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STRATEGIES FOR LARGE SCALE INOCULUM DEVELOPMENT FOR SOLID STATE FERMENTATION SYSTEM: CONIDIOSPORES OF *Trichoderma harzianum*

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SUMMARY

The suitability of disc fermenter for efficient production of conidiospores by *Trichoderma harzianum* is limited to a working capacity of 0.6 l agar medium due to large decrease in spore production per cm^2 of the culture surface area with further increase in the capacity. In contrast, Zymotis, a large scale solid state fermenter designed at ORSTOM, France and use of inert solid support for absorbing nutrients offer many advantages for production of conidiospores in quantity sufficient enough to inoculate pilot and larger fermenters. Five times higher production of conidiospores in Zymotis, as compared to the agar medium in flask, constitutes a success in the development of large scale inoculum.

INTRODUCTION

No worthwhile newer commercial exploitation of solid state fermentation (SSF) system in the last decade is evident inspite of intensive research and development efforts throughout the world. One of the major reasons for this situation is the lack of efficient techniques for development of active spore inoculum which is required in large quantity in SSF system. The form, age and ratio of inoculum are of critical importance in SSF system which rely on larger inoculum ratio to control contamination (Lonsane *et al.*,1991). The spores are usually preferred over vegetative or mycelial cells in SSF system due to ease in mixing of the inoculum with autoclaved moist solids (Lonsane *et al*, 1985). The productivity of the system is also influenced by the age of the inoculum (Lonsane and Ramesh,1990). Moreover, the viability of the spores is of great significance. The development of large scale inoculum has been specified as one of the areas which poses problems in scale-up of submerged fermentation processes (Bank, 1984). In fact, this will be more problematic in SSF system due to involvement of lower water activity, complex medium constituents, use of water insoluble polymeric substrates and high heterogeneity (Mitchell and Lonsane, 1991). Work was, therefore, undertaken to develop efficient strategies for large scale inoculum development on agar media and also in the SSF system involving the use of bagasse as support. *Trichoderma harzianum* has been selected for the studies due to its significant industrial importance in the production of cellulases (Deschamps *et al.*, 1985), biopesticides (Elad *et al.*, 1982), antibiotics (Fujiwara *et al.*, 1982), protein enrichment of cassava flour (Muindi and Hanssen, 1981) and flavour compounds (Okuda *et al.*, 1982).

MATERIALS AND METHODS

Microorganism. *T. harzianum CCM F-470,* obtained from Czechoslovak Collection of Microorganisms, Brno, was maintained on potato-dextrose agar at 4°C and by subculturing once every four months. The spores from freshly grown slants were suspended in sterile water containing 0.01% Tween 80 (10 ml/slant) for inoculum preparation. The inoculation was with 8×10^7 spores per 100 ml or g of the conidiospore production media in all the cases. This provides 3×10^7 spores/g cassava flour initially present in these media

Conidiospore production on agar medium. Erlenmeyer flasks, 250 ml, and disc fermenters (Roussos, 1987) of four different sizes (Table 1) were used as bioreactors. The disc fermenter D1 consists of a cylindrical glass column with a flanged open end, which can be closed by using two stainless steel plates, "O" rings and winged nuts for aseptic operation. A central shaft with sterilizable bushings and gaskets, inlet and outlet ports were provided on the plates. Each disc, which provides surface for the agar medium, consists of a pair of metallic sieves (each of 2 mm diam) and a ring of 10 mm thickness between the two sieves. Both the sieves and the rings fit closely on the shaft. The central shaft accomodates 10 discs and its end can be screwed so that all the sieves and rings are in one fixed position and do to get disturbed during agitation of the fermenter. The diameter of the discs is marginally smaller than that of the glass vessel. The fermenter was kept in horizontal position using an appropriate stand.

Table 1 : Details of the design of the disc fermenters

Attributes	Disc fermenters			
	D1	D2	D3	D4
Length of the glass cylindrical fermenter (cm)	25.0	80.0	65.0	60.0
Internal diameter of the glass cylindrical fermenter (cm)	9.2	9.2	20.3	30.3
Total capacity (l)	1.5	5.0	20.0	42.0
Working capacity (l)	0.3	0.6	0.8	1.0
Number of discs	10.0	35.0	25.0	20.0
Disc diameter (cm)	9.0	9.0	20.0	30.0
Surface area of disc (cm^2)	127.0	127.0	628.0	1413.0
Total surface area of the discs (cm^2)	1270.0	4445.0	15700.0	28260.0

The agar medium for conidiospores production contained (g/l): cassava flour 40; KH_2PO_4 2; $(NH_4)_2SO_4$ 4; urea 1 ; $CaCl_2$ 1; agar 15; distilled water 1 l and the pH was adjusted to 5.6 using 5 M HCl. The medium in desired quantity was charged in the bioreactors for autoclaving at 110°C for 30 min. The medium was then cooled to about 45°C and inoculated. In case of Erlenmeyer flasks, the well-mixed inoculated medium was allowed to solidify undisturbed. On the other hand, the disc fermenters were rotated at 30 rpm, till the temperature reduced to 25-29°C, for uniform mixing of inoculum and solidification of agar on the discs. Subsequently, the rotation was stopped. The excess medium in the disc fermenters was drained off when it was still in liquid state.

The growth and sporulation of the culture were allowed to take place at ambient temperature (28±1°C) without any pH control. However, the disc fermenters were aerated at the rate of 40 l of humidified and sterilized air/h during the entire duration of 7 days of the

fermentation. The air supply from compressor was regulated at 0.5 pressure, freed from oil in oil separator, filtered in glass-wool filter, humidified by passing through water held in a glass column and fed to the fermenters.The exit air was filtered through glass-wool. The conidiospores, at the end of 7 days of fermentation, were harvested by adding 5 parts of sterilized 0.01% Tween 80 solution to the bioreactors. The content was mixed well by mechanical agitation in disc fermenters and by magnetic stirring in case of Erlenmeyer flaskes. The resulting spore suspension was collected in sterilized containers and stored in refrigerator at 4°C up to 53 days. In some cases it was subjected to vacuum drying under different temperatures and with or without the use of bagasse as absorbing agent. The sterilized bagasse of 1.5 cm particle size was used at 1.5% level to absorb the spores, before vacuum drying.

Except for the size of the vessel, the number of discs and diam of discs as shown in Table 1, the other details of disc fermenters D2, D3 and D4 and the culturing technique were same as described for disc fermenter D1. In addition, glass columns used in the disc fermenters D3 and D4 were open at both ends and therefore were provided with plates at each end.

Conidiospore production by SSF system. The moist solid medium used in column fermenter and Zymotis (a large scale solid state fermenter designed at ORSTOM, France) contained (g): bagasse 80, cassava flour 30, $(NH_4)_2SO_4$ 3 , urea 0.75, KH_2PO_4 1.5 , $CaCl_2$ 2, feather meal (pulverized feathers of poultry birds, manufactured by Energéticos para Alimentos Balanceados, S.A. de C.V., Iztapaluca, México) 10.75 and tap water 100 ml. The ingredients were mixed thoroughly and the moist medium was charged in about 6 kg wet weight quantities in cloth sacks for autoclaving at 121°C for 15 min. After cooling to about 30°C, it was mixed thoroughly with the liquid inoculum obtained from disc fermentor D2 so as to provide $3x10^7$ spores/g cassava flour initially present in the autoclaved medium. The final moisture content of the medium was 75%. The inoculated medium was charged in 18 g wet weight quantities in the column fermenter. The Zymotis compartments were charged to occupy 5 and 10 cm length with 50 cm height as well as 10 cm length with 30 cm height. In another case, Zymotis was charged with 21 kg moist medium and the medium in this case contained bagasse: cassava flour at 80:20 instead of at the ratio of 70:30 used in other experiments. Moreover the medium was autoclaved at 110°C for 90 min in the cloth sacks in this case.

The column fermenter assembly and the design of Zymotis have been described elsewhere with the operating procedures (Raimbault and Alazard, 1980; Gonzalez-Blanco *et al.*, 1990). In both the cases, the fermentation was carried out at 29°C for 6 days, with aeration by humidified air at a rate of 4 l/h/column and 300 l/h/kg dry solids in Zymotis. At the end of the fermentation, the fermented solids were removed from the bioreactors and the conidiospores were harvested from 10 g sample in 0.01% Tween 80 solution as per the methodology described for conidiospores production on agar medium.

Studies on conidiospores produced. The samples of the vacuum-concentrated spore suspensions, with or without absorption on bagasse, were studied for viability determination. In another set of experiments, the effect of the storage of the conidiospore suspensions at 4°C was examined up to 53 days. The comparative production of cellulases in column fermenters was also evaluated by using the conidiospores produced in different bioreactors. The medium used contained bagasse:wheat bran at 80/20 (w/w) ratio while the methodology followed was as described elsewhere (Roussos *et al.*,1989). All the above studies were confined to the conidiospores grown in flask and disc fermenters D1 and D2.

Analytical methods. The viability of the conidiospores was studied using the medium of Douglas *et al* (1979) with the methodology of Roussos (1987). The conidiospores were counted using haemocytometer. Carboxymethyl cellulase (CMCase) and filter paper activity (FPA) of the cellulolytic enzymes were estimated as per the methodology of Mandels *et al.*(1976). The enzyme concentration is expressed as international unit (IU) which denotes the micromoles of glucose released per min of the reaction (Roussos, 1987).

RESULTS AND DISCUSSION

Conidiospore formation on agar medium. The extent of the conidiospore formation in disc fermenters D1 and D2 is similar to that on the agar medium in Erlenmeyer flasks (Table 2). It, however, decreased considerably in disc fermenters D3 and D4 probably due to various limitations which usually are imposed when reactor volume is increased geometrically. Moreover, the conidiospore formation per cm^2 of the culture surface area in the fermenters D3 and D4 is extremly lower because only a smaller portion of the disc in these reactors gets covered with the medium as compared to that in disc fermenters D1 and D2. The data indicate that the conidiospore production on agar medium in disc fermenters is efficient only upto the bioreactors of D2 size.

Table 2 : Productivities of different bioreactors in the formation of conidiospores of *T. harzianum*

Bioreactor	Conidiospore production	
	per g cassava flour	per cm^2 of the culture surface area
Erlenmeyer flask	1.1×10^{10}	1.7×10^{8}
Disc fermenter		
D1	9.3×10^{9}	2.2×10^{8}
D2	3.3×10^{10}	1.5×10^{8}
D3	2.2×10^{9}	1.4×10^{5}
D4	2.0×10^{9}	0.7×10^{5}
Column fermenter (18 g moist solids)		
No feather meal	1.0×10^{10}	8.8×10^{8} *
With feather meal	5.0×10^{10}	4.0×10^{9} *
Zymotis		*
5 cm l x 50 cm h compartment, 3.6 kg moist solids	3.8×10^{10}	3.5×10^{9} *
10 cm l x 50 cm h compartment, 7.2 kg moist solids	4.1×10^{10}	3.3×10^{9} *
10 cm l x 30 cm h compartment, 4.3 kg moist solids	4.6×10^{10}	3.7×10^{9} *
With load of 21 kg moist medium	5.0×10^{10}	7.7×10^{8} *

* The counts are per cm^3

The conidiospores from disc fermenter D2 are sufficient for inoculation of 100 kg moist cassava flour medium for protein enrichment of cassava or 100 kg moist bagasse plus wheat bran medium for cellulases production. The calculations are based on the rate of inoculation of these media at 10^{10} spores/kg moist solid medium, the standardized values (Raimbault and Alazard, 1980; Roussos, 1987). Larger inoculum will be required for larger pilot or village level plant of 5-8 tons moist medium per day capacity. Using disc fermenter of size D2, the inoculum for such plants can be produced but it will require resortion to the use of 20 fermenters working in tandem. However, such strategy is highly unthinkable to put

into practise and it will be cost-intensive in respect of both the capital and operating expenses. It is also not practicable as the use of agar medium at such large scale is highly laborious and agar itself is expensive. It is, therefore, necessary to search for alternative strategy of simple and inexpensive nature for large scale production of conidiospores.

Conidiospore production in SSF system. The use of inert solid support for absorbing liquid medium for fermentation in SSF system has been pioneered by Raimbault and Alazard (1980) for facilitating selective and homogenous development of mycelia as well as study of the physiology and growth of the fungi. Two different types of bioreactors for SSF system have also been developed and include column fermenter (Raimbault and Alazard, 1980) and Zymotis (Roussos, 1987). The data in Table 2 indicate that these bioreactors offer excellent potential for conidiospore production.

The use of bagasse as support to absorb starch containing liquid medium in column fermenter gave nearly equal conidiospore production as compared to that on agar medium in flasks as well as disc fermenters D1 and D2. The inclusion of feather meal in the medium (10.75 g in 100 g mixture of dry bagasse and starch) resulted in an increase of 5 times in the conidiospore production (Table 2).

The productivities of the conidiospores in Zymotis, operated at four different substrate load conditions, were equal to that in the same medium in column fermenters (Table 2). In fact, the conidiospore production in Zymotis per g cassava flour is 5 times higher than that on the agar medium in flasks and this constitutes a success in development of large scale inoculum. The conidiospores formed in 21 kg moist medium in Zymotis are sufficient to inoculate 5 tons of cassava flour or bagasse + wheat bran media in SSF processes. The full working capacity of Zymotis is 42 kg moist medium and it will provide inoculum for 10 tons of wet cassava flour or bagasse + wheat bran media.

The productivity per g cassava flour is higer in Zymotis with 21 kg moist medium load as compared to other loads, though the productivity in terms of conidiospores/cm^3 of the culture surface area is comparatively lower. This is because of the use of cassava flour:bagasse at 20:80 (w/w) in the former as compared to 30:70 (w/w) in latter cases. Consequently, the effective surface area contained more of the inert solid support. It may be possible to achieve better productivities by using higher cassava flour concentration.

Studies on conidiospores produced. The conidiospore suspension from the disc fermenters is highly dilute due to the need for use of large volume of liquid to recover the conidiospores completely from the agar surfaces, the efficiency of recovery achieved being 98%. Unless used immediately , the preservation at 4°C becomes essential. The viabilities of the conidiospores after such preservation for 1, 26 and 53 days were 97, 84 and 83 %, respectively. The vacuum concentration of the suspension at 40°C to reduce the bulk volume, however, resulted in merely 9.4% viability. The viability reduction was more drastic at 50°C and 60°C vacuum concentration. The conidiospores of *T. harzianum* are very sensitive to temperature (Roussos, 1987). Its optimum temperature for growth is 29°C and it does not grow at 35°C. It is interesting to note that the viability was 38.8% when bagasse was added to the suspension before vacuum concentration at 40°C. The bagasse probably absorbs the conidiospores and impart protection during vacuum concentration. The conidiospores, when used for production of cellulases in column fermenters and Zymotis, performed equally and produced 19 IU of FPA and 200 of CMCase activities per g substrate dry matter, in a number of experiments.

It is emphasized that the use of SSF system for production of the conidiospores can overcome the problems associated with dilute nature of the conidiospore suspensions from the disc fermenters In addition, the need for recovery of the conidiospores can be avoided and the spore containing fermented mass can be used directly as is done in a number of food fermentations (Lonsane and Krishnaiah, 1991). Alternatively it can be dried at 40°-50°C to a moisture content of 8-12%, without any appreciable loss of spore viability, for use at a latter date. In this respect, Zymotis offers advantages as it will be possible to dry the fermented solids *in situ* by passing dried hot air through the loop which was used during fermentation for supplying humid air. The inoculum grown on wheat bran and other solid substrates has been stored up to 6 months after drying without appreciable loss of the viability of the spores (Mitchell and Lonsane, 1991).

The data indicate high potential and many advantages of producing large scale conidiospores (inoculum) in Zymotis for use in pilot and large scale SSF systems. Most of the strategies available so far are based on conventional methods of using trays and the medium in shallow depth. They became too unwieldy and laborious at larger scale and are also prone to contamination. The other available methods are of small sizes. The fermenters similar to Zymotis design, bagasse impregnated with nutrients and incorporation of feather meal in the medium do not seem to have been used earlier for large scale inoculum development in SSF system. The conidiospores thus produced can also be used as such as inoculum in SmF processes or if necessary, after recovery in sterile water. The dried fermented solid with its high load of conidiospores of *T.harzianum* can also be used directly as biopesticide.

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