the culture filtrates. This bioassay was incubated at 37°C per 6 days. The turbidimetry values obtained in bioassays were compared with the values of the control (an assay containing only the *M. tuberculosis* inoculum and the culture medium). It was quantified the absorption in 660 nm from the culture filtrates, culture medium and initial inoculum in order to allow an adequate quantification (without interferences) of the bacterial growth.

RESULTS

In order to determine the influence of glucose (g/L), potato infusion (% v/v) and *S. reticulata* infusion (% v/v) content in the production of culture filtrates that present inhibitive properties to *M. tuberculosis* growth, a 2^3 experimental design was used. Table 2 shows the results of the experimental design. The inhibition of *M. tuberculosis* growth, revealed by the 2^3 experimental design, ranged from 38.6 to 76.4%.

The main effects and their respective interactions calculated from the data of Table 2 are shown in Table 3. The standard errors and the estimated effects are shown in Table 3. Barros et al. (1995) only considers significant (for 95% confidence) the effects with values higher than $tv \times \mu$. The tv value is t test for v freedom degree. In this study the t test, for 2 freedom degree (95% confidence), was 4.3025.

The linear effects of glucose (A), potato infusion (B) and *S. reticulata* infusion (C) were significant and a linear model was adjusted (Equation 1).

Inhibition of *M. tuberculosis* growth (%) = 45.4318 + 0.838333* (Glucose] + 0.16* (Potato infusion) – 0.177* (*S. reticulata* infusion) (Equation 1). Table 4 shows the variance analysis, ANOVA, for the linear effects shown in Equation 1.

The ANOVA test was used to evaluate the regression and the lack-of-fit of the model (Barros et al., 1995). The P-value for all the considered factors was near or inferior to 0.05, showing that these effects have significant

Table 2. Results of the 2^3 experimental design with three repetitions on the central point.

Test	Glucose (g/L)	Potato infusion (% v/v)	*S. reticulata* infusion (% v/v)	*M. tuberculosis* growth inhibition (%)
1	0	50	50	43.6
2	0	0	0	41.6
3	30	0	50	57.6
4	30	50	50	70.1
5	0	50	0	53.5
6	0	0	50	38,6
7	30	50	0	76.4
8	15	25	25	60.6
9	15	25	25	59.4
10	30	0	0	73.8
11	15	25	25	58.2

Table 3. Variables affecting the production of culture filtrates that present inhibitive properties to *M. tuberculosis* growth as revealed by the 2^3 experimental design.

Variables	Estimated effects ± Standard error
Average	57.5818 ± 0.361814
A: Glucose*	25.15 ± 0.848528
B: Potato infusion*	8.0 ± 0.848528
C: *S. reticulata* infusion*	-8.85 ± 0.848528
AB	-0.45 ± 0.848528
AC	-2.4 ± 0.848528
BC	0.75 ± 0.848528

Standard error estimated from pure error with 2 f.d. *Significant effects at the 5% level (t = 4.3025).
[A] = dextrose content; [B] = sulfate ammonium content.

Table 4. Analysis of variance for evaluation of the model (Equation 1).

Source of variation	Sum of squares	Df	Mean square (MQ)	F-ratio	P-value
A: Glucose	1265.04	1	1265.04	878.50	0.0011
B: Potato infusion	128.0	1	128.0	88.89	0.0110
C: *S. reticulata* infusion	156.645	1	156.645	108.78	0.0091
Lack-of-fit	61.9664	5	12.3933	8.61	0.1074
Pure error	2.88	2	1.44		
Total (corr.)	1614.54	10			

R-squared = 95.9836%, R-squared (adjusted for d.f.) = 94.2623%.

regression. The P-value of lack-of-fit was 0.1074 which shows that the lack-of-fit was not significant. The absence of lack-of-fit, the significant regression and the high variance percentage explained, showed that the model presented in Equation 1 could be used to explain the studied region. The surface response created using the model (Equation 1) is shown in Figure 1.

DISCUSSION

Bacterial and viral drug resistance and the spread of fungal and parasitic diseases necessitate the search for additional antibiotic compounds that present activity at low concentrations and with reasonably low toxicity to humans. It has been demonstrated that the search for these compounds among endophytic bacteria and lichens has been effective since they represent biological associations that provide protection against competing organisms (Francolini et al., 2004; Hoffman et al., 2007).

Specifically, endophytic fungi have been described as good producers of substances with antimicrobial activity (Hoffman et al., 2007; Rukachaisirikul et al., 2008). During the screening for antibiotic producers, the strain

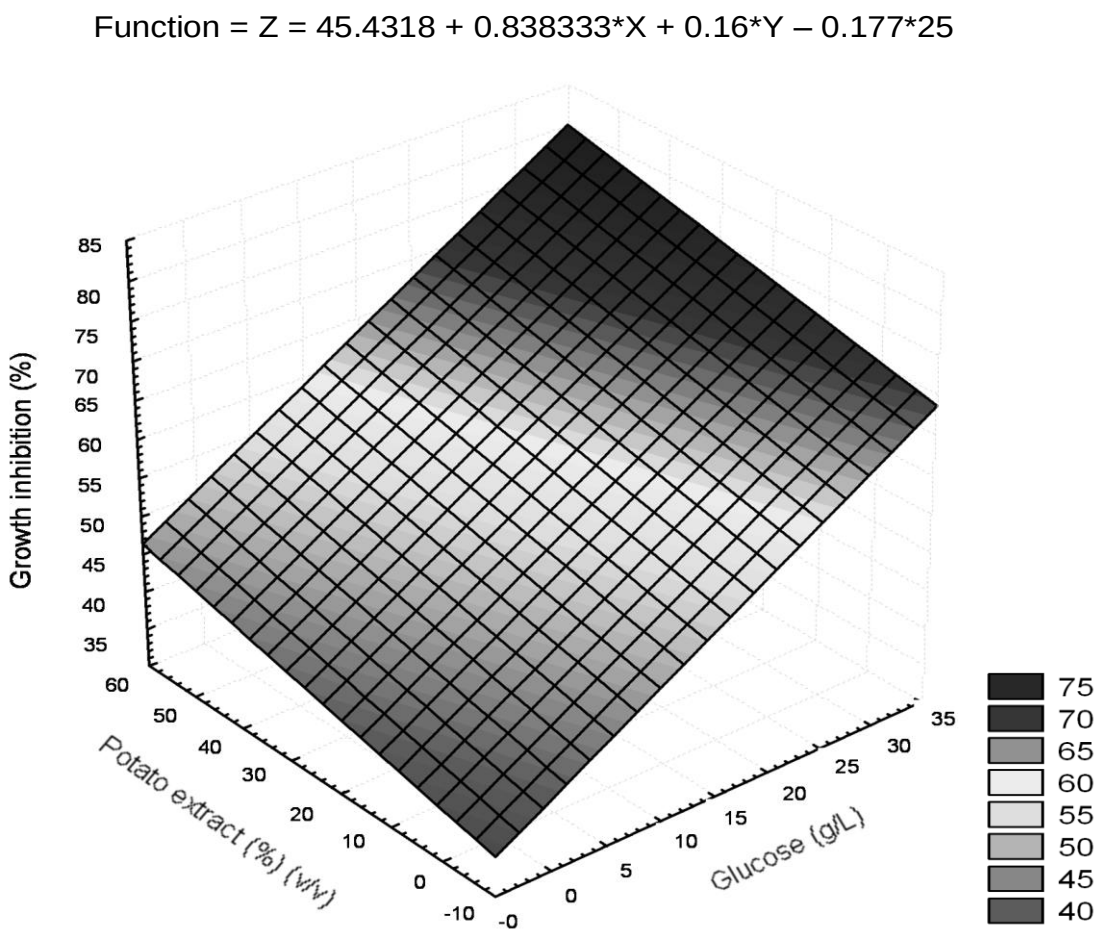

Figure 1. Surface response demonstrating the influence of glucose (g/L) and potato infusion (% v/v) in the production of culture filtrates that have inhibitive properties to *M. tuberculosis* growth.

C10 (that was isolated from *S. reticulata*) produced culture filtrate that was able to inhibit the growth of *M. tuberculosis*. However, it has been necessary to stimulate the production of these interesting substances to make their purification, identification and characterization easier.

The inhibition assays presented results that ranged from 38.6 to 76.4%, showing that the compositions of the medium used in the bioprocess have a significant influence in the production of anti-mycobaterium substances. This result was expected, because the literature has shown many works demonstrating the influence of the culture medium in the production of metabolic substances (Berdy, 1998; Brizuela et al., 1998; Lu et al., 2000).

In the presented experiments, the glucose and potato infusion had positive influence on the production of inhibitive substances. Conversely, the *S. reticulata* infusion had negative influence. The highest production of inhibitive substances was reached using 30 g/L glucose and 50% v/v potato infusion. That composition is similar to the conventional Potato Dextrose Agar medium.

The influence of *S. reticulata* infusion was investigated in order to find out if the plant components were necessary for the production (or the stimulation of the production) of culture filtrates that causes inhibition in *M. tuberculosis.* The data (Table 3) showed that the plant infusion caused a decrease in the production of the inhibitive substances. This result could be due to the presence of substances in the *S. reticulata* infusion that interfere in one, or more, of the phases involved in the fungal bioproduction of the antimicrobial substances. Another explanation could be the existence of anti-fungal metabolites in the *S. reticulata* infusion.

On the other hand, it is important to remember that literature shows (Borba and Rodrigues, 2000; Ryan et al., 2003; Girão et al., 2004) that some strains lost the ability to produce antimicrobial substances after being taken away from their natural botanic sources. So, it is necessary to indentify substances that are essential for the stimulation of the metabolic paths related to the production of the antimicrobials.

No statistically significant result was observed involving the interaction of glucose, potato infusion or *S. reticulata*

infusion during the experiments. The three investigated components provide different nutrient sources to the microorganism: a) the glucose provides a fast carbon source for immediate utilization, b) the potato infusion provides starch that is used as a secondary carbon source and also provides protein and mineral sources that could be used as nitrogen sources, c) and the *S. reticulata* infusion provides soluble components present in the plant that could activate the production of antimicrobials.

The factorial design experiments and the surface response methodology demonstrated to be useful statistical tools that allowed for the calculating of the importance of each factor and their interaction. It also produced a mathematical model that, after validation, could be used to produce statistically significant data. Other factors such as: aeration, pH, and inoculums size should be included in a new experimental design, in order to identify increases in the production of substances that have bioactivity.

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