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A N I N T E R N A T I O N A L J O U R N A L

Spore Production of *Beauveria bassiana* From Agro-Industrial Residues

Herta Stutz Dalla Santa[1], Osmar Roberto Dalla Santa[1], Débora Brand[2], Luciana Porto de Souza Vandenberghe[2] and Carlos Ricardo Soccol[2*]

[1]Laboratório de Microbiologia; Departamento de Engenharia de Alimentos; Universidade Estadual do Centro-Oeste; Camargo de Varela de Sá, 03; Av. Cascavel; 85040-080; Guarapuava - PR - Brazil. [2]Laboratório de Processos Biotecnológicos; Unidade de Biotecnologia Industria; Universidade Federal do Paraná; C. P. 19031; 81531-970; Curitiba - PR - Brasil

ABSTRACT

The purpose of this work was to produce Beauveria bassiana by Solid-State Fermentation using agro-industrial residues and optimizing the cultivation conditions. Refused potatoes, coffee husks and sugar-cane bagasse were tested. The blend of refused potatoes and sugar-cane bagasse (60-40%) with particle size in the range of 0.8-2 mm was used in the fermentation experiments. In Erlenmeyer flasks the best spore production was achieved with the following conditions: incubation temperature 26º C; initial pH 6.0; inoculum concentration 10^7 spores.g^{-1}.dw and initial moisture 75%. In the column type reactor using forced aeration under the optimized conditions, the maximum production ($1.07x10^{10}$spores.g^{-1}.dw) was obtained at the 10^{th} day of fermentation. The respirometric analyses of the fermentation showed a strong correlation between fungal growth and spore production.

Key words: Agro-industrial residues, *Beauveria bassiana,* solid-state fermentation

INTRODUCTION

The genus Beauveria is a parasite of a great number of arthropods, occurring in more than 200 species of insects and acaridae. These entomopathogenic fungi may occur in enzootic and epizootic forms in field or produced *in vitro* through fermentative processes (Alves, 1998). Solid-State fermentation (SSF) may be defined as the growth of microorganisms in solid substrates in the absence of free water. The free water is found in the complexes form in the interior of a solid matrix (Pandey, 1992; Lonsane et al., 1985; Soccol, 1994).
SSF may be classified by the function of the solid phase; it can serve only as a support for the growth of microorganisms and be inert for nutritional purposes; and in such case the nutritive sources necessary for the growth of microorganisms are adsorbed by the support. The solid phase may be the support and at the same time the substrate for fermentation. In this case, the support gives also the nutrients required for the growth of microorganisms (Soccol, 1994). SSF shows advantages for the production of spores in short period of time, due to its simplicity in comparison with submerged cultivation. To make the production of fungal spores process at semi-industrial scale viable, it is necessary to obtain an ideal, cheap and highly productive culture media, which maintain morphological, pathogenical and virulogical characteristics.

*Author for correspondence

These are several studies on the efficient utilization of agro-industrial residues with value addition (Soccol and Vandenberghe, 2003; Soccol, 1994; Pandey, 1992). The residues could be utilized as substrates and support for the production of citric acid (Vandenberghe, 1999); biological detoxification of coffee husk for the production of animal feed (Brand et al. 2000), edible mushrooms (Leifa et al., 2000), enzymes and ethanol; reducing in this way environmental pollution problem that the disposal of this residues may cause (Pandey et al., 2001).

Diverse raw materials have been tested for the production of entomopathogenic fungi, such as caupi, sorgo, broad bean, beans, cassava bagasse, rye flour, cassava flour, different types of rice, and residues such as sugar-cane bagasse enriched with cane syrup and torula residues, or still refused potatoes are utilized (Burtet et al., 1997; Soccol et al., 1997; Vilas Boas et al, 1996; Calderon et al., 1995). With high carbohydrates, proteins and significant amounts of salts and vitamins, potato has a high nutritional value (Trindade, 1994). During the processing of potatoes significant losses occur. Refused potatoes were utilized as substrate/support for the production of spores of *Beauveria bassiana* (Ayala, 1996). In this way, the fermentation process reduces the pollutant potential of this residue, in which the starch is utilized as carbon source by the fungi for its development and for the production of spores.

The aim of the present work was to verify the possibility of using agro-industrial residues for the production of the entomopathogenic fungi *Beauveria bassiana* LPB by solid-state fermentation, and to optimize cultivation conditions aiming the highest spore production.

MATERIAL AND METHODS

Raw materials

Refused potatoes were obtained from the local market; coffee husk was gently donated by Café DAMASCO, Curitiba -PR. Sugar-cane bagasse was obtained from Usina Santa Terezinha, Maringá, PR. The residues were dried at 55°C for 48 h, milled and sieved to obtain particles size between 0.8 and 2.0 mm.

Microorganism

The strain utilized in this work was *Beauveria bassiana*- LPB 01, maintained in the collection of Laboratório de Processos Biotecnológicos, UFPR. The strain was maintained at 4º C in agar slants cultivated in potato dextrose agar (PDA) and sub-cultured every three months.

Inoculum

B. bassiana was produced in Erlenmeyer flasks (250 mL) containing 50 mL of PDA, incubated at 26º C for 10 days under static condition. The spore suspension was prepared by the addition of 40 mL sterile distilled water, 15 g of glass beads and Tween 80 (0.1%) and stirred for 30 minutes on a magnetic stirrer. The spores were counted in Neubauer chamber.

Solid-State Fermentation in Erlenmeyer flaks

Selection of substrate

In order to select the best substrate/support for spore production, the following formulations were tested: 1- mixture of refused potatoes and sugar-cane bagasse (50-50%); 2- coffee husk (100%); 3- mixture of refused potatoes and coffee husk (50-50%); and 4- refused potatoes (100%). The initial moisture content (São Paulo, 1985) for every residue was of 69, 65, 63 and 60%, respectively. SSF was carried out in Erlenmeyer flasks (250 mL) with 10 g of substrate/support with particles size between 0.8 and 2 mm. The pH of the substrates was measured by utilizing 5 g of the sample diluted in 50 mL of distilled water; the obtained suspension was well homogenized and pH was determined with a potentiometer (Soccol, 1994). In order to make a pre-gelatinization of refused potatoes, 30% (w/v) distilled water was added in substrate/support and sterilized at 121º C for 15 minutes. The experiments were conducted with two replicates. The substrate/support was inoculated with a spore suspension (10^7 spores.g^{-1} of dry matter, DM) and the pH was adjusted to 5.8 - 6.2 with NaOH (1 M). The flasks were incubated at 26º C for 10 days. After this 1g of the fermented substrate/support was mixed with of 30 mL distilled water, Tween 80 (0.01%) and 15 g of glass beads. After 30 min agitation the mixture was filtered through a nylon sieve of 200 µm and the spore concentration was evaluated using a Neubauer Chamber.

Effect of particle size and percentage composition of refused potatoes for spore production

Based on the results of substrate/support selection, two experiments were realized in order to verify the effect of particle size and percentage composition of refused potatoes in a blend with sugar-cane bagasse. The software STATISTICA version 5 (Barros Neto et al, 1995) was utilized as a tool for the statistical design of the first assay. A factorial plan 2^{2-0} was employed and the factors particle size and percentage composition of refused potatoes in the substrate/support were evaluated. They were distributed in two levels with one central point. The experiments were realized with two replicates and four repetitions of the central point, resulting in a total of 12 assays. The evaluated parameters and respective values are demonstrated in Table 1.

Table 1 - Experimental design for particle size and percentage composition of refused potatoes in spore production of *Beauveria bassiana.*

Parameters	Values		
Particle size (mm)	< 0.8	0.8 a 2	>2
Refused potatoes (%)	30	50	70

Table 2 - Refused potatoes and sugar-cane bagasse content in the substrate/support mixture for spore production of *Beauveria bassiana.*

Assays	Refused potatoes (%)	Sugar-cane bagasse (%)
1	80	20
2	70	30
3	60	40
4	50	50
5	40	60

Table 3 - Experimental design for the study of the physical parameters influence in the spore production of *Beauveria bassiana,* by utilizing refused potatoes and sugar-cane bagasse (60:40%) in Erlenmeyer flasks.

Parameters	Values		
Initial pH	5.5	6.0	6.5
Temperature (º C)	22	26	30
Initial moisture content (%)	60	70	80
Inoculum size (spores.g^{-1} of dry matter)	106	107	108

The best percentage of refused potatoes in mixture with sugar-cane bagasse was investigated by means of a second experiment (Table 2).

The experiments were realized with two replicates, resulting in a total of 10 fermentation assays. The procedures of sterilization, inoculation, spore evaluation, initial pH measurement of substrate/support, inoculum size and incubation temperature were the same as described above.

Effect of inoculum concentration, initial pH and initial moisture content for spore production

This experiment had objective to determine the best physical conditions for spore production by *B. bassiana* utilizing Erlenmeyer flasks as bioreactors. The software Statistica was utilized and in this case a complete factorial design $2{:}2^4$ with four experimental values: initial pH, inoculum concentration, incubation temperature and initial moisture content of the substrate/support was established. These were distributed in two levels with a central point. The experiments were conducted with two replicates, and two repetitions of the central point, resulting in a total of 20 assays. The values employed in this experiment are demonstrated in Table 3.

Solid-State Fermentation in column type bioreactor with forced aeration

Influence of initial moisture content of the substrate/support and aeration rate

The experiments were conducted in vertical fermentation columns with 4 cm of diameter x 20 cm length, bed height of 12 cm (Raimbault and Alazard, 1980). Each column was packed with a known quantity of substrate/support previously inoculated (inoculum size of 10^7 spores.g^{-1} of dry matter, pH 6.0) and incubated for 10 days at 26º C in a water bath. Saturated air was passed through the columns. The assays were done with two replicates and the evaluated parameters were: aeration rate (40, 60 and 80 ml.min^{-1}); and initial moisture content (55, 65 and 75%).

Spore production and respirometric analysis

The optimized conditions for spore production in a column type bioreactor by utilizing refused potatoes and sugar-cane bagasse (60:40%) were: pH 6.0; aeration rate 60ml min^{-1}; inoculum concentration 10^7 spores.g^{-1} of dry matter; and initial moisture content of 65%. These were utilized to follow the respiratory metabolism of the fungi during the spore production in column type bioreactor. The experiments were realized until the 20th day of fermentation at 26º C, with two replicates. Samples were collected every 48 h. The respirometric analysis for gases were passed through silica gel columns and analyzed by gas chromatography (SHIMADZU- GC- 8A), interfaced to a computer (COMPAQ- XT 386), following mathematical model developed by Rodriguez Léon et al (1988). The conditions utilized in the gas chromatography system were: detector and column temperature - 60°C; gaseous phase -helium with flux of 30 mL.min^{-1} and pressure of 1 bar; catarometer current of 120 mA and injection volume of 300 μL. The gases utilized for system calibration were: air: CO_2 (0.0) / O_2 (21.0) / N_2 (79.0); mixture 1: CO_2 (5.0) / O_2 (5.0) / N_2 (90.0) and mixture 2: CO_2 (10.0)/ O_2 (15.0)/ N_2 (75.0). The respirometric analysis was conducted every two h, with three replicates. The spore production was correlated with the respiratory metabolism of the microorganism.

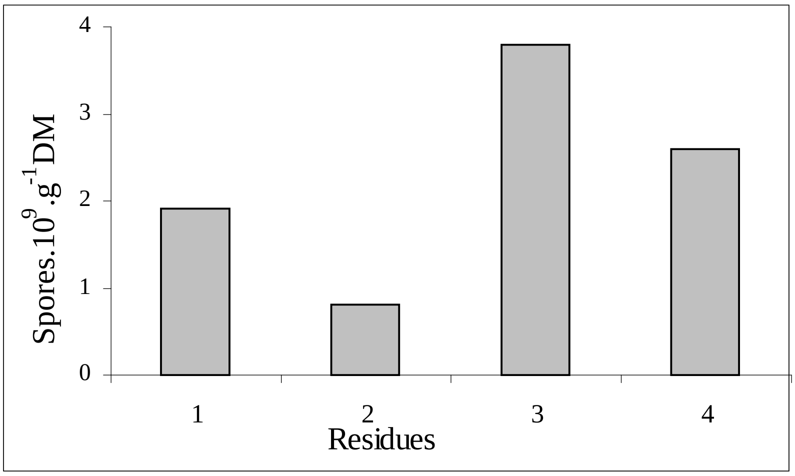

Figure 1 - Influence of different residues in spore production of *Beauveria bassiana:* 1) refused potatoes; 2) coffee husk; 3) refused potatoes and sugar-cane bagasse (50:50%), refused potatoes and coffee husk (50:50%).

RESULTS AND DISCUSSION

Solid-State fermentation in Erlenmeyer flasks

Spore production in different agro-industrial residues

The results demonstrated in Fig. 1 allow comparing the tested residues. Refused potatoes enhanced the spore production, confirming studies of Ayala (1996). The fungus grew better and produced higher quantity of spores in a mixture of residues.

This could be due to the presence of cellulosic substrate/support, which according to Lonsane et al. (1985) provided better aeration, less compactation problems and greater growth surface for spore production.
The solid substrate/support that resulted highest spore production (3.8x10 spores.g^{-1} of dry matter) was the mixture of refused potatoes and sugar-cane bagasse. In this system the refused potatoes were used as carbon source for fermentation due to its carbohydrate, protein, mineral salts and vitamins; and sugar-cane bagasse was only the support.

Effect of particle size and percentage composition of refused potatoes in spore production

The results obtained in the first experiment were submitted to statistical analysis ($p \leq 0.05$) and by means of the contour graph (Fig. 2) it was observed that the quantity of refused potatoes was the factor that influenced the production of spores of *B. bassiana*. The results demonstrated in Fig. 3 showed that the maximum quantity of spores (3.4×10^9 spores.g^{-1} of dry matter) was obtained with solid material particle size varying from 0.8 to 2 mm and with the blend of 60% refused potatoes and 40% of sugar-cane bagasse.

Effect of inoculation rate, initial pH, temperature and initial moisture content in the spore production

By means of the analysis of Pareto Chart of Effects (Fig. 4), it was verified that none of the studied factors were significant ($p \leq 0.05$), because the tested levels were inside the optimal range for spore production by the fungus.

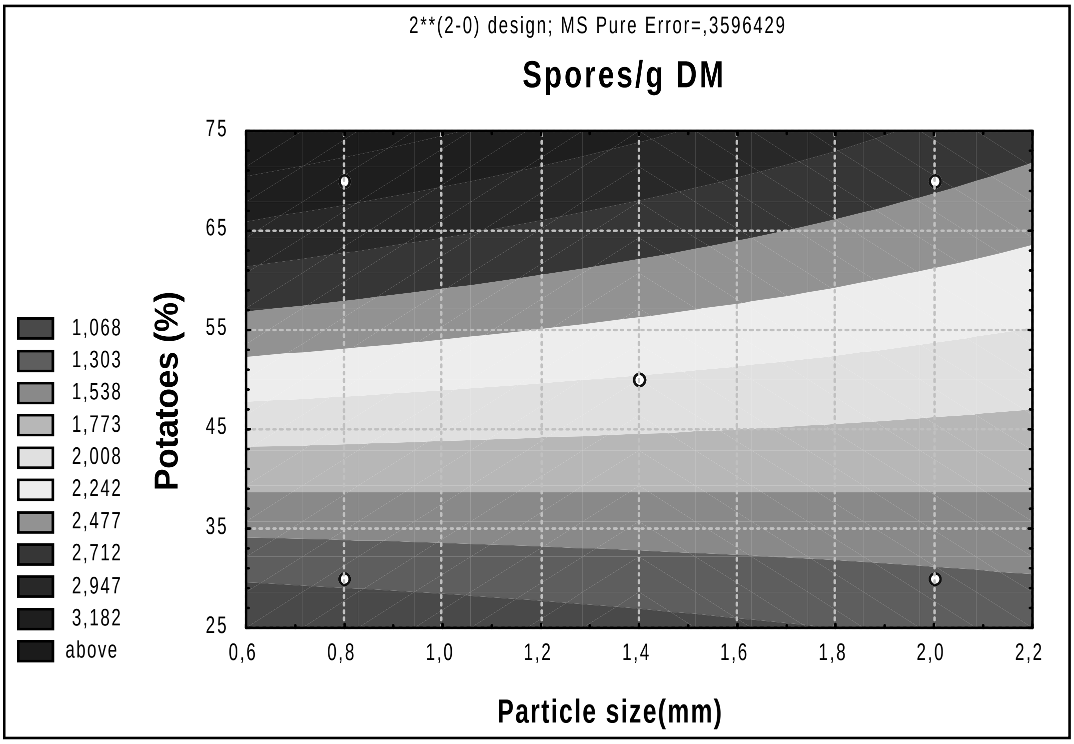

Figure 2 - Contour graph for spore production of *Beauveria bassiana.*

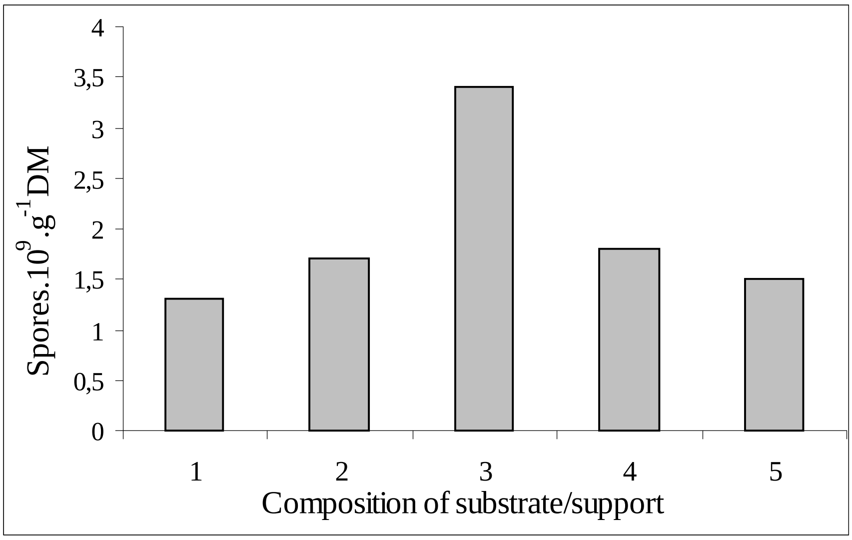

Figure 3 - Influence of refused potatoes and sugar-cane bagasse content in the mixture of substrate/support for spore production of *Beauveria bassiana*: 1) 80:20, 2) 70:30, 3) 60:40, 4) 50:50 e 5) 40:60%, respectively.

The value of 26º C as optimal temperature was in accordance with the results published by other authors (Diehl-Fleig et al., 1988; Fargues et al., 1997). pH is one of the factors that most influences the microbial development in solid-state fermentation (Doelle, 1985).Values close to 6.0 were also found by Ayala (1996) in studies with refused potatoes. The initial moisture content of 75% of the substrate/support was considered optimal in this study, since cellulosic materials adsorbed more water than starchy materials. According to Raimbault (1998), values of initial moisture content varying from 35 to 80% were utilized in solid-state fermentation, depending on the microorganisms and on the substrate/support employed. In relation to the inoculum rate, the best concentration of 10^7 spores.g^{-1} of dry matter found in this study was similar to the one reported by Soccol et al. (1997).

Solid-State Fermentation in column type bioreactor with forced aeration

Influence of initial moisture content of the substrate and aeration rate

The high spore production ($9.8x10^9$ .g^{-1} of dry matter) was obtained with 65% initial moisture content of substrate/support (Fig. 5). This value differed from the one obtained in fermentation utilizing Erlenmeyer flasks (75%) for the same substrate/support. Substrate in column bioreactors was provided aeration with saturated air, which might have helped in maintaining the moisture in it, not needing high initial moisture content.

As demonstrated in Fig. 6 the airflow of 60 mL.min^{-1} gave better results to spore production ($9.9x10^9$ .g^{-1} of dry matter), similar to the one found by Ayala (1996). This confirmed the importance of forced aeration in the maintenance of temperature, initial moisture content and of the aerobic conditions in the bioreactor.

Spore production and respirometric analysis

Data obtained from the kinetics of spore production until the 20th day of fermentation allowed verifying a maximum production of spores at the 10th day of fermentation, which was $1.07x10^{10}$ .g^{-1} of dry matter. A mass balance was realized to estimate the oxygen uptake rate (OUR) and the evolution of CO_2 production in terms of volumetric flow (L/h). The evolution of the O_2 consumption showed that the peak of greater consumption was between 10 and 19 h of fermentation (data not shown). This could be probably due to a substantial enhancement in biomass production in the substrate/support. Through the respirometric analysis, it was possible to verify a correlation between the development of the fungus and spore production (Fig. 7).

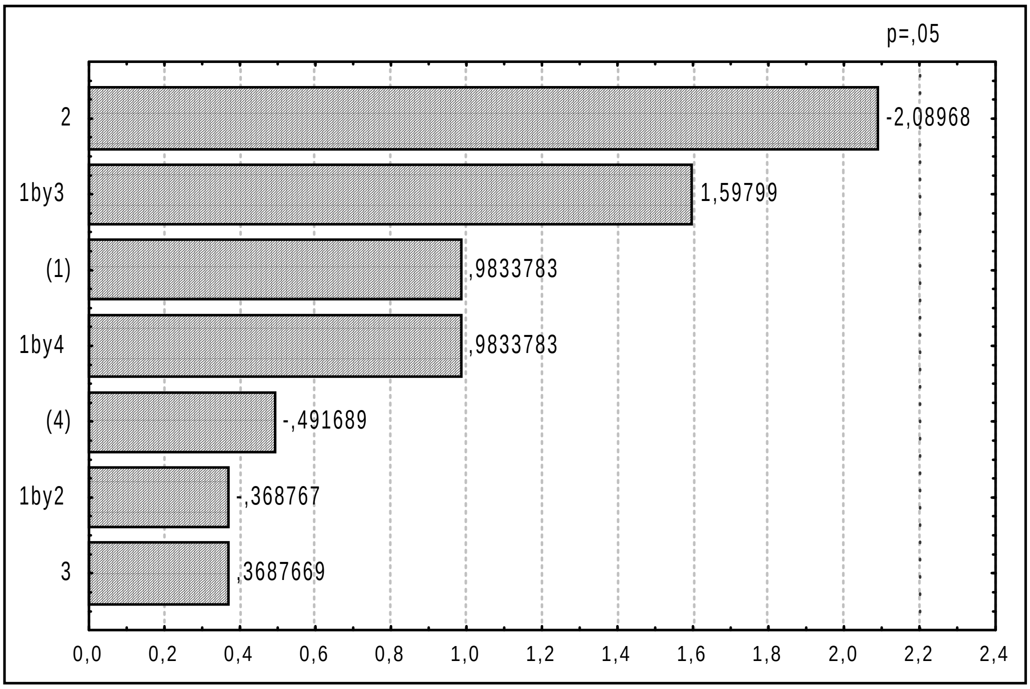

Figure 4 - Pareto Graph of Effects of the factors that have influence in spore production of Beauveria bassiana in Erlenmeyer flasks: 1) inoculation rate; 2) pH; 3) initial moisture content of substrate/support; 4) temperature.

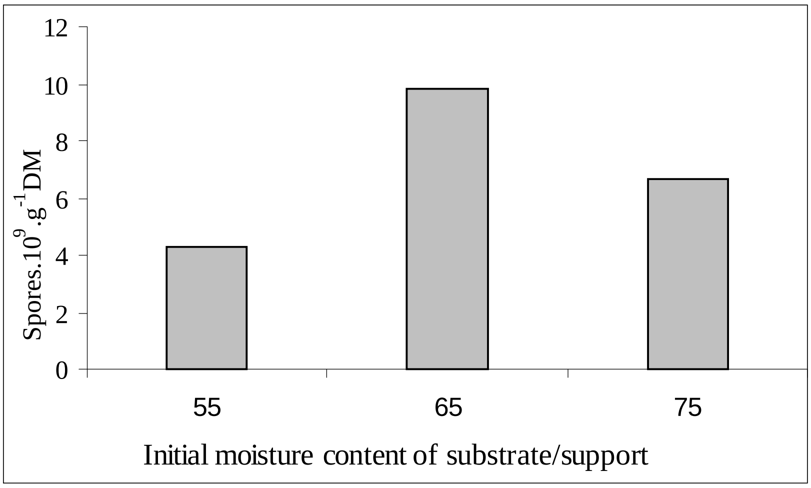

Figure 5 - Influence of initial moisture content of substrate/support (refused potatoes and sugar-cane bagasse: 60-40%) in column type bioreactor in spore production of Beauveria bassiana.

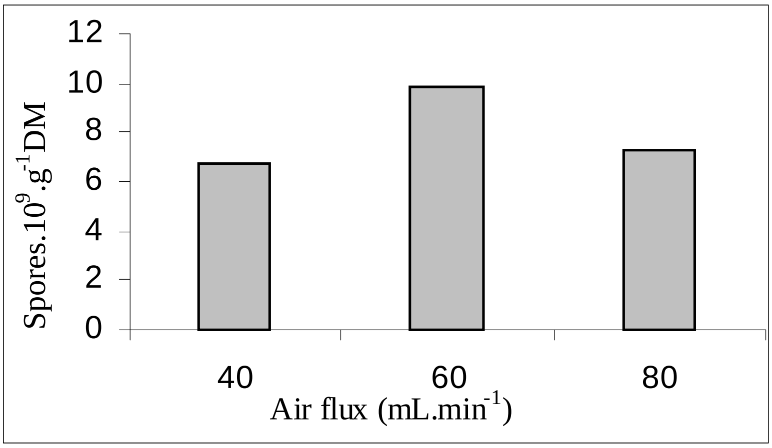

Figure 6 - Influence of aeration rate in the substrate/support (refused potatoes and sugar-cane bagasse 60:40%) in column type bioreactor in spore production of *Beauveria bassiana.*

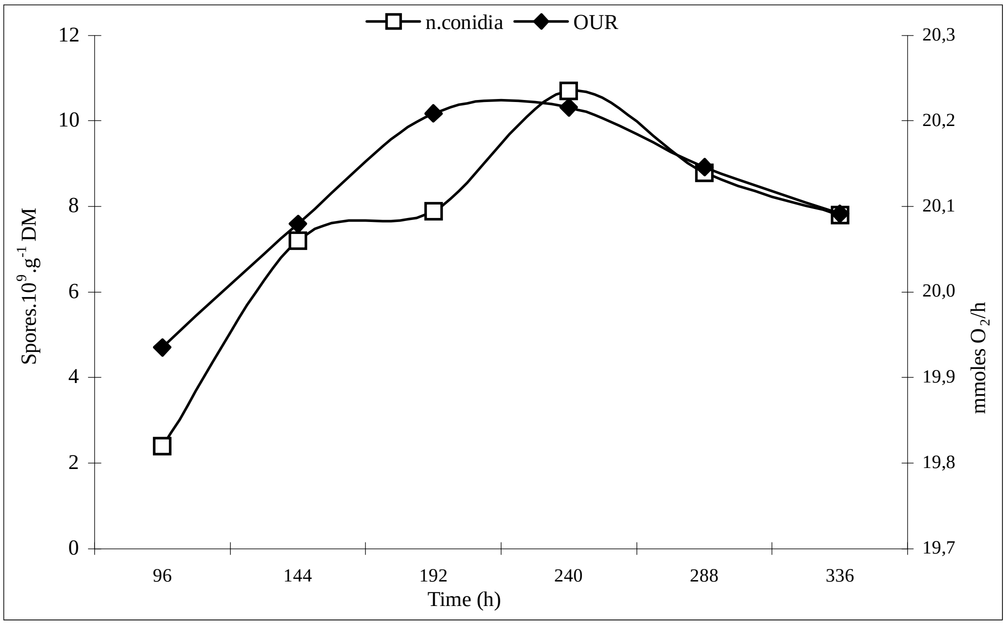

Figure 7 - Spore production and oxygen uptake rate (OUR) during the development of *Beauveria bassiana* in column type bioreactor (substrate/support refused potatoes and sugar-cane bagasse: 60:40%).

CONCLUSIONS

The production of spores of *Beauveria bassiana* LPB-01 by solid-state fermentation by utilizing refused potatoes, as substrate/support was found viable. The results employing different agro-industrial residues (refused potatoes, coffee husk, refused potatoes + sugar-cane bagasse 50:50, and refused potatoes + coffee husk 50:50) demonstrated that the best substrate/support was the mixture of refused potatoes and sugar-cane bagasse, resulting $3.8x10^{7}$ spores.g^{-1} of dry matter. The analysis of the quantity of refused potatoes and sugar-cane bagasse showed that a higher spore production ($3.4x10^{9}$ spores.g^{-1} of dry matter) was obtained with the proportion of 60:40%, respectively.

The studies on the effect of physical parameters of the process in Erlenmeyer flasks demonstrated that the best conditions for spore production were temperature of 26º C, pH 6.0, and initial moisture content of the substrate/support 75% and inoculation rate of 10^7 spores.g^{-1} of dry matter. In column type bioreactors best result of spore production was $9.9x10^9$ spores.g^{-1} of dry matter and was achieved with optimized conditions of initial moisture content (65%) and aeration rate (60 ml. min^{-1}). Finally, a kinetic study of spore production with optimized conditions, concomitant with a respirometric analysis during the development of the fungus showed a correlation between the spore production and the total O_2 consumption.

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RESUMO

O objetivo deste trabalho foi produzir *Beauveria bassiana* por fermentação no estado sólido em resíduos agro-industriais e otimizar as condições de cultivo. Batata-refugo, polpa de café e bagaço de cana de açúcar foram testados. A mistura de batata-refugo e de bagaço de cana de açúcar (60:40%), com granulometria de 2 a 0,8 mm foi escolhida como melhor substrato/suporte. Em frascos de Erlenmeyer a produção de esporos foi maior com as seguintes condições: pH 6,0; temperatura de incubação de 26º C; taxa de inóculo de 10^7 esporos.g^{-1} de matéria seca; e umidade inicial de 75%. Em bioreator do tipo coluna com aeração forçada, as condições otimizadas possibilitaram uma produção máxima de esporos no 10º dia de fermentação, obtendo-se $1,07x10^{10}$ esporos.g^{-1} de matéria seca. A análise respirométrica desta fermentação permitiu correlacionar o desenvolvimento do fungo com a produção de esporos.

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