Journal of Industrial Microbiology & Biotechnology (1997) 19, 163–168
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Large-scale production of chlamydospores of *Gliocladium virens* strain GL-21 in submerged culture

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SoilGard™ is a commercial microbial fungicide containing chlamydospores of *Gliocladium virens* strain GL-21. The formulation is registrated with the US Environmental Protection Agency (EPA) by Thermo Trilogy Corporation, Columbia, Maryland. The biocontrol agent was developed in cooperation with the Biocontrol of Plant Diseases Laboratory (BPDL), US Department of Agriculture (USDA), and is targeted for controlling damping-off diseases caused by *Rhizoctonia solani* and *Pythium ultimum* in vegetable and ornamental greenhouses. Formulation requires production of chlamydospore biomass in liquid fermentation which was carried out in 20-L, 1500-L and 4000-L submerged liquid fermentors. Key economic process parameters, fermentation kinetics and the reproducibility of the fermentation at this scale were evaluated and analyzed. Starter culture quality and maturity of chlamydospores were identified as key issues for obtaining successful fermentations.

Keywords: biological control; antagonist; kinetics

Introduction

The fungus *Gliocladium virens* Miller, Giddens & Foster (= *Trichoderma virens* Miller, Giddens, Foster & von Ark) [9] has been commercially developed as SoilGard™ by Thermo Triology Corp (formerly Grace Biopesticides), Columbia, MD, USA. A formulation of this fungus (strain GL-21) has been marketed in several states and is intended for use as a pre-plant amendment to soilless mixes to prevent damping-off diseases caused primarily by the soil-borne plant pathogens, *Pythium ultimum* Trow and *Rhizoctonia solani* Kuhn [5,7].

Quality biomass of this and other fungal biocontrol agents must be produced gnotobiotically in large quantities, with timely and economic methods, and with certain characteristics that assume maximum biological control effectiveness [3]. Submerged liquid fermentation is a preferred system for producing biomass of *G. virens* [8], which promotes production of chlamydospores of the fungus almost exclusively [6].

Several factors have been identified as influencing the characteristics of fungal biocontrol agents. The nutritional environment provided by the culture medium is important [1,2,10], especially the carbon nitrogen ratio (C/N) as it affects the ability of the mycoherbicidal fungus, *Colletotrichum truncatum* to infect its weed host *Sesbania exultata*. Also, specific factors have been examined to define optimum conditions for production of conidia of the antagonist *Trichoderma harzianum* [3]. These specific conditions included fermentation medium, stir rate, pH, and age of the fermentation.

The objective of this study was to determine key economic process parameters, fermentation kinetics, and reproducibility of the fermentation for *G. virens* as it related directly to the morphology of the fungus when examined by electron microscopy.

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Received 7 February 1997; accepted 27 May 1997

Materials and methods

Microorganism

Strain GL-21 of *Gliocladium virens* was used in all fermentation experiments. This strain was isolated from a sclerotium of *Sclerotinia minor* [4,6].

Inoculum and seed fermentation

During the fermentation studies, two culture sizes were used for seeding the fermentors; 0.2% (2-L shake flask) and 2% (20-L fermentor, Chemap AG, Mannedorf, Switzerland).

Shake flasks: Yeast extract broth (30 g L^{-1}) with the addition of anti-foam (P-2000 0.8 g L^{-1}) was adjusted to pH 7.0 with 6 M NaOH. Baffled 500-ml shake flasks containing 200 ml of broth were started with one cryovial containing stock culture made of conidia. Culture flasks were incubated at 26°C and 200 rpm. The flasks were incubated for 40–48 h.

Seed fermentor: A 20-L fermentor was used for seed preparation. Proprietary medium was used for the seed fermentor. The fermentor was inoculated with a 200-ml seed shake flask prepared as described above.

Fermentation conditions

The fermentation studies were carried out in 20-L, 1500-L and 4000-L (Chemap AG, Mannedorf, Switzerland) stirred tank fermentors mounted with 4–90° baffles and a 3-disk stirrer impeller system with blade width to stirrer diameter of 1/5. The working volumes of the fermentors were 14 L, 1000 L and 3200 L. Complex proprietary production fer-

mentation medium containing glucose as a carbon source was used. Glucose was added in a semi-fed batch mode, with 30 g L^{-1} added initially, followed by an additional 30 g L^{-1} toward the middle and end of the exponential growth of cells.

Temperature was controlled at 26°C in all fermentations, and pH was controlled at 6.0 by the automatic injection of anhydrous ammonia gas directly into the inlet air stream (before the air filter) or by the automatic addition of 6 M H_2SO_4. The air flow was kept constant at 1.0 vvm. Dissolved oxygen was measured using polarographic Ingold probes. Agitation speed (150–300 rpm) was slightly adjusted to maintain levels of dissolved oxygen above 10% of air saturation, especially during the beginning of the exponential phase. The fermentation was constantly monitored by taking biomass samples every 8 h. For each sample, the following analytical tests were performed: sterility, pH conformation, glucose determination, dry cell weight, spore count, spore viability and colony-forming units (CFU) ml^{-1} determination.

Cell mass determination (dry weight)

A weigh pan and filter were dried and tarred. Whole culture biomass was filtered (0.45-μm Nucleopore filter), and then washed with distilled water. The filter was then transferred to the weighing pan. The pan, the filter and the washed cells were then dried to constant weight and weighed on an analytical balance.

Glucose determination

A glucose analyzer (model 23, Yellow Springs Instruments, Yellow Springs, OH, USA) was used to determine glucose concentration. Fermentation samples were diluted appropriately with deionized water. A 1-μl diluted sample was then injected directly into the instrument which displayed the glucose measurement directly in mg dl^{-1}. The instrument was calibrated frequently with a standard glucose solution.

Spore counts

Biomass (1 ml of medium) was aseptically diluted until it reached a countable level of 10^3–10^5 chlamydospores. Spore counts were performed by using a hemacytometer.

CFU determination

Whole culture biomass (0.1 ml) was diluted in duplicate (10^4–10^6 dilutions) and plated on Tryptic Soy Agar plates (40 g L^{-1} Bacto Tryptic agar). The diluted sample was incubated at 25°C for 3 days and the CFU ml^{-1} of the sample were determined.

Spore viability

Fermentation biomass was serially diluted aseptically in 0.05% sterile Tween 80, until 10^5 chlamydospores were obtained. One hundred microliters of sterile Sabouraud Dextrose broth were pipetted into two wells of a sterile 96-well plate. One hundred microliters of the spore suspension were added to one column and the remaining column was used as a contamination control. The plate was incubated for 24 h at 25°C and evaluated using an inverted Zeiss microscope at 32× for the presence of a germ tube. All spores seen in one microscope field for each well were counted and the number of germinated and non-germinated spores were recorded. The percentage of viable spores was determined by dividing the number of germinated spores by the total number of spores counted.

Transmission electron microscope (TEM) analysis of chlamydospores

Fermentation biomass at the beginning of the stationary phase was centrifuged and the pellet obtained was washed twice with sterile 0.05% Tween 80 solution. The washed biomass pellet was fixed in osmium tetroxide, stained with uranyl acetate, dehydrated in an ethanol series, and infiltrated and embedded in Spurr's plastic resin. The embedded resin was ultra-thin sectioned and the sections were collected on grids. The grids were post-stained with lead citrate and extensively examined in a transmission electron microscope.

Viscosity determination

The rheological properties of *Gliocladium virens* fermentations were determined by using a Brookfield Spindler viscometer Spindle No. 4 (Brookfield, USA) at the rates of 30 and 60 rpm.

Results and discussion

Fermentation kinetics and biomass formation

Biomass measurements were made in a 500-L fermentor using 0.2% inoculum size for seven fermentation runs in a row. All fermentations were started with identical starter culture concentrations and were run under the same working conditions. After a lag phase of 24 h, the culture entered the exponential phase of growth which lasted for a further 70 h (Figure 1). During the exponential phase, the morphology of the biomass changed from mycelia to immature chlamydospores. The pH began to drop during this period as the fungus grew. Around 40–50 h into the fermentation the heaviest glucose uptake occurred (2.0–2.5 g L^{-1} h^{-1})

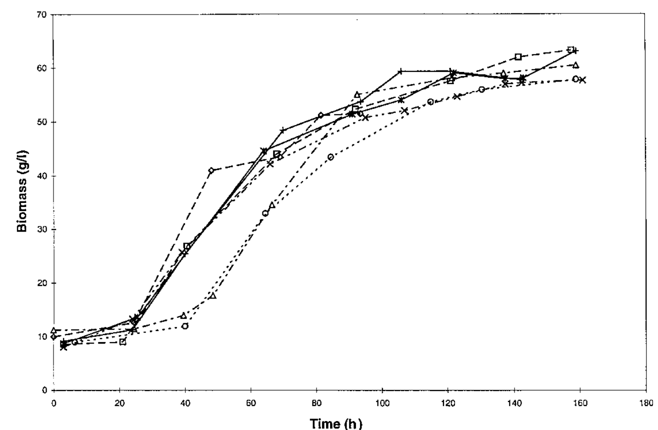

Figure 1 Biomass concentration (expressed as grams dry weight per liter) of *Gliocladium virens* GL-21 in seven 1500-L submerged fermentation runs. Each point represents the means of three replications for dry weight determination. All fermentations were harvested at 160 h. --◇-- 310; --○-- 312; --△-- 313; --×-- 314; --*-- 315; --□-- 316; —+— 317.

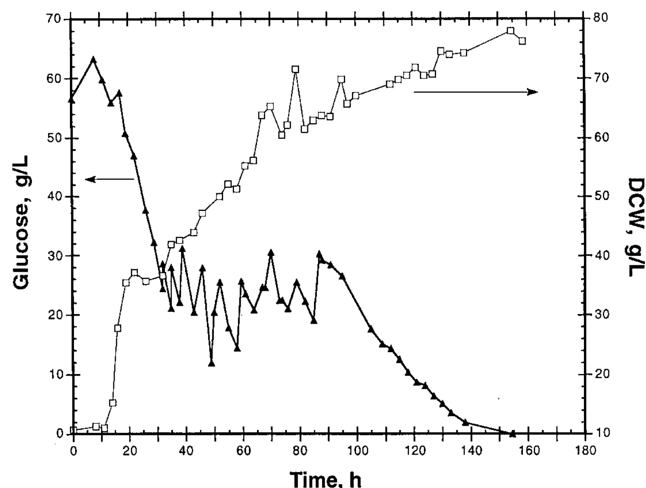

Figure 2 Grams per liter glucose uptake (-▲-) and gram dry cell weight (DCW) (-□-) in 1500-L fermentor using 2.0% starter culture of *Gliocladium virens*.

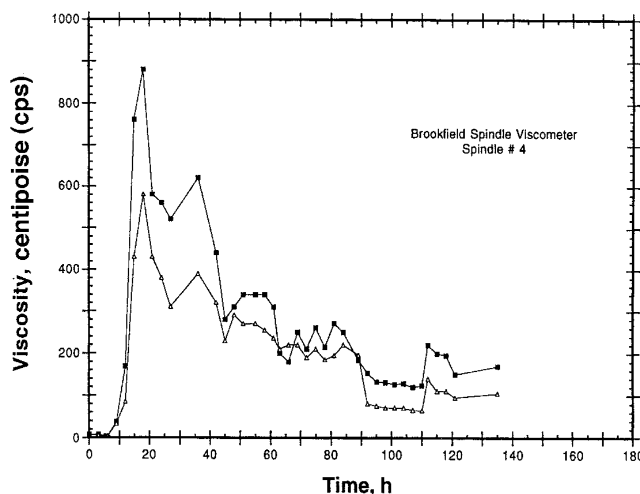

Figure 4 Viscosity of fermentation during the cultivation of *Gliocladium virens* in 1500-L fermentation. -■- 30 rpm; -△- 60 rpm.

(Figure 2). During this period, chlamydospores were beginning to form at the ends of the hyphae. The maximum specific growth rate for this phase was evaluated at 0.047 h^{-1} (Figure 3). During the late exponential phase, dissolved oxygen rose and it was maintained above 10% of air saturation in all runs (Figure 3). However, at the beginning of the exponential stage, between 20 h and 45 h, the fermentation was at the oxygen limitation regime, as can be seen in Figure 3, and the viscosity of the fermentation broth was at a peak (Figure 4). Once the sporulation had started at around 60 h the dissolved oxygen rose (Figure 2). At 75–96 h, the pH began to drift upwards. When the fermentation reached 96 h, sporulation became heavy but there was still a fair amount of mycelium present and the chlamydospores were still immature. Only during the stationary phase (120–160 h) were chlamydospores beginning to mature and the glucose uptake rate began to drop off. During the first 20–40 h the viscosity of the fermentation biomass increased (Figure 4). After mycelium buildup was finished, the viscosity dropped until the remaining mycelium completely lysed. At around 120–140 h of fermentation the chlamydospores were mature and ready to be harvested and formulated (Figure 5). Viscosity at this stage was at its lowest point (Figure 4). The above fermentation kinetics description was almost identical for all seven runs in the 1500-L fermentors as well as the four runs in the 4000-L fermentor.

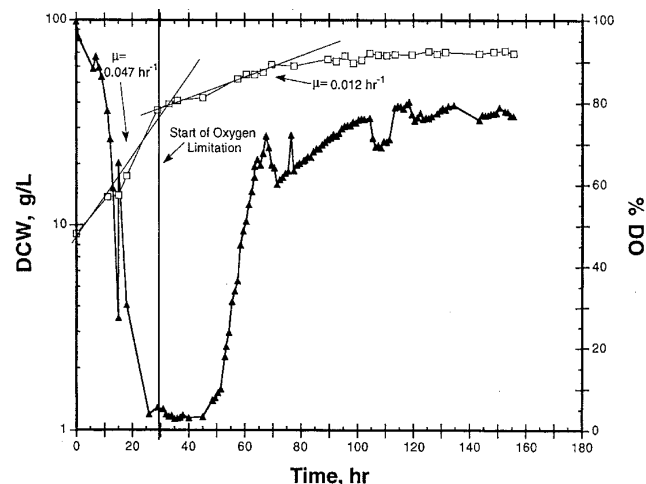

Figure 3 Oxygen limitation (% saturation) and gram dry cell weight (DCW) per liter in 1500-L fermentor using 2.0% starter culture of *Gliocladium virens*. -□- DCW; -▲- %DO.

Harvesting time

Typical Transmission Electron Microscope (TEM) micrographs showed different morphological variability of different types of chlamydospores of *G. virens* taken from the fermentor at 92 h of cultivation (Figure 5). From TEM studies, it was concluded that mature chlamydospores could only be achieved during the stationary stage when mycelium lysed completely and defined walls formed around each chlamydospore.

In order to determine at which stage of maturity of chlamydospores the fermentation should be harvested, viability of the spores was measured and determined as a function of the shelf-life of the final formulated product. Chlamydospores from different stages of the fermentation were used to prepare final formulations and the stability of this preparation was measured as a function of temperature and storage time. Initial CFU mg^{-1} of the formulated chlamydospores were determined and after 300 days of storage at 4°C and 25°C the preparations were evaluated for their viability in terms of CFU g^{-1} dry weight. As can be seen from Table 1, mature spores at the end of the stationary phase retained their viability (expressed in CFU g^{-1}) much better than mycelium or immature chlamydospores harvested during the exponential phase or at the beginning of the stationary phase.

Effect of starter culture

The effect of starter culture size on the fermentation yield (expressed in g dry cell weight ml^{-1}) was tested at 0.2–2%. The higher rate of 2% resulted in obtaining the higher biomass yield. The 2% culture rate resulted in faster dry cell rate of accumulation (Figure 6). However, there was no increase in the number of chlamydospores at the begin-

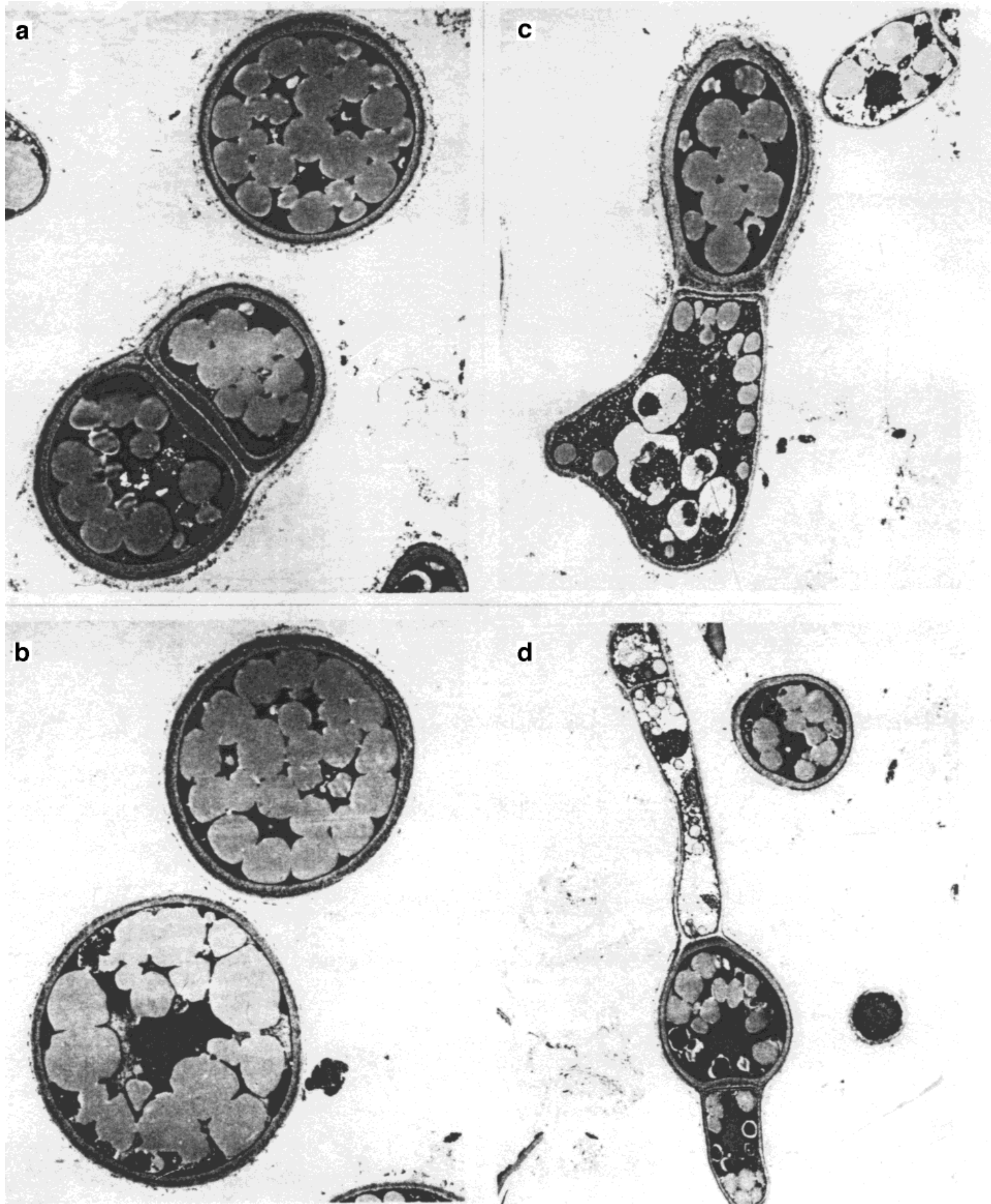

Figure 5 Transmission electron micrographs of chlamydospores of *Gliocladium virens* (strain GL-21) demonstrating morphological variability. Figures 5a and 5b represent mature chlamydospores. Figures 5c and 5d represent immature chlamydospores.

ning of the stationary phase (data not shown). The effect of inoculum size on percent saturation of dissolved oxygen is shown in Figure 7. Sporulation started earlier at the inoculum size of 2%, around 50 h, compared to 60 h with the 0.2% inoculum size (data not shown). These results indicate that the main impact of using low inoculum on the fermentation was a lengthening of the fermentation cycle.

A more important issue than inoculum size was the quality of the seed inoculum. From earlier studies conducted in small fermentors (14 L), we learned that inoculum inconsistency and culture instability play a key role in producing quality fermentation biomass. Using a strict preservation protocol for preparing standardized inoculum ensures that the starter seed culture for all fermentations was identical

Table 1 The effect of harvest time on chlamydospore viability of *Gliocladium virens*

Time of harvest from fermentor (h)	CFU ml^{-1}	% Viability after 300 days storage at:	
		4°C	25°C
43	0	0	0
64	2.5×10^6	18.6	12
87	5.2×10^7	41	16.8
90.5	6.1×10^7	58.8	28.6
113	1.0×10^8	78.4	62
120	1.2×10^8	86.8	73
135	1.2×10^8	98	82
160	1.25×10^8	98	88

The viability was assessed by formulating chlamydospores from different stages of the fermentation and analyzing each sample for shelf-life stability at 4°C and 25°C. Viability was determined by calculating colony-forming units (CFU) g^{-1} at day 0 and at day 300, and expressing the drop in CFU g^{-1} per time of harvest.

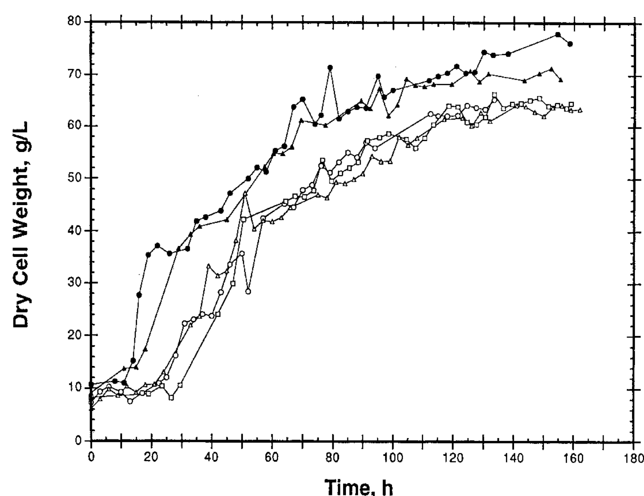

Figure 6 The effect of starter culture (0.2% and 2.0%) on dry cell weight of *Gliocladium virens*. -○- F319, 0.2%; -□- F320, 0.2%; -△- F321, 0.2%; -●- F322, 2.0%; -▲- F323, 2.0%.

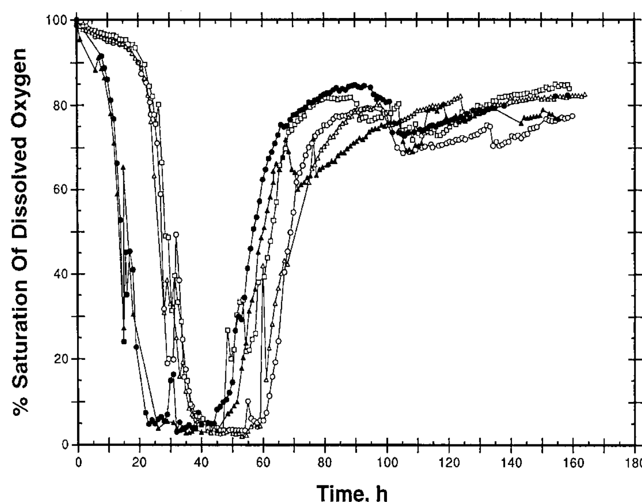

Figure 7 The effect of *Gliocladium virens* starter culture (0.2% and 2.0%) on dissolved oxygen consumption during fermentation. -○- F319, 0.2%; -□- F320, 0.2%; -△- F321, 0.2%; -●- F322, 2.0%; -▲- F323, 2.0%.

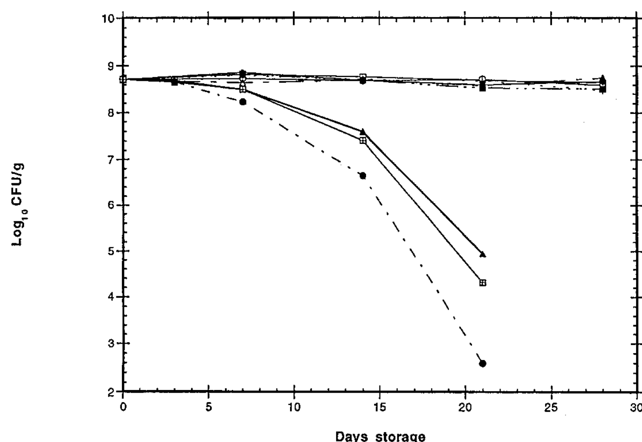

Figure 8 The effect of temperature and pH during storage on the stability of fermentation biomass after harvesting. -▲- pH 5.5, 23°C; -□- pH 5.5, 10°C; -○- pH 5.5, 4°C; -⊞- pH 5.0, 23°C; -+- pH 5.0, 10°C; -△- pH 5.0, 4°C; --●-- pH 4.5, 23°C; --■-- pH 4.5, 10°C; -◆- pH 4.5, 4°C.

in concentration and history (unpublished data, J Eyal). All fermentations which were carried out consecutively in the 1500- and 4000-L fermentors resulted in full sporulation and high quality mature chlamydospores.

Storage stability of biomass

An important parameter which can influence the economics of preparing a formulation was the stability of the chlamydospore biomass after harvesting, therefore the effect of temperature and pH on the stability of the chlamydospores after harvesting and concentration was examined (Figure 8). Storage at 23°C influenced the stability of chlamydospores. However, storage of biomass at a temperature range of 4°C to 10°C at a pH range of 5.5–4.5 did not affect the viability of the spores stored for as long as 30 days. Storing the chlamydospores at low pH and low temperature also limited bacterial contamination and growth during formulation.

Conclusion

Reproducible results are essential for process development parameters on fermentation performance and for success during commercialization. For GL-21 fermentations, two key issues may influence the reproducibility and quality of the production of chlamydospores of *G. virens* in submerged fermentation. The first key issue is the stability and consistency of the seed culture. Inconsistency in inoculum may impact the quality of the chlamydospores, delay sporulation and formation of chlamydospores in the fermentor and impact the production plant performance and economics during commercialization (unpublished data, J Eyal). Implementing strict preservation protocols for storage, preparation and maintenance of standardized inoculum, ensured that the starter GL-21 seed culture for all fermentations was identical in concentration and history, which resulted in reproducible fermentations. The second key issue is the determination of the correct harvest time for each biomass fermentation. Harvesting the biomass before

chlamydospores have completely matured may result in degradation of the biomass during the formulation process and will limit the shelf-life of the product.

Understanding the *G. virens* fermentation cycle and the process of formation of chlamydospores during the fermentation is critical in determining when mature spores in a high yield will be produced and when the fermentation should be harvested with resulting high quality chlamydospores. Our results clearly demonstrate that only mature chlamydospores which are formed between 120–160 h are capable of providing a product with a long shelf-life.

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