Production of Spores of *Trichoderma harzianum* on Sugar Cane Molasses and Bagasse Pith in Solid State Fermentation for Biocontrol

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ABSTRACT

Solid state fermentation was carried out for the production of spores from Trichoderma harzianum *No 53 using sugar cane bagasse pith as solid matrix and sugar cane molasses as carbon and energy source. Different nitrogen sources such as urea, $(NH_4)_2SO_4$, $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ were added in the media to test their effect on spores production. Among these, urea was found most useful that resulted high no of spores ($1x10^9$/gDM). The influence of temperature and initial moisture of the substrate was studied through a 2^2 experimental plan design. No statistical differences were found within the range of 30-35^oC and 60-70% for temperature and moisture respectively. The biotechnological parameters of the process were derived from the Oxygen Uptake Rate (OUR) pattern, which corresponded to the order of 10^9spores/g moist material. The specific growth rate, maintenance coefficient and the yield based on O_2 consumption were 0.108 h^{-1}, 0.001 g.O_2/g.biomass.h and 2.7 g biomass/g O_2 consumed, respectively.*

Key words: *Trichoderma harzianum*, solid state fermentation, sugar cane pith and molasses, biocontrol, kinetics

INTRODUCTION

In the last few years, the significance of biological control and pest management has been well illustrated by several authors (Elad & Chet, 1995; Hokkanen, 1994; Lacey & Goettel, 1995; Leggett & Gleddie, 1995; Moore & Prior, 1993; Sutton &. Peng, 1993). There are associated benefits and the risks of the employment of micro-organisms against unwanted organisms (target pests). Although the products from *Bacillus thuringiensis* are by far the most applied nowadays, there are some biofungicides that are marketed at present. Such a product has been prepared from *Trichoderma* sp also (Hokkanen, 1994; Fokkema, 1996).

There has been rapid development on the procedures for the production of micro-organisms to be employed in biocontrol. *Trichoderma* sp. is considered as a promising source for the production of bioplaguicides for the control of different plagues like *Botrytis cinerea, Fusarium* sp., *Phytophtora* sp., *Rhizoctonia solani* and *Sclerotium rolfsii* (Belanger et al., 1995; Bonilla, 1990; Dickinson et al., 1995; Echemendía et al., 1995; Goldman et al., 1994; Lewis & Lumsden, 1995; Lorito et al., 1996; Mao et al., 1997; Orlikowsi, 1995). However, in case of fungi, there is general lack of efficient technologies for commercial production. One of the main reasons for this being their tendency is to form pellets in submerged fermentation, which is traditionally employed to produce spores.

Solid state fermentation (SSF) offers numerous advantages over liquid fermentation (Pandey, 1991, 1992, 1994) and could be an attractive alternative for this. SSF has been

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considered a useful system for spores production (Cliquet & Scheffer, 1997; Muñoz et al., 1995; Roussos, 1987). However, little is known about the kinetics of SSF processes that hinders further development and scale-up of particular processes and their consequent technologies. In that sense, it seems to be of paramount importance to determine the process parameters to assess system charac-terization for complete process description. In case of fungal cultures like *Trichoderma* sp., if to be used for spore production, it would be important to use such media that does not induce fungal activity of enzyme formation but support mycelial growth and sporulation.

In this paper we report our results on the cultivation of *Trichoderma harzianum* No 53 in a medium, balanced as a function of sugar present as well as on the supplementation of medium with different nitrogen and phosphorus sources in an aerated solid state fermentation on bagasse pith matrix enriched with sugar cane molasses. Process kinetics were determined through Oxygen Uptake Balance (OUR), as has been previously described (Rodríguez *et al*., 1998; Sato *et al*., 1983; Soccol *et al*., 1993).

MATERIALS AND METHODS

***Microorganism*:** The study was carried out with the strain of *Trichoderma harzianum* No 53 obtained from the National Institute for Research on Plant Health (INISAV, Cuba).

Inoculum: Inoculum was prepared by growing the culture in Czapek medium at 30°C for 48 h under static conditions. Mycelia (10%, wet wt. basis), so obtained, were used as inoculum.

***Substrate*:** Sugar cane molasses (density 1.43 g/ml, sugar concentration 53%) was mixed with bagasse pith in 1:1 (w/w) ratio. This amount was calculated to prevent percolation to achieve a final concentration of 37% of total sugars based on dry bagasse pith (21% based on initial dry total weight). Bagasse pith was previously sieved through a 3-mm sieve. Initial pH and moisture of the substrate were adjusted to 5.1 and 60%, respectively.

Fermentation: Fermentation was carried out in glass columns (100-ml) as described by Raimbault (1981). It was aerated with a saturated air at 1 VKgM (l air/substrate kg/m). The studied nitrogen sources were urea, $(NH4)_2SO_4$, $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$. Their concentration was decided by the amount of nitrogen required for 50% uptake of total sugars and 30% of true protein (on dry matter basis) in microbial biomass. All fermentations were carried out at 30°C.

Influence of temperature and moisture: A factorial experimental design (2^2) with a substrate formulation, which gave the best results in the experiments with different nitrogen sources, was carried out to study the influence of temperature and moisture. Independent variable levels were 30°C (low level) and 35°C (high level), and 60% (low level) and 70% (high level) for temperature and moisture, respectively. Runs were carried out in triplicate in random blocks corresponding to the temperature of 30^0C and in duplicate for the block corresponding to 35°C.The time of fermentation in all the cases was 5 days with aeration saturated with water vapours.

Determination of the process kinetics: The consumed O_2 and the CO_2 evolved was monitored during the whole process by gas chromatography (Gow-Mac equipment) with a thermal conductivity detector and a concentric column CTR-1 (Alltech, USA).

Counting of spores: Spores counting was done in a Neubauer chamber.

***Protein determination*:** Protein was determined by Barnstein method (Winton & Winton, 1944). A sample of 0.7 mg was treated with concentrated H_2SO_4 and after digestion $Cu(OH)_2$ was added. The protein precipitate was washed thoroughly and nitrogen was determined by the Kjeldahl method.

RESULTS AND DISCUSSION

Table I shows the results obtained for spores counting after 5 days of fermentation in the substrates with different nitrogen sources tested. As is apparent, there was no statistical difference between urea and ammonium dihydrogen-phosphate with a 95% of confidence level. This suggested a positive effect of both of these compounds on the microbial culture for spore production. The low values exhibited by ammonium sulfate and diammonium hydrogen phosphate could probably be due to a sharp decrease of pH regardless its possible metabolic influence.

Table 1. Final counting of spores as a function of the nitrogen source employed for the spore production from *Trichoderma harzianum* No. 53

	No. Spores/g dried matter
Urea	1.6×10^9
$(NH_4)_2SO_4$	1.0×10^8
$(NH_4)H_2PO_4$	0.9×10^9
$(NH_4)_2HPO_4$	0.9×10^8

The response matrix obtained from the factorial design 2^2 respect to the final spore counting as a function of temperature and initial moisture was: 6.91×10^9, 5.18×10^9 ,4.06×10^9 ,384×10^9.

The discrimination of the coefficients corresponding to the model of the experimental design was carried out by the Fischer's test (Bacon, 1971), considering a $s_p = 2{,}06 \times 10^9$ calculated from the replicated runs, with 6 degrees of freedom and at a 95% confidence level (for a $C_{ritical} = 5{,}99$). The results are shown in Table II, where b_o is the mean value of the block, b_1 and b_2 are the corresponding coefficients for temperature and initial moisture, respectively. b_{12} is the coefficient that represents the interaction between the former factors studied.

From this design, it could be deducted that although none of the two factors were significant, it was convenient to work at the lowest levels of moisture (60%) and temperature (30°C) tested for two reasons; first to prevent bacterial contamination that could occur at higher moisture levels, and secondly, keeping due to the fact that uncontrolled processes the temperature increase could delay the normal development of the culture.

Table 2. Factor discrimination for spore counting corresponding to the 2^2 factorial design as function of temperature and moisture

	b_i	$F = b_i^2 / ¼\ s_p^2$	$F/F_{critical}$
b_o	$5{,}00 \times 10^9$	23.564	4
b_1	$- 0{,}49 \times 10^9$	0,23	0.04
b_2	$- 1{,}05 \times 10^9$	1,04	0.17
b_{12}	$0{,}38 \times 10^9$	0,14	0.07

The conditions tested here were far to be optimum provided that the interaction between temperature and initial moisture has no significance and showed the lowest coefficient value (b_{12}). These results suggested that the optimum value for initial moisture could be below 60% and for temperature less than 30°C (taking into account the negative which showed declining trends afterwards to a minimum around 37-38 h when the micro-organism started to sporulate.

To solve this equation, only the values obtained within the first 38 h were considered. The values corresponding to yield based on oxygen consumption (Y_{ox}), maintenance coefficient (m) and the estimation of biomass at each point (X_i) that corresponded to this process were obtained by a trial and error procedure as reported by Rodriguez-León *et. al*. (1998) and Soccol *et al.* (1993). This trial and error was made from an initial biomass (X_0) of 2.5 g estimated by protein determination, considering 30% protein concentration in biomass (dry basis) and a final biomass concentration at 38 h as 8.9 g.

The Oxygen Uptake Rate (OUR) during fermentation is shown in Figure 1. A lag phase of about 15-h was observed, and before this time no oxygen uptake was detected. OUR profile exhibited a maximum at 21 h, which showed declining trends afterwards to a minimum around 37-38 h when the micro-organism started to sporulate. To solve this equation, only the values obtained within the first 38 h were considered. The values corresponding to yield based on oxygen

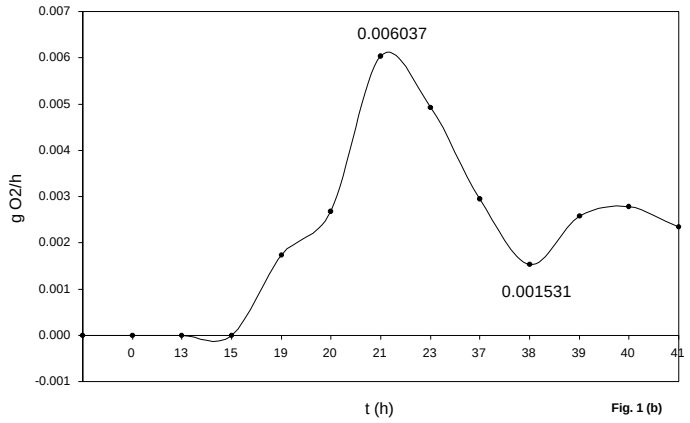

Figure 1. Kinetics of OUR during SSF of sugarcane bagasse by *T. harzianum*

The kinetic parameters were determined by means of the equation:

$$X_n = \frac{Y_{ox}\ \Delta t[1/2(R_o + R_n) + \sum_{i=1}^{n-1} R_{i\,j} + (1 - \frac{a}{2})\ X_o - a \sum_{i=1}^{n-1} X_i}{1 + a/2}$$

Where:
$a = Y_{ox}\ m\ \Delta t$
X_o: initial biomass (g).
X_i: biomass at time t_n (h).
Y_{ox}: yield based on consumed oxygen (g biomass / g O_2 consumed).
R_o: initial oxygen uptake rate (g O_2 / h).
R_i: oxygen uptake rate at time t_1 (g O_2 / h).
m: maintenance coefficient (g O_2 / g biomass / h).
Δt: time interval (h).

Table 3. Kinetics parameters corresponding to solid state fermentation of sugar cane molasses on bagasse pith by the strain *Trichoderma harzianum* No 53

Y_{ox}:	2.7	g biomass/g O_2 consumed
m:	0.001	g O_2 consumed/g biomass/h
μ:	0.108	h^{-1}
r:	0.996	
X_n determined at 37 h analytically	8.9	g
X_n estimated by equation (1) at 37 h	8.7	g

consumption (Y_{ox}), maintenance coefficient (m) and the estimation of biomass at each point (X_i) that corresponded to this process were obtained by a trial and error procedure as reported by Rodriguez-León *et. al*. (1998) and Soccol *et al*. (1993). This trial and error was made from an initial biomass (X_0) of 2.5 g estimated by protein determination, considering 30% protein concentration in biomass (dry basis) and a final biomass concentration at 38 h as 8.9 g.

The results obtained by the trial and error process are reported in Table 3. The specific

growth rate, (μ) was estimated from the values of X_i for each time corresponding to the log phase. The regression coefficient (r) reported corresponded to this estimation.

These results indicated good values for the yields based on consumed oxygen ($Y_{x/o}$), as well as for maintenance coefficient (m). Such a low value for (m) could be related with a process in which no inducible enzymes were produced, in spite of the fact that *Trichoderma* sp. are well known as producers of cellulolytic and xylanolytic enzymes. The whole microbial energy was employed to grow from the sugars. This behavior seemed to be related to initial repression of those enzymes, by sugars content and the corresponding nitrogen balance as function of the initial sugars. This meant that if the nitrogen was not balanced, it could be expected that the strain would produce cellulases or xylanases and therefore the time to reach the appropriate spore counting could be much longer and maintenance coefficient would be higher with the concomitant decrease of oxygen based yield.

CONCLUSIONS

The results demonstrated that a good sporulation from *Trichoderma harzianum* could be achieved in only 5 days. It could be interesting to further study the sporulation behavior at moisture levels below 60%, which could result higher productivity of spores in aerated solid state fermentation system.

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RESUMO

Esporos de *Tricoderma harzianum* N^o 53 foram produzidos por fermentação no estado sólido (FES) utilizando bagaço de cana como suporte e melaço de cana como fonte de carbono. Diferentes fontes de nitrogênio foram testadas (uréia, $(NH_4)_2$ SO4 , $NH_4H_2PO_4$ e $(NH_4)_2HPO_4$) na produção de esporos. As mais elevadas concentrações de esporos (10^9 esporos/g de suporte úmido) foram obtidas utilizando a uréia como fonte de nitrogênio. O efeito da temperatura e umidade inicial foram estudadas através da utilização da planificação experimental utilizando um modelo 2^2. Não foi encontrada diferença estatística na produção de esporos na faixa de temperatura compreendida entre 30-35 °C e umidade inicial de 60-70%. Os parâmetros biotecnológicos foram determinados através da taxa de oxigênio consumido (OUR) correspondente a uma produção de 10^9 esporos/g de suporte úmido. A taxa de crescimento especifico, coeficiente de manutenção e rendimento foram calculados em função do O_2 consumido, cujos valores foram 0.108 h-1 , 0.001 g O_2/g biomassa/h e 2.7 g biomassa/g O_2 consumido respectivamente.

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