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Short Communication

Comparison of *Aspergillus niger* spore production on Potato Dextrose Agar (PDA) and crushed corncob medium

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Minimizing the number of generations through which a culture passes from the initial culture until its recovery at the final stage of fermentation is a general principle. For filamentous fungi, a large number of generations can cause the appearance of low-yield producing variants and mutant strains, which are generally genetically unstable. The use of successive sub-cultures can decrease the degree of relationship, causing a decrease in productivity. In order to reduce problems with strain deterioration, all cultures maintained as stock-cultures must be prepared from only one spore of the parental strain. From stock-culture, the microorganism is inoculated in tubes containing agar solidified in an inclined position, resulting in a first-generation culture. For preparation of working-inocule, spores of the first-generation are suspended in a Tween solution and used to inoculate Petri plates containing agar. The inoculation must be carried out over the total agar surface; otherwise growth will be irregular and a uniform culture will not be obtained. Spores produced in the plates are then recovered in Tween solution and used for inoculations of working-media (McNeil and Harvey, 1990).

Several articles have applied agarized medium as an inoculum to *Aspergillus niger* (Ates et al., 2006; Dinu et al., 2007; Djekrif-Dakhmouche et al., 2006; El-Enshasy et al., 2006; Mlakar and Legiša, 2006). The disadvantages in applying agar medium are low spore production and difficulties in obtaining spores, such as defixing of agar from plates during mechanical shaking and the loss of material when transferring the suspension to another recipient.

Solid-state fermentation allows the production of aerial conidia of biological control fungic agents (Ye et al., 2006). Semi-solid media has been developed and tested in strains of different genera, such as *Beauveria*, *Trichoderma* and *Metarhizium,* and this technology was considered more appropriate to large-scale production of fungic spores (Cavalcante et al., 2008; Kang et al., 2005; Prakash et al., 2008; Roussos et al., 1991). Semi-solid culture media are predominantly used in Brazil in large-scale production of entomopathogenic fungi, such as *Metarhizium anisopliae*, which was traditionally done using cooked rice as the substrate (Faria and Magalhães, 2001; Loureiro et al., 2004). Bianchi et al. (2001) have studied the production of

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Aspergillus niger spores in corncob moistened with a sucrose solution for inoculum formation for the production of citric acid.

The objective of this work was to test a low-cost medium for production of *Aspergillus niger* spores and compare it with an agarized medium.

Aspergillus niger CNPAT 001, isolated from cashew apple by the Laboratory of Phytopathology of Embrapa Tropical Agroindustry (Fortaleza/CE, Brazil), was used in this work. The stock culture was maintained in sterile soil at −18°C before use (Martin, 1964).

Potato Dextrose Agar (PDA) was obtained from Himedia Laboratories (India). Bacteriological agar, aniline blue, glycerin and lactic acid were obtained from Vetec. A commercial concentrated suspension of thiabendazole (485 g/L) (Tecto SC) was obtained from Novartis Biociências S.A.

PDA powder (39.0 g) was dissolved in 1 L of distilled water, according to the product recommendations. Water-agar (WA) was prepared by dissolution of 20 g of bacteriological agar in 1 L of distilled water. After dissolution, 10 μl of commercial concentrated suspension of thiabendazole was added. All agarized media were autoclaved at 121°C for 15 min.

Corncob was ground using a knife mill with a 6 mm mesh sieve after drying in an aerated stove at 100°C for 4 h. To 100 g of crushed corncob, 130 ml of 5.6% peptone solution was added. After vigorous homogenization, 10.5 g of medium was aliquoted in 125 ml Erlenmeyer flasks, and then sterilized at 121°C for 30 min.

A solution of aniline blue was prepared by dissolution of 0.5 g aniline blue and 62 ml of distilled water, followed by addition of 875 ml of 80% lactic acid and 63 ml of glycerin according to the method described by Weber et al. (2004).

The microorganism was aseptically transferred from stock-culture into tubes containing inclined PDA. The inoculated tubes were incubated at 30°C for 7 days and were conserved at 4°C.

A 0.3% sterile solution of Tween 80 was added to a tube containing activation culture. Using a platinum loop, spores were manually removed from the agar surface. A sample of 0.1 ml of the suspension was transferred to 90 mm Petri dishes containing PDA, while 1.0 ml of the same solution was added to corncob medium, a solid-state medium. Media were incubated in the dark at 30°C for 10 days. Four samples of each culture were collected every 2 days.

A 0.3% sterile solution of Tween 80 was used to recover the spores. An amount of 40 ml of the Tween 80 solution was added to the Erlenmeyer flasks containing corncob culture and 20 ml of the Tween 80 solution was added to the plates containing PDA.

The volume of spore suspension was measured using a 100 ml graduated cylinder. Spore concentration was determined by counting in a Neubauer Chamber, with the help of an optical microscope under a magnification of 400× (Bier et al., 2001). The viability of the spores was determined by inoculating and spreading 0.1 ml of the spore suspension in 90 mm Petri dishes containing WA, which was incubated in the dark at 21°C for 24 h. After this period, 1.0 ml of aniline blue staining solution was added and gently spread on the entire plate surface. Using an optical microscope Olympus CBA (400× magnification), 100 spores were analyzed. Hyphae were considered germinated when the hyphae size was equal to or greater than that of the spores (Horaczek and Viernstein, 2004).

The concentration and the number of spores in the suspensions recovered from PDA and crushed corncob media, after 2 to 10 days under incubation, were compared through Tukey's test at the 5% significance level.

Volumes of suspensions recovered from each medium were significantly different, at all analyzed times (Table 1). An increase from 7.5 ± 0.6 to 10.8 ± 2.5 ml in the average volume recovered from PDA medium was observed, but the difference was not statistically significant. It is not possible to increase the volume added to the Petri plate because of its shape, and higher volumes may cause overflow or may impede the mechanical release of spores from the agar surface. The volume of *Aspergillus niger* spore suspension recovered from ground corncob was 28.6 ± 1.5 ml until 6 days of fermentation. After this period, the volume decreased to 21.9 ± 4.6 and 17.9 ± 1.9 ml, respectively in 8 and 10 days of experimentation. This reduction may be caused by a loss of humidity in the medium, resulting in higher absorption of Tween solution.

No significant difference was observed in spore concentration among the suspensions recovered from PDA medium during the 10 days under fermentation. However, a significant increase was observed in the concentration of spores recovered from corncob medium on the 4th day. The concentration of spores remained practically constant thereafter until the 10th

Table 1. Spore production and viability in the recovered suspensions from PDA and crushed corncob media during incubation.

Day	Volume of suspension (ml)		Concentration (spores/ml)		Number of spores/volume		Viability (%)		Total viable spores	
	PDA	Corncob	PDA	Corncob	PDA	Corncob	PDA	Corncob	PDA	Corncob
2	7.5 ± 0.6^{a}	29.4 ± 1.1^{a}	$(2.0 \pm 1.1) \times 10^{6\,a}$	$(4.4 \pm 1.0) \times 10^{7\,a}$	$(1.5 \pm 0.7) \times 10^{7\,a}$	$(1.3 \pm 0.3) \times 10^{9\,a}$	64.0 ± 13.0^{a}	64.0 ± 10.0^{a}	$(9.8 \pm 6.3) \times 10^{8\,a}$	$(8.0 \pm 0.9) \times 10^{10\,a}$
4	9.8 ± 2.9^{a}	27.7 ± 1.6^{a}	$(2.3 \pm 1.0) \times 10^{6\,a}$	$(1.6 \pm 0.3) \times 10^{8\,b}$	$(2.3 \pm 1.3) \times 10^{7\,a}$	$(4.4 \pm 0.6) \times 10^{9\,b}$	42.0 ± 6.0^{b}	62.0 ± 8.0^{a}	$(9.7 \pm 5.3) \times 10^{8\,a}$	$(2.8 \pm 0.6) \times 10^{11\,b}$
6	9.5 ± 1.0^{a}	28.6 ± 1.8^{a}	$(6.5 \pm 0.7) \times 10^{6\,a}$	$(1.5 \pm 0.1) \times 10^{8\,b}$	$(6.2 \pm 1.1) \times 10^{7\,a}$	$(4.3 \pm 0.4) \times 10^{9\,a}$	21.0 ± 7.0^{c}	26.0 ± 10.0^{b}	$(1.3 \pm 0.6) \times 10^{9\,a}$	$(1.2 \pm 0.5) \times 10^{11\,a}$
8	10.2 ± 2.9^{a}	22.0 ± 4.6^{b}	$(6.2 \pm 5.3) \times 10^{6\,a}$	$(1.5 \pm 0.5) \times 10^{8\,b}$	$(7.3 \pm 8.3) \times 10^{7\,a}$	$(3.1 \pm 0.7) \times 10^{9\,a}$	14.0 ± 11.0^{c}	28.0 ± 13.0^{b}	$(7.2 \pm 3.7) \times 10^{8\,a}$	$(8.7 \pm 3.8) \times 10^{10\,a}$
10	$10.8 \pm 2,5^{a}$	17.9 ± 1.9^{b}	$(4.6 \pm 1.9) \times 10^{6\,a}$	$(1.5 \pm 0.6) \times 10^{8\,b}$	$(5.1 \pm 3.3) \times 10^{7\,a}$	$(2.7 \pm 0.9) \times 10^{9\,a}$	14.0 ± 5.0^{c}	27.0 ± 8.0^{b}	$(7.3 \pm 5.4) \times 10^{8\,a}$	$(7.1 \pm 3.1) \times 10^{10\,a}$

Different superscripts within a column indicate significant difference at the 95% confidence level ($p < 0.05$).

day. The highest concentration in suspension recovered from this medium was 1.6×10^{8} spores/ml, at day 4. This concentration was two orders of magnitude higher than the concentration recovered from PDA medium (Table 1). Total spore number in the suspension recovered from corncob, calculated by multiplying the volume obtained by the concentration of the suspension, showed that the period between the fourth and the sixth day was the most appropriate to production, with values ranging from 4.3×10^{9} to 4.5×10^{9} spores.

In a study on the production of inocule in large-scale fermentation, Roussos et al. (1991) compared the spore production of *Trichoderma harzianum* in agar medium and in a solid-state fermentation system using cane bagasse as support. Spore production in the inert medium (cane bagasse) and substrate composed by cassava flour and nutrient solution, during 6 days, was up to 5.0×10^{10} spores/g of medium. The quantity of spores produced in the semi-solid medium was 5 times higher than in the agarized medium.

In the present work, spore viability was also evaluated. An accentuated reduction in spore viability was observed from the second day on in the PDA medium, while in corncob medium the reduction was observed only from the fourth day on. Until that time the viability was higher than 60% (Fig. 1).

The use of the solid-state crushed corncob medium was better than the agarized medium because of the higher concentration of spores of the suspensions recovered from it. For the production of the same quantity of total viable spores (concentration of spores of the suspension × volume of the suspension × viability of spores of the suspension) of one Erlenmeyer, it would be necessary to use 217 Petri plates, the comparison made at the best time of fermentation of each

Fig. 1. Viability of spores in the suspensions recovered from PDA and crushed corncob media during incubation.

medium, being the 4th for the crushed corncob, and the 6th for the agarized medium. Using crushed corncob medium as inoculum demands less laborious work, since fewer laboratory flasks are necessary; the medium is easier to prepare, more economical and more environmentally correct because the demand of reagents is smaller and they are cheaper than agar; the handling of wastes is easier; and it provides bigger amounts of spores, which make it more suitable for work on a large scale.

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