Appl Microbiol Biotechnol (1997) 48: 714–719

ORIGINAL PAPER

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Liquid fermentation to produce biomass of mycoherbicidal strains of *Fusarium oxysporum*

Received: 24 April 1997 / Received revision: 6 August 1997 / Accepted: 29 August 1997

Abstract Conditions for optimizing spore production, especially chlamydospores, by host-specific mycoherbicidal strains of *Fusarium oxysporum* causing vascular wilts in coca (*Erythroxylum coca*) and poppy (*Papaver somniferum*) were studied in 2.5-1 fermentors. The fermentor dissolved oxygen and pH had significant effects on the growth characteristics of *F. oxysporum* strains. The effect of the fungal strain, however was not significant for most of the variables studied except for chlamydospore formation. After 14 days of fermentation, the spore types produced were microconidia and chlamydospores, with very little production of macroconidia. While the total viable counts were significantly higher under high than under low dissolved O_2, the chlamydospore counts were significantly higher under low than under high dissolved O_2. The percentage of chlamydospores obtained, as a proportion of total viable was significantly higher when the fermentor pH was increased, than when it was not. Scaling-up the liquid fermentation to 20 l, yielded $\log_{10} c = 6.8$ (where c = chlamydospores ml^{-1}) after 14 days' fermentation, with biomass viable counts of $\log_{10} v \sim 8.0$ (where v = viable counts g^{-1} air-dried biomass). A single-step liquid fermentation reported in this study increased chlamydospore yields and reduced the time required for their production with techniques currently available from 5 weeks to less than 2 weeks.

Introduction

Biological control of plant pests, fungal pathogens and weeds has been used as an alternative to chemical control with varying degrees of success. Recently, in addition to saprophytic strains of *Fusarium oxysporum*, which have been investigated for their potential as suppressers of plant fungal pathogens (Alabouvette 1990; Gullino et al. 1995), pathogenic strains of *F. oxysporum* causing vascular wilts have been identified as potential mycoherbicides of several weeds (McCain and Noviello 1985; Boyette et al. 1993; Abbasher et al. 1995; Sands et al. 1995; Pilgeram et al. 1995). Preliminary results from the above studies in greenhouses under controlled conditions and in small field plots are encouraging. The pathogenic strains of *F. oxysporum* causing vascular wilts have been reported to be highly specific to their host plants (Armstrong and Armstrong 1981).

However, a critical requirement for successful application of *F. oxysporum* as a mycoherbicide is the large-scale production of biomass, preferably chlamydospores, which are resistant to desiccation and temperature extremes (Schippers and Van Eck 1981). Fermentor-produced chlamydospores of the biocontrol fungi, *Gliocladium virens*, and *Trichoderma* spp. (Papavizas et al. 1984) have been formulated into dry preparations such as alginate prill (Lewis and Papavizas 1985) with various food bases, and also with pregelatinized starch granules (Lewis et al. 1995) based on the research of Dunkle and Shasha (1988).

Recently some research has been done on liquid and solid-based formulations with the mycoherbicidal strains of *F. oxysporum* (Boyette et al. 1993), but none using chlamydospores obtained by liquid fermentation. Currently, for large-scale production of chlamydospores of *F. oxysporum*, a two-step method, semi-solid fermentation procedure followed by drying of the biomass obtained, is utilized (Hildebrand and McCain 1978). This method requires at least 5 weeks before substantial numbers of chlamydospores are obtained.

Recently, it was shown that water extract of soya bean hull fiber, an agricultural byproduct, was an inexpensive and suitable substrate for chlamydospore formation in mycoherbicidal strains of *F. oxysporum* (Hebbar et al. 1996). The purpose of this work was to

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optimize conditions required for the production of chlamydospores by mycoherbicidal strains of *F. oxysporum* using liquid fermentation in bench-top fermentors and in 20-l carboys.

Materials and methods

Fungal strains

The fungal strains used (deposited at Agricultural Research Service Culture Collection, Peoria, Ill.) were *Fusarium oxysporum* f. sp. *erythroxyli* EN4-S isolated from diseased coca (*Erythroxylum coca* var. *coca*) (Sands et al. 1995) and a phenotypic variant EN4-FT (Hebbar et al. 1996), and *Fusarium oxysporum* f. sp. [*papavari*] CP3A isolated from a diseased poppy (*Papaver somniferum*) (Pilgeram et al. 1995). Suspensions of the single-spore-derived cultures, which included a mixture of spores and mycelia, were maintained at −20 °C in 50% glycerol.

Fermentor medium and preparation of starter cultures

The medium for bench-top fermentors contained aqueous extracts of soya bean hull fiber (Dietfiber-Soyfiber, TU 20070, Lauhoff, Danville, Ill.), prepared by autoclaving a 1% (w/v) suspension of the fiber at 121 °C for 30 min and then filtering the extract through a Whatman no. 4 filter-paper. The filtrate (2 l) was poured into the fermentor vessel (2.5 l, Bioflo IIC, New Brunswick, N.J.) and the unit re-autoclaved. Large volumes (15 l) of fungal fermentations were performed in 20-l autoclavable polypropylene carboys using a previously published method (Papavizas et al. 1984) with nonfiltered aqueous suspensions of 1% soya bean hull fiber as the growth medium. The carboys were autoclaved for 1 h at 121 °C on each of two consecutive days before they were inoculated with starter flasks of the fungus.

Starter flasks (100 ml) of 1% filtered soya bean hull fiber medium were inoculated with 100 µl glycerol-preserved stock cultures and incubated on a shaker at 100 rpm for 3 days at room temperature (22–25 °C). The starter flasks were tested for bacterial contamination by plating aliquots of 100 µl on nutrient agar medium (Difco, Detroit, Mich.) 1 day before they were inoculated into the fermentors. Contaminated starter cultures were discarded.

Bench-top fermentation

The bench-top fermentor was set up according to the manufacturers instructions and inoculated with 100 ml starter culture. The fermentor medium temperature was maintained at 25 °C, the agitation speed adjusted to 50 rpm and the fermentor was run for 14 days. The three treatments (T) used for the fermentor runs were as follows: T1, high dissolved oxygen (DO) conditions, wherein 2 l filtered air h^{-1} was bubbled (sparged) into the fermentor from day 0 to day 14; T2, low DO conditions wherein 2 l h^{-1} filtered air was introduced into the fermentor only as a surface stream (non-sparged) from day 0 to day 14; T3, nonsparged from day 0 to day 14 with pH artificially raised to 9.0–10.0 on day 4 and maintained. The calibration of the DO probe (to 100%) and pH electrodes (with pH 7.0 standard) was carried out according to the manufacturer's instructions at the start of the fermentation run. The DO and pH readings of the fermentor medium, read on the digital display, were recorded daily. All the treatments were repeated twice for the three strains (EN4-S, EN4-FT, CP3A) tested.

The fermentors were sampled (approx. 20 ml) once every 2 days for 14 days and the total number of viable propagules was determined by plating serial dilutions on potato dextrose agar (PDA; Difco, Detroit, Mich.). The sample was then blended with a 15-ml tissue grinder (Pyrex, Ten Broeck), mainly to disperse the chlamydospores. Subsequently, the clearly distinguishable oval-shaped one-celled microconidia, four-celled spindle-shaped macroconidia and the single-celled, thick-walled and rounded chlamydospores were counted with a hemocytometer. Fermentor samples were tested for bacterial contamination under a light microscope (×100) and also by plating on nutrient agar. Fermentor runs with bacterial contamination were discontinued. The pH of the medium was also recorded throughout the experiment. At the end of the 14-day run, the fermentor medium was filtered through no. 4 Whatman filter-paper and dried overnight in a 80 °C oven, and the total dry weights were recorded.

Fermentation in 20-l carboys

For large-scale fermentation, the carboys, maintained at room temperature (22–25 °C), were aseptically inoculated with 100 ml starter inoculum of strain EN4-S and stirred by sparingly sparging (approx. 8 l h^{-1}) sterile filtered air for up to 4 days of fermentation to avoid complete anaerobiosis in the lower regions of the medium (Papavizas et al. 1984). This was followed (from day 4 to day 14) by increased sparging (approx. 250 l h^{-1}) and the one-time (on day 4) addition of 150 ml 1 NaOH. The carboys were aseptically sampled (approx. 25 ml) on days 0, 2, 4, 7, 10, and 14, chlamydospores and the numbers of viable cells (total) were estimated, and the pH was recorded as above. At the end of the run, the biomass (contents of the carboy) was concentrated by centrifugation at 5000 rpm and filtered using no. 4 Whatman filter-paper. The numbers of viable colony-forming units in the air-dried biomass on the filter-paper was estimated by plating serial dilutions on PDA. The fermentation was repeated twice with only strain EN4-S.

Statistical analyses

Statistical analyses used the repeated-measures analysis of variance with factorial treatment structure and interactions (SAS Institute, 1990). The mean separation was accomplished using Duncan's multiple-range test. All tests of significance were conducted at $P < 0.05$ or $P < 0.01$ using log transformed values (eg. $1 \times 10^6 = \log\ 6.0, 3 \times 10^6 = \log\ 6.5, 5 \times 10^6 = \log\ 6.7$). For both bench-top (with strains EN4-S, EN4-FT, CP3A) and 20-l carboy fermentation (with strain EN4-S), two replicate fermentor runs were used per treatment.

Results

Bench-top fermentation

In bench-top fermentation, the treatments (DO/pH) controlled by the sparging air and/or adding NaOH had a significant effect not only on microconidial, chlamydospore, and total viable counts, but also on the percentage of chlamydospores as a proportion of total counts; there was no significant effect on macroconidial count (Table 1). The fungal strain used did not affect microconidial, macroconidial, or total viable, but did affect chlamydospore numbers significantly. The strain × treatment interaction was significant for the microconidial and chlamydospore counts but did not affect the other variables. The sampling time × strain interaction was significant for the chlamydospore counts but did not affect the other variables. As expected, the sampling time and its interaction with the other parameters (treatment or treatment and strain) were significant for most of the variables studied. These interactions indicated that the various strains and treatments responded differently over time for several variables.

Table 1 *F* values from repeated-measures analysis of variance and interaction analysis for growth characteristics of *Fusarium oxysporum* strains in 2.5-l bench-top fermentors. *df* degrees of freedom, *NS* not significant

Parameters	*df*	Variables				
		Micro-conidial counts	Macro-conidial counts	Chlamydospore counts	Viable counts	Chlamydospores as a proportion of viable counts (%)
Strain	2	2.73 NS	3.63 NS	10.12**	0.754 NS	0.3 NS
Treatment	2	154.56**	1.74 NS	8.68**	50.47**	5.8**
Sampling time	7	66.33**	40.38**	999.67**	60.32**	14.68**
Strain × treatment	4	7.60**	0.95 NS	4.36**	1.98 NS	0.8 NS
Sampling time × strain	14	0.64 NS	1.13 NS	5.61**	0.80 NS	0.3 NS
Sampling time × treatment	14	2.65 **	0.83 NS	25.86**	4.35**	3.7**
Strain × treatment × time	28	1.67 *	1.68 *	5.93**	2.14**	0.7 NS

*$P \leq 0.05$, **$P \leq 0.01$

The two physical factors controlled in this study were dissolved oxygen (DO) concentration and the pH of the fermentor medium. The DO concentration in the fermentor medium under sparged (80%–100%) and non-sparged treatments (0–40%) were significantly different for the first 10 days of fermentation (Fig. 1a). However, after the active initial growth phase (4 days), the DO in the non-sparged treatments increased with or without addition of NaOH, and reached values similar to that of the sparged treatment. The pH increased in the medium from an initial value of approximately 5.5 to above 7.0, and the rate of increase was faster when the fermentor was sparged (treatment T1) than when it was not sparged (treatment T2) (Fig. 1b). The pH was adjusted to between 9.0 to 10.0 from the fourth day of fermentation in T3 treatment by the addition of 1 M NaOH.

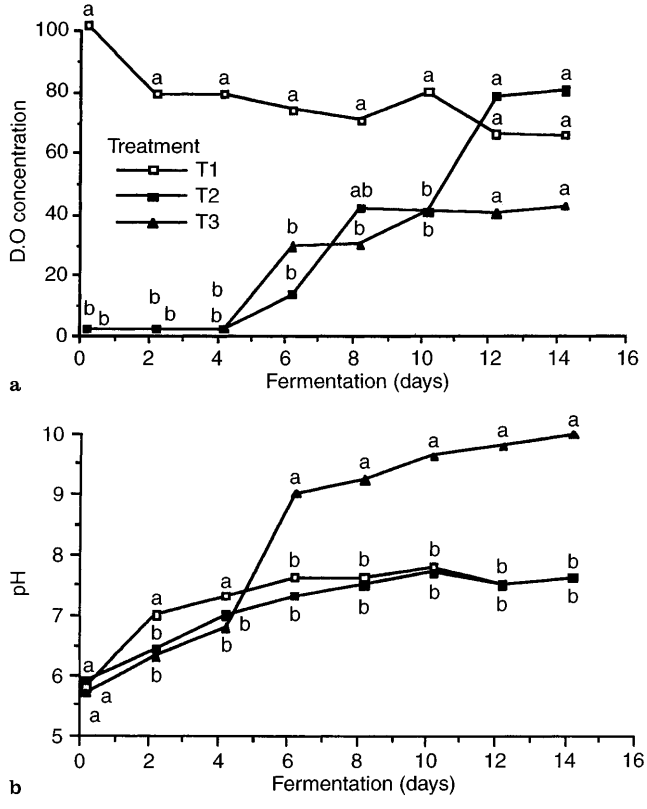

Fig. 1a, b Effect of fermentor treatments on (**a**) dissolved oxygen (*DO*) concentration, and (**b**) on pH of the fermentor medium. Treatments: *T1* sparged 0 day to 14 days; *T2* nonsparged 0 day to 14 days; *T3* nonsparged 0 day to 14 days, pH raised after 4 days and maintained between 9.0 to 10.0. Points on a given sampling day (means from two replicate fermentor runs) followed by the same letter are not significantly different from each other at the 5% level by Duncan's multiple-range test

To clarify the effects of the treatments on fungal strains used, the data from each of the three strains were also analyzed separately. After 14 days of fermentation, the major spore types produced in the medium were microconidia ($\log_{10} mi = 6.4$–7.6, where *mi* is the concentration of microconidia ml^{-1}), and chlamydospores ($\log_{10} c = 5.7$–6.6), but fewer macroconidia ($\log_{10} ma = 1.7$–4.5 where *ma* is the concentration of macroconidia ml^{-1}) were produced (Table 2). Microconidial counts were significantly ($P \leq 0.05$) higher by $\log_{10} mi = 0.5$ (5 fold) under higher DO (T1) than under lower DO, with (T3) or without (T2) pH adjustments, for all three strains. However, macroconidial counts were not affected by the treatments. The effect of DO on chlamydospore yields was strain-dependent. Chlamydospore counts were significantly higher (by 5 fold, $\log_{10} c = 0.5$ ml^{-1} medium) only in strains EN4-S and CP3A under lower DO without pH adjustments (T2) than under higher (T1) DO conditions. Viable counts were not significantly different for higher (T1) and lower DO (T2); however, pH adjustments (T3) significantly reduced viable counts, for all three strains.

Although the pH of the fermentor medium changed from an initial value of 5.5 to above 7.0 for all the three strains studied, higher numbers of chlamydospores could not be obtained by increasing (T3) the fermentor pH from 7.0 to between 9.0 and 10.0 (Table 2). However, the percentage of chlamydospores obtained as a proportion of the total viable, after a 14-day fermentor run, was higher for strain EN4-FT when the fermentor pH was artificially increased than when it was not. The percentage of chlamydospores obtained for treatments

Table 2 Growth characteristics of *F. oxysporum* strains under three different fermentor conditions. Treatments: *T1* sparged 0 day to 14 days; *T2* nonsparged 0 day to 14 days; *T3* nonsparged 0 day to 14 days, pH raised after 4 days and maintained between 9.0 and 10.0. Microconidial, macroconidial, chlamydospore counts determined using a hemocytometer, and viable counts assayed on potato dextrose agar (PDA) are means of two replicate fermentor runs after 14 days of growth. The percentage of chlamydospores was calculated as a proportion of the total viable counts determined on PDA after 14 days of growth. Values (*) in each column for each strain followed by the same superscript are not significantly different at 5% level by Duncan's multiple-range test

Strain treatment	log spores (counts ml^{-1})				Chlamydospores (%)
	Microconidia	Macroconidia	Chlamydospores	Viable count (PDA)	
EN4-S					
T1	7.4^{a*}	2.1^{a}	6.1^{b}	7.4^{a}	5.0^{b}
T2	6.8^{b}	1.7^{a}	6.6^{a}	7.0^{ab}	43.9^{a}
T3	6.7^{b}	3.5^{a}	6.0^{b}	6.6^{a}	30.3^{a}
EN4-FT					
T1	7.6^{a}	4.2^{a}	5.7^{a}	7.6^{a}	2.9^{b}
T2	7.1^{ab}	3.9^{a}	6.1^{a}	7.0^{ab}	14.0^{b}
T3	6.4^{b}	2.3^{a}	6.1^{a}	6.4^{b}	56.2^{a}
CP3A					
T1	7.4^{A}	4.5^{A}	5.9^{B}	7.2^{A}	5.5^{B}
T2	6.8^{B}	4.1^{A}	6.3^{A}	6.8^{AB}	37.4^{A}
T3	6.5^{B}	4.0^{A}	6.3^{A}	6.7^{B}	56.6^{A}

T1, T2, and T3 for strain EN4-FT was, 2.9%, 14%, and 56.2% respectively.

In general, studies on the kinetics of chlamydospore formation indicated that, although chlamydospore formation was faster (4–6 days) under higher DO (T1) than under lower DO (T2, T3), significantly ($P \leq 0.05$) higher numbers of chlamydospores could be obtained in the latter treatments after 10–14 days of incubation (Fig. 2a). The percentage of chlamydospores obtained as a proportion of viable showed that this percentage was also higher and was achieved earlier under low DO (T2 and T3) than under high DO (T1) (Fig. 2b). Although the percentage of chlamydospores obtained after pH treatment (T3), at the end of the 14 days of fermentation, was significant only for strain EN4-FT (Table 2), mean values from all three strains showed that the overall effect was significant (Fig. 2b). These differences were significant after 10 days of fermentation with the nonsparged pH-adjusted treatment T3 showing maximum production (approx. 60%) by day 12. The differences in final yield (dry weight l^{-1}) of fungal biomass due to the various treatments were not significant (data not presented).

Fermentation in 20-l carboys

When fermentation was scaled-up using 20-l carboys, viable and microconidial counts of strain EN4-S reached their peak by day 7 and remained constant at $\log_{10} mi = 7.4$ ml^{-1} medium at least until day 14 of incubation (Table 3). However, macroconidia ($\log_{10} ma = 3.7$) or chlamydospores ($\log_{10} c = 6.7$) were formed only after 10 days of fermentation, and not before the sparging had been increased and the fermentor pH artificially raised (day 7) by a one-time addition of 150 ml 1 M NaOH. There was a slow increase in the pH of the fermentor medium from an initial value of pH 5.0 at day

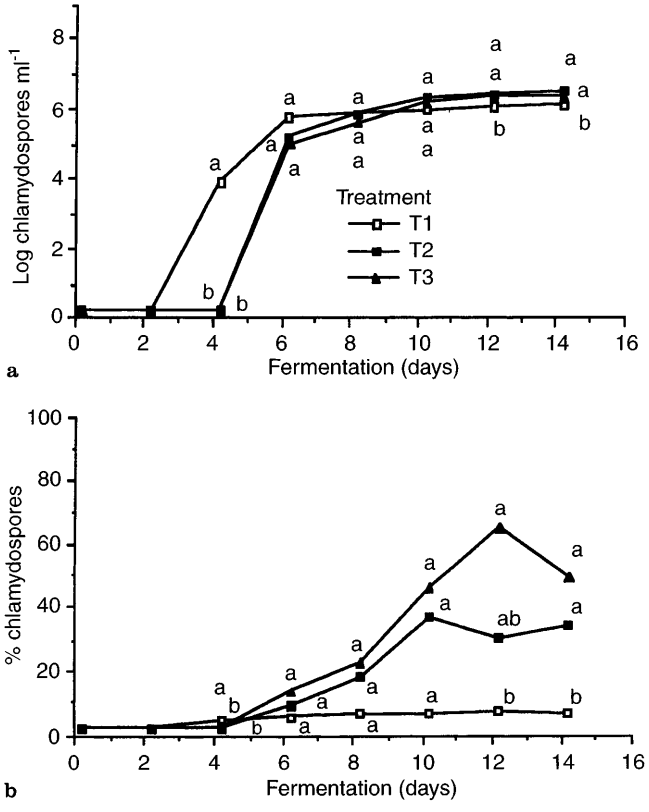

Fig. 2a, b Kinetics of chlamydospore formation in bench-top fermentors as affected by various treatments. **a** Chlamydospore numbers [$\log_{10} c$(counts ml^{-1})] versus fermentation days as affected by three different treatments. **b** Percentage (%) chlamydospores as a proportion of viable counts plotted against fermentation time as affected by three different treatments. Treatments: *T1* high DO, sparged 0 day to 14 days; *T2* low DO nonsparged 0 day to 14 days; *T3* low DO with pH increases, nonsparged 0 day to 14 days, pH raised after 4 days and maintained between 9.0 and 10.0. Points on a given sampling day (which are means of chlamydospore counts from two replicate fermentor runs) followed by the same letter are not significantly different from each other at the 5% level by Duncan's multiple-range test. Chlamydospore counts were determined with a hemocytometer and viable counts were determined on PDA

Table 3 Growth characteristics of *F. oxysporum* strain EN4-S in 20-1 carboys with 1% soya bean hull fibre growth medium. Hemocytometer counts (microconidia, macroconidia, and chlamydospores), viable counts (on PDA), and pH are mean values (±standard error) from two replicate carboys. Growth was at room temperature. *ND* not determined. Zero values represent numbers below detectable levels.

Growth (days)	Hemocytometer counts: ($\log_{10}$ spores $ml^{-1} \pm SE$)			Viable count ($\log_{10} v$ ml^{-1})	pH of medium
	Microconidia	Macroconidia	Chlamydospores		
0	ND	ND	ND	4.6 ± 0.09	5.3 ± 0.07
2	5.8 ± 0.03	0	0	5.9 ± 0.01	6.0 ± 0.07
4	7.2 ± 0.14	0	0	7.2 ± 0.20	6.0 ± 0.20
7*	7.4 ± 0.03	0	0	7.4 ± 0.04	6.0 ± 0.20
10	7.4 ± 0.03	3.7 ± 0.00	6.7 ± 0.15	7.3 ± 0.21	7.7 ± 0.14
14	7.6 ± 0.10	3.9 ± 0.27	6.8 ± 0.15	7.3 ± 0.10	7.7 ± 0.21

*Air flow was increased and 150 ml of 1 M NaOH was added after 7 days of growth.

0 to pH 6.0, and this temporarily increased to pH 9.9 when NaOH was added. Because the pH was not kept constant, it had decreased to 7.7 by day 10. Chlamydospores as a proportion of viable counts amounted to 32% after 14 days of fermentation. In fermentor runs where either the pH was not raised or the sparging was not increased, chlamydospores were not observed by day 10 and $\log_{10} c = 5.5$ was obtained by day 14 (data not presented). Viable counts from filtered and dried biomass was approximately $\log_{10} v = 8.0$ g^{-1}. Similar numbers were obtained for the other two mycoherbicidal strains (EN4-FT, CP3A) tested (data not presented).

Discussion

The present study, in contrast to previous reports, shows that substantially higher [$\log_{10} c$(counts ml^{-1}) = 6.6] chlamydospore numbers can be obtained with mycoherbicidal strains of *F. oxysporum* within 2 weeks in a one-step liquid fermentation in a soya bean hull fiber medium. This method is clearly more advantageous than the two-step semi-solid fermentation used previously (Hildebrand and McCain 1978). This latter approach required a total of 5 weeks of incubation to obtain chlamydospores. However, it is necessary to optimize fermentation conditions for individual strains in view of the effect of the treatments (DO and pH), fungal strains, sampling time and their interactions on chlamydospore production.

The present study also demonstrated the importance of physical factors such as DO and pH of the liquid medium on the growth characteristics of *F. oxysporum*. Fewer chlamydospores were formed under higher DO concentration (treatment T1) than under lower (treatment T2) DO. The opposite was true for microconidial formation. This may be partly explained by the production of more mycelial filaments without the formation of conidia under the less-agitated microaerophilic conditions. This situation eventually results in the formation of more chlamydospores, which are entirely of mycelial origin. Nash and Huber (1971), while investigating antibiotic synthesis by *Cephalosporium acremonium*, showed that medium agitation has an effect on fungal morphology.

Although final numbers were lower, chlamydospore formation was faster under sparged than under non-sparged conditions. As CO_2 levels were not monitored, we can only speculate that the slower rate of chlamydospore formation observed in the absence of aeration was probably due to the inhibitory effect of CO_2 in the medium. The formation of chlamydospores in the non-sparged treatments also corresponded with increases (above 20%) in the DO of the medium, which eventually reached levels similar to that of the sparged treatment. This increase was probably due to the lower utilization of the dissolved air introduced into the surface of the fermentor medium. In other studies too, CO_2 was shown to be inhibitory to chlamydospore formation in *F. oxysporum* (Newcombe 1960) and *F. solani* (Bourett et al. 1965).

Previous growth kinetic studies in soya bean hull fiber medium with mycoherbicidal strains of *F. oxysporum* have shown that exposure for at least 48 h at pH 7.0 predisposed the fungi to form chlamydospores, and that chlamydospores were not formed in an acidic potato dextrose broth culture (Hebbar et al. 1996). The same study also showed that chlamydospore formation also coincided with an increase in the pH of the soya bean hull fiber medium from 5.5 to above 7.0. In the present study, it was shown that, although raising the fermentor pH beyond pH 7.0 to 9.0–10.0 by adding NaOH (T3) did not increase the total numbers of chlamydospores, it did significantly increase, after 12 days of fermentation (Fig. 2), the percentage of chlamydospores formed as a proportion of the total viable. In view of the reduction in viable propagules in treatments with NaOH, the percentage increase in chlamydospores formed as a proportion of the total viable fungus seems to be an indirect effect. In addition, as a slight reduction in the percentage of chlamydospores was recorded at the end of the fermentor run, the optimum incubation period required under high pH conditions will have to be standardized.

Although substantial numbers [$\log_{10} c$(counts ml^{-1}) > 5.0] of chlamydospores were formed by all the

strains under the three conditions tested, the ideal conditions were non-sparged low DO, with (T3) or without (T2) pH adjustments. In addition, peak values could be obtained faster under these conditions, thereby reducing the fermentation period by at least 2 days, which could be beneficial in a commercial production system. According to Ryu and Oldshue (1977) aeration and agitation, which are interdependent, accounted for a significant part of the operating cost in microbial fermentation.

Although high chlamydospore counts and viability, obtained from dried fungal biomass with a simple, easy-to-operate 20-l capacity carboy, indicates the potential for scaling-up the fermentation, more work needs to be done to optimize conditions for large-scale fermentation. When the three conditions similar to those of the bench-top fermentation were applied to the 20-l carboy (unpublished data), better chlamydospore yields were obtained by sparging sparingly, sterile filtered air up to day 4 of fermentation, and then increasing sparging with the one-time (on day 4) addition of 150 ml 1 M NaOH.

In conclusion, the methodology standardized in this work enabled reduction in the time required for the production of chlamydospores, from 5 weeks with the existing technology to 2 weeks in a single-step liquid fermentation procedure applicable to all three strains tested. Preliminary data suggest (unpublished) that the fermentation method used in this study is also applicable for obtaining chlamydospores from saprophytic strains of *F. oxysporum* useful for the biocontrol of soil-borne plant pathogens. The present study indicates that a liquid fermentation method is now available to obtain chlamydospores from mycoherbicidal strains and potentially other beneficial biocontrol strains of *F. oxysporum*. Attempts to scale-up fermentation and develop methods to formulate the chlamydospores are in progress.

Acknowledgements The authors thank Robert Larkin for his assistance in statistical analysis of the data and FAS/ICD-USDA for providing the visiting fellowship for the first author. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply approval to the exclusion of other products or vendors that may also be suitable.

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