J Ind Microbiol Biotechnol (2006) 33:941–952
DOI 10.1007/s10295-006-0164-6

ORIGINAL PAPER

Dissolved oxygen as principal parameter for conidia production of biocontrol fungi *Trichoderma viride* in non-Newtonian wastewater

M. Verma · Satinder K. Brar · R. D. Tyagi · R. Y. Surampalli · J. R. Valéro

Received: 17 January 2006 / Accepted: 23 July 2006 / Published online: 15 August 2006

Abstract Dissolved oxygen (DO) concentration was selected as a principal parameter for translating results of shake flask fermentation of *Trichoderma viride* (biocontrol fungi) to a fermenter scale. All fermentations were carried out in a 7.5 l automated fermenter with a working volume of 4 l. Fermentation performance parameters such as volumetric oxygen transfer coefficient (k_La), oxygen uptake rate (OUR), rheology, conidia concentration, glucose consumption, soluble chemical oxygen demand, entomotoxicity and inhibition index were measured. The conidia concentration, entomotoxicity and inhibition index were either stable or improved at lower DO concentration (30%). Variation of OUR aided in assessing the oxygen supply capacity of the fermenter and biomass growth. Meanwhile, rheological profiles demonstrated the variability of wastewater during fermentation due to mycelial growth and conidiation. In order to estimate power consumption, the agitation and the aeration requirements were quantified in terms of area under the curves, agitation vs. time (rpm h), and aeration vs. time (lpm h). This simple and novel strategy of fermenter operation proved to be highly successful which can be adopted to other biocontrol fungi.

Keywords Biocontrol agents · Conidia · Dissolved oxygen · Rheology · Starch industry wastewater · *Trichoderma viride*

Nomenclature

τ	shear stress (mPa)
τ_0	yield stress (shear stress at 0 rpm of spindle, mPa)
γ	shear rate (s^{-1})
K	consistency index (mPa s^n)
n	flow behaviour index (dimensionless)
μ_P	plastic viscosity (mPa s)

Introduction

The biocontrol agents (BCAs) market has been growing continuously over the last few decades due to the adverse environmental impacts of chemical pesticides [11]. Additional factors such as production cost, resistance development in pests, and stricter government policy have also inhibited the utilization of chemical pesticides. In particular, fungal BCAs share considerable market due to their broad spectrum of biological activity and environmental safety [29]. Amongst fungal BCAs, conidial formulations of many *Trichoderma* spp. have been shown to be effective in killing and/or preventing growth of several plant pathogens, namely *Rhizoctonia*, *Pythium*, *Fusarium*

M. Verma · S. K. Brar · R. D. Tyagi (✉) · J. R. Valéro
INRS-ETE, Université du Québec,
490, de la Couronne, Quebéc, Canada G1K 9A9
e-mail: tyagi@ete.inrs.ca

R. Y. Surampalli
US EPA, P.O. Box-17-2141, Kansas City,
KS 66117, USA

J. R. Valéro
NRCan Canadian Forestry Services,
Laurentian Forestery Centre des 1055 du P.E.P.S,
P.O. Box 3800, Québec, Canada G1V 4C7

and *Cylindrocladium* [7, 10]. In addition to the production of antimicrobial compounds, conidia of *Trichoderma* fungi are preferred over mycelia and chlamydospores due to their high rate of production [18]. However, a significant amount of conidia are lost during the formulation step and soil application due to air-drying and mechanical stress during handling. Thus, higher *Trichoderma viride* conidia concentrations would be required at the end of the submerged fermentation process in the formulated product for field application [15]. The biological control properties of *Trichoderma* spp. can be further enhanced by applying them in combination with existing novel crop protection strategies [28]. Moreover, production cost has been a major obstacle to the success of many biopesticides [15], including *Trichoderma* spp. This stimulated the exploration of cheap and waste-based raw materials, e.g. wastewater sludge [31] and food-processing waste [35], to minimize the overall production cost. However, none of the studies using waste as a substrate were carried out in fermenter, a pre-requisite for mass-scale production.

Although conidiation in submerged fermentation is a difficult phenomenon [15, 26], it offers several advantages over solid state fermentation. *Trichoderma* spp. fermentation is an aerobic process and oxygen mass transfer therefore is a prominent obstacle. Moreover, conidiation and production of antimicrobial compounds are highly affected by oxygen transfer (aeration and agitation). Furthermore, the oxygen transfer in submerged fermentation is also largely affected by viscosity and morphology (mycelia and pellet formation) [5, 25]. These parameters create heterogeneity in the medium, resulting in compartmentalization of the fermentation broth in terms of dissolved oxygen concentration, pH, and substrate availability [14, 36]. Therefore, scaling up is a function of rheological parameters that needs to be studied explicitly. Further, more complex rheology of a fermentation medium necessitates study on bioreactors for the optimization of critical parameters such as agitation and aeration (oxygen transfer), which have a direct impact on operating costs [14, 26]. It has also been reported that high shear on fungal cells resulted in sporulation and cell rupture, which hampered the conidia production rate [12]. Therefore, a critical analysis of operating parameters would be a vital step in economizing the mass production of *Trichoderma* sp.-based BCAs.

This study was conducted for process development of *Trichoderma viride* conidia and antagonist metabolites production by using starch industry wastewater (SIW) as raw material. The optima of aeration and agitation intensities and incubation time were determined, so as to decrease the final cost of these BCAs. Bioassay of fermented broth was also conducted on larvae of a forest pest, spruce budworm (*Choristonuera fumiferana*), and on a fungal phytopathogen, *Cylindrocladium floridanum*, to assess the biocontrol efficacy of the fermentation process.

Materials and methods

Chemicals

The analytical grade chemicals were purchased from Sigma-Aldrich or BDH (Toronto, Canada). Microbiological media and fermentation-related chemicals (e.g., anti-foam, acid and base) were of commercial grade.

Raw material (starch industry wastewater)

Starch industry wastewater (SIW) used in this study was obtained from a local starch industry (ADM-Ogilvie, Candiac, Quebec, Canada). Table 1 represents the physio-chemical characteristics of SIW, which showed metal concentrations in accordance with Québec guidelines for agricultural application [21]. The SIW was stored for a maximum of 3 weeks at 4 ± 1°C to minimize microbial degradation.

Table 1 Characteristics of starch industry wastewater

Parameter	Concentration ± σ[a] (mg/kg, unless stated otherwise)
Total solids (g/l)	16.7 ± 0.872
Total volatile solids (g/l)	13.1 ± 0.601
Suspended solids (g/l)	2.1 ± 0.060
Suspended volatile solids (g/l)	1.9 ± 0.104
pH	4.0 ± 0.01
Total carbon	401012.0 ± 25161
Total nitrogen	59187.6 ± 2753
Total phosphorus	12014.7 ± 808
N-NH_3	578.9 ± 47
N-NO_2^- , N-NO_3^-	42.8 ± 2.7
P-PO_4^{3-}	782.6 ± 56
Al	195.8 ± 8.8
Ca	26063.7 ± 1768
Cd	0.9 ± 0.06
Cr	9.6 ± 0.64
Cu	100.5 ± 6.56
Ni	12.6 ± 1.06
Fe	5623.3 ± 306
K	1785.3 ± 141.7
Pb	15.3 ± 0.883
Mn	54.2 ± 2.8
S	684.2 ± 51.5
Zn	612.1 ± 35
Na	6809.6 ± 50

[a] σ Standard deviation

Starter culture, pathogens and inoculum

The *Trichoderma viride* fungus was a commercial strain [31] isolated from soil. It was found to be active against phytopathogenic fungi, *Fusarium* sp. and *Cylindrocaldium floridanum* and spruce budworm (SB) insect larvae, as verified in our laboratory. For fungal pathogen testing, *Cylindrocladium floridanum*, pathogenic to spruce trees was obtained from the Laurentian Forestry Centre (LFC, Quebec, Canada). The insecticidal bioassay was carried out using third instar larvae of eastern spruce budworm (SB) (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) provided by Natural Resources Canada (Sault Ste. Marie, Ontario).

The *Trichoderma viride* strain was grown on potato dextrose agar (PDA) plates for 4–7 days, under dark conditions at 28 ± 1°C and 35 ± 2% relative humidity. It was subsequently maintained at 4 ± 1°C and subcultured monthly. For starter culture, $a \approx$ ½ in. × ½ in. scraped piece of 32–36 h old mycelial mat of subculture was taken. Afterwards, it was aseptically homogenized in 4 ml of sterile tryptic soya broth (TSB, Difco) with a Micro Tissue Grinder® (VWR, Canada) submerged in an ice bath. The starter culture was inoculated into 500 ml Erlenmeyer flasks containing 150 ml of sterile TSB at pH 6.0 ± 0.01. Subsequently, the flasks were incubated in a rotary shaker at 28 ± 1°C and 250 ± 5 rpm for 48 h, and used immediately as inocula for the fermenter.

Fermentation

Fermentations were carried out in a bench top glass fermenter (7.5 l capacity) (LABFORS 3, INFORS AG, Switzerland). The fermenter was equipped with a programmable logic controller for pH, agitation, antifoam, dissolved oxygen and temperature. A data acquisition and process control software (IRIS version 5.01; Infors, Switzerland) was utilized for process automation. The agitator system consisted of two equally spaced Rushton turbine-type impellers and three baffles. Sterilization of 4 l of SIW was carried out for 30 min at 121 ± 1°C. The inoculum comprised 10% v/v starter culture. The pH during fermentation was maintained at 6.0 ± 0.1 by using 2 N H_2SO_4 and 2 N NaOH, whereas the temperature was kept constant at 28 ± 1°C. Three dissolved oxygen (DO) levels (≤ 80, ≥ 40 and ≥30%) were maintained in separate experiments in duplicate by varying the agitation speed (PID control) and aeration rate (manually). A maximum of 75–100 ml samples were drawn aseptically at regular intervals and stored at 4 ± 1°C for subsequent analyses. The significance of the fermentation data was based on the average of the duplicate set of fermentations.

Spore assessment

A modified CFU plating method was utilized to measure the *Trichoderma* spore count. In this method, the fungal conidia was separated from mycelia and media agglomerates, as described elsewhere [31].

For CFU plating, appropriately diluted 100 µl samples of the separated conidia of *Trichoderma viride* were plated on tryptic soya agar plates. The agar plates were incubated at 28 ± 1°C and 35 ± 2% relative humidity for 30–36 h in the dark. For statistical significance of data, five replicates for three different dilutions were used for ANOVA enumeration. Standard deviation for CFU count was 8–10%.

Microscopy

Smear preparation was carried out with 20–50 µl of fresh samples using a pipette tip cut at the end. Morphological examination of the mycelia and conidia were carried out to assess the effect of agitation during mixing in the fermenter. To this end, a computer-coupled optical microscope (Zeiss Axiolab) equipped with a digital camera (Axiocam HRC Zeiss) was utilized. Also, contamination was checked by qualitative observation of all samples.

Soluble chemical oxygen demand (SCOD)

The closed reflux method as described in Standard methods [2], was used for SCOD determination. Standard deviation was 3–5%, based on triplicate samples of two fermentation runs.

Volumetric oxygen transfer coefficient (k_La), oxygen uptake rate (OUR), and power consumption measurements

The dynamic gassing-out method was used for k_La measurements [1]. The DO control was momentarily stopped during the dynamic gassing-out method to facilitate correct k_La measurements. Oxygen uptake rates were obtained by measuring the slope of DO decrease during air-off of the dynamic gassing-out protocol. For calculation of the cumulative oxygen consumed, the area under OUR curves was manually quantified by approximating the area with rectangles and triangles. The oxygen probe used in this study was based on the Clark polarographic sensor. The probe

was equipped with a temperature compensation circuit and a response time ≤30 s to attain 90% of the final value at 25°C.

For measurement of power consumption in terms of revolutions per min × h (rpmh) and l per min × h (lpmh), the area under the agitation and aeration profiles, respectively, was measured. To this end, the rectangles between two adjacent *Y*-ordinates of respective parameters were calculated with the Excel spreadsheet program.

Rheological measurements

Rheological analyses were carried out for fresh samples by using a rotational viscometer (DVII+, Brookfield) equipped with small sample adapter spindle (SC4 34, Brookfield). The calibration and the rheological testing procedure used for the spindle were carried out according to the instrument's manual. The viscosity data were examined by using the software Rheocalc V2.6 (Brookfield Engineering Labs 1999). The Ostwald–deWaele power law, $\tau = k\gamma^n$, Bingham equation, $\tau = \tau_0 + \mu_P\gamma$, and Casson equation, $\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{\mu_P\gamma}$, were investigated to describe the rheological characteristics of all samples.

Enzyme activity

Protease activity (PA) was measured in centrifuged supernatant (7,650 *g* for 20 min at 4 ± 1°C) of fermented broth. The supernatant was appropriately diluted in borate buffer at pH 8.2 ± 0.01 and used as enzyme aliquot. A modified method [17] was employed for PA measurements in IU/ml.

Cellulase activity was measured as described by Wang et al. [32]. Amylase activity was determined by measuring the appearance of total reducing sugar in the reaction mixture by utilizing the dinitrosalycylic acid reagent method [23]. The enzyme reaction mixture consisted of 40 µl of 0.5 M sodium acetate buffer (pH 6.0), 100 µl of 0.5% soluble starch, 0 to 60 µl of enzyme solution, and ultrapure water to a final volume of 200 µl [8]. The reaction was carried out for 30 min at 50°C and stopped by heating at 96°C for 5 min. One amylase unit was defined as the amount of enzyme which releases 1 mmol of glucose per min (saccharifying activity) from soluble starch.

Standard deviations for all enzyme measurements were up to 9%, based on triplicate samples of two fermentation runs.

Insecticidal bioassay

Entomotoxicity (Tx) of the fermented samples was determined using diet incorporation bioassay [4]. Sample preparation and diet protocol are described elsewhere [6]. Industry standard contained spores and crystals of the bacterium *Bacillus thuringiensis* at a potency of 20.1×10^9 IU/l (International Unit) measured against cabbage looper (*Trichoplusia ni*). By comparison, Tx as spruce budworm unit (SBU) of the *Trichoderma*-fermented sludge reported in this study was 20–25% higher than Tx reported as IU. Tx of sample preparations was expressed in spruce budworm units/µl (SBU/µl) with 8–10% of standard deviation.

Fungicidal bioassay

Two important fungal pathogens of the forestry sector were investigated by slightly modified scored response bioassays as described by Mischke [24]. This study involved submerged fermentation, and therefore no extraction of metabolites was needed as explained by Mischke [24]. In this procedure, serial dilutions of 1/2, 1/4, 1/8 and 1/16, were used in 24-well tissue culture plates. The standard times for scoring and for the ordinal scale of inhibition were similar to those of the original study [24]. The phytopathogen used in this study was *Cylindrocladium floridanum.* The inhibition effects were quantified as inhibition index *I* in Eq. 1:

$$I = \frac{\sum_{i=1}^{n}\left[\left(S_{1,i}.D_i.T_1\right) + \left(S_{2,i}.D_i.T_2\right)\right]}{M}, \qquad (1)$$

where *S* is the degree of inhibition assigned as numerical score for well "*I*" at time *T* in days. *D* is the dilution factor for well "*I*", e.g., 2, 4, 8, 16, respectively for wells 1, 2, 3, 4 and so on. *M* is the maximum possible *I* for each row "*i*", i.e., *S* is presumed to be equal to 2 for every well in each row "*i*" at every time so that; $M = \sum_{i=1}^{n}\left[(2.D_i.T_1) + (2.D_i.T_2)\right]$.

Results and discussion

Fermentation: conidium growth kinetics

In order to evaluate the feasibility of *Trichoderma viride* conidia production using SIW as substrate, minimum DO concentration was examined. The constant range of DO levels varied between ≥30% and ≤80% in independent experiments (Figs. 1, 2, 3). The

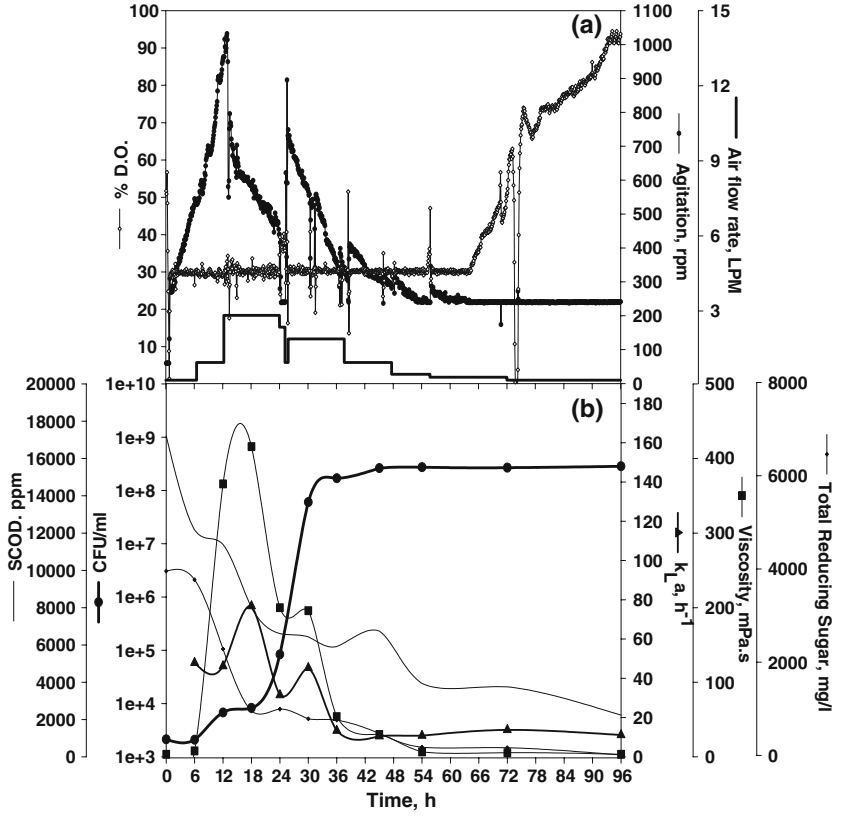

Fig. 1 Fermentation profile of *T. viride* in SIW at ≥30% DO; **a** operational parameters, **b** growth parameters

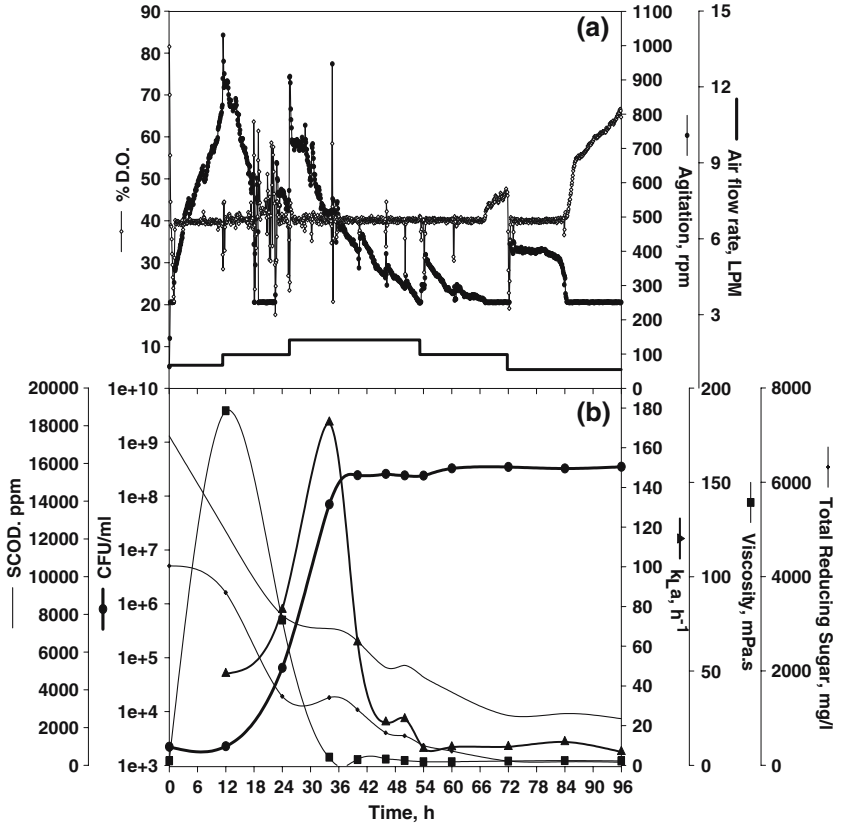

Fig. 2 Fermentation profile of *T. viride* in SIW at ≥ 40% DO; **a** operational parameters, **b** growth parameters

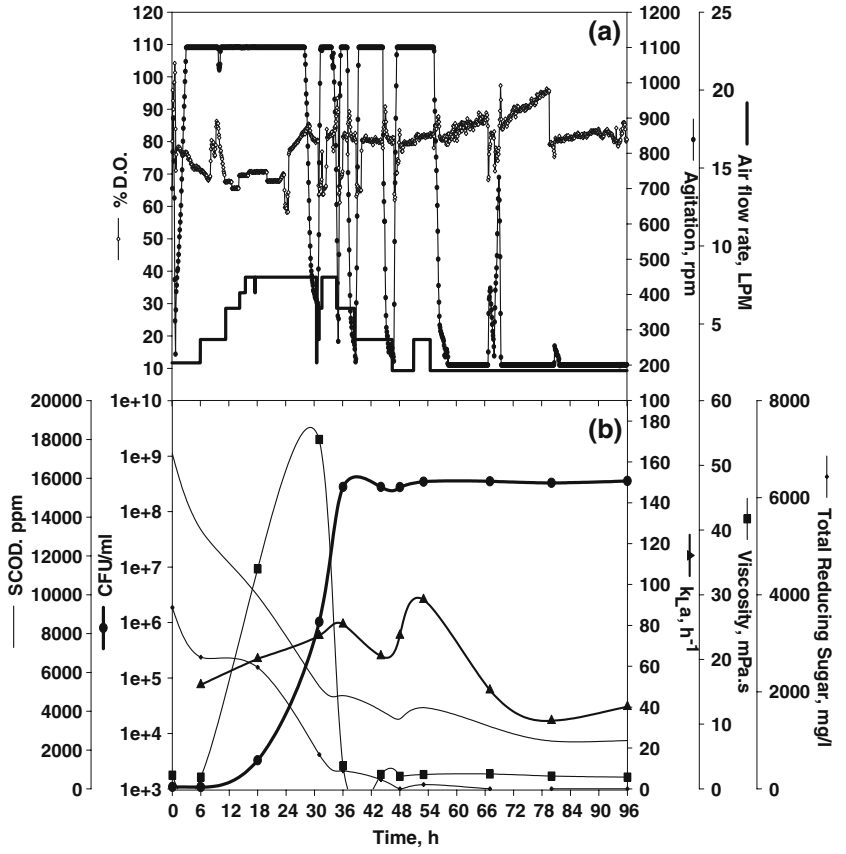

Fig. 3 Fermentation profile of *T. viride* in SIW at≤80% DO; **a** operational parameters, **b** growth parameters

DO outside this limit was extremely deleterious for fermentation (data not shown). In case of DO below 30%, problems like wall growth, very high viscosity due to mycelia, and channeling of air bubbles were observed. However, it was extremely difficult to maintain DO above 80% due to design limitations of the fermenter. In addition, higher agitation to maintain DO>80% caused damage to the mycelial mass and consequently resulted in lower conidia build up. The detrimental effect of agitation on *Trichoderma* spp. has already been reported by many researchers [12, 33]. Nevertheless, inhibitory effects of oxygen on fungal physiology could not be ruled out. For example, high oxygen concentration resulted in thinning and fragmentation of fungal mycelia, which consequently affected rheological behaviour [20]. Therefore, it was decided to maintain DO levels at ≤80, ≥40 and ≥30% in three independent experiments.

Conidium growth kinetics for all fermentations were interpreted in terms of aeration and agitation requirements, CFU production, reducing sugar consumption, and SCOD utilization (Figs. 1, 2, 3). At ≥30% DO, conidia production reached a maximum (2.84×10^8 CFU/ml) after approximately 45 h of cultivation. Total reducing sugar (TRS) depleted sharply (3,982 to 984 mg/l) for 18 h, followed a by slow decrease towards the end of fermentation (Fig. 1b). The TRS concentration decrease was also evident from the SCOD decrease (17,212 to 2,236 mg/l), which accounted for all soluble constituents (including TRS) of SIW. Furthermore, the decrease in TRS and SCOD was concurrent with an increase in mycelia growth and aeration requirements (0–24 h, Fig. 1a), a well-known fact about *Trichoderma* fungi [26]. Conidiation of *Trichoderma* reached its maximum at ≈ 45 h so that the batch kinetics at ≥30% DO suggested the fermentation batch time to be ≈ 45 h. However, TRS and SCOD consumption and BCA efficacy (discussed later) in terms of bioassay suggested 96 h as cultivation time. In fact, at around 96 h, TRS and SCOD decreased to a minimum level, and Tx and inhibition index (*I*) attained their maxima. Meanwhile, micrographs showed that the mycelial mass diminished gradually from 18 to 96 h, leaving sparse fragments at the end (Fig. 4). This observation suggested that conidiation after 96 h was not possible (nearly complete absence of mycelia), thereby rendering it as batch time.

Figure 2 shows the batch kinetics of *Trichoderma* at≥40% DO. The profiles of conidiation, TRS consumption and SCOD reduction were similar to batch

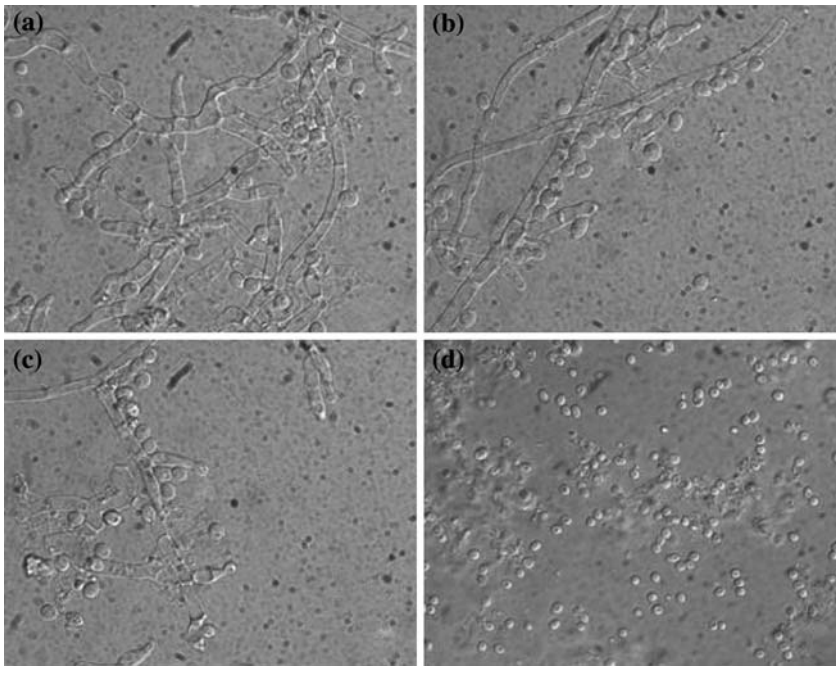

Fig. 4 SIW ≥ 30% DO; **a** vegetative growth as mycelia at 18 h, **b** partial conidiation at 36 h and **c** 45 h, and **d** almost complete conidiation at 96 h

kinetics at≥30% DO. However, conidiation (≈2.42×10^8 CFU/ml at 40 h), the decrease in TRS (4,225 to 88 mg/l at 72 h) and SCOD (17,453 to 2,637 mg/l at 72 h) were faster with respect to batch kinetics at≥30% DO. Similarly, at≤80% DO (Fig. 3), the incubation time decreased to reach maximum conidiation (≈2.77×10^8 CFU/ml at 36 h). In addition, a decrease in TRS (3,736 to 0 mg/l, at 48 h) and SCOD (17,261 to ≈ 2,800 mg/l at 72 h) was also observed. Thus, conidiation, TRS and SCOD results showed that the batch time of 4 days was sufficient in each case. Nevertheless, it was observed that the increment in final CFU concentration was only marginal at ≥40% and ≤80% DO, in comparison to ≥30% DO (an increase by a factor of 1.22–1.25). Furthermore, the bioassay of final fermentation broth suggested superiority of ≥30% DO over ≥40 and ≤80% DO (discussed later).

Thus, DO ≥30% was found to be better for conidia formation in *Trichoderma viride* and it resulted in higher BCA activity in the fermented medium.

Rheology and mass transfer phenomena

Trichoderma fermentation is a highly aerobic process [12, 18], and therefore, any increase in broth viscosity can hamper oxygen transfer rates. It would consequently be pertinent to examine the volumetric oxygen transfer coefficient (k_La), broth viscosity, aeration and agitation as a function of time [4]. This would facilitate predicting and ensuring a sufficient amount of oxygen for *Trichoderma viride* growth during fermentation. This would also help increase conidia production on the one hand, and develop a strategy for scaling-up of the bioreactor design for mass production on the other hand [3]. Previously, some authors have reported broth rheology and mass transfer limitations in *Trichoderma* fermentation [16, 35]. However, none of them considered conidia production as a main product, which is very important for the production of fungi as biocontrol agent.

Viscosity of SIW was a function of *Trichoderma* fermentation, as observed in Figs. 1b, 2b and 3b. Broth viscosity varied between 416 and 1.82 mPa.s for ≥30, ≥40 and ≤80% DO. The highest viscosity was observed at DO concentrations in the increasing order as 30 > 40 > 80%. Irrespective of % DO, a major change in viscosity took place during the first 36 h of cultivation. This was also visualized under microscope and was apparent from the broth appearance inside the glass bioreactor. Visual observation showed an increase in mycelial mass during the first 36 h, followed by a decrease until the end of fermentation (96 h). Moreover, mycelial growth of fungi is well known to impart a viscous behaviour during fermentation [26]. In general, k_La decreases with viscosity; however, in the case of ≥30% DO, the profiles of k_La and the viscosity were similar until 36 h (Fig. 1b). This was probably due to an increase in aeration and agitation rates to maintain ≥30% DO (Fig. 1a). For ≥40% DO, k_La varied inversely to viscosity until 36 h of fermentation. Subsequently, the decrease in k_La was independent of

viscosity and followed aeration and agitation variations. At ≤80% DO, variation in k_La until 36 h was largely dependent on agitation and aeration rates. The k_La decrease between 36–54 h was possibly be due to anti-foam addition.

The OUR profiles of ≥30, ≥40 and ≤80% DO are shown in Fig. 5. It shows that the oxygen consumption at ≥30 and ≥40% DO reached maxima around 12 h and, at ≤80% DO, around 18 h. This was also apparent from mycelial mass formation during this period. The OUR profiles also collectively showed that once significant conidiation had occurred, the system did not need higher oxygen supply. This explains the decrease in OUR values after ≈ 36 h in all cases. Moreover, it was evident (Fig. 5) that the cumulative oxygen consumption was approximately 158.8 mmol/l at ≥ 30 ≈ 40% DO and 106.1 mmol/l at ≤80% DO. The higher OUR and oxygen consumption at lower DO (≥ 30 and 40%) with respect to ≤80% DO was probably due to the detrimental effect of agitation on mycelial biomass [12, 33]. This was also apparent in micrographs (Fig. 4) and through visual observation of the glass bioreactor. In addition, the viscosity change (in the order of 30 > 40 > > 80% DO) was the indirect indicator of mycelial growth. This should explain the higher BCA efficacy of ≥ 30 and 40% DO fermentation broths (discussed later).

Broth rheology was also analyzed for its non-Newtonian behaviour as shown in Fig. 6. The time dependent rheological profiles of *Trichoderma*-fermented SIW at different fermentation times showed a decrease in viscosity with time (thixotropic behaviour). The fermentation samples obeyed the Ostwald-deWaele power law with a confidence of fit of ≥75–93%, which was the highest in comparison with Casson and Bingham laws. The complexity of rheology was evident from the variation in K and n, irrespective of % DO. It was noticeable that the respective initial and final values of K and n were almost similar in each case. The range of K varied as 30 > 40 > 80% DO, and variation in n closely followed the change in viscosity. The characteristic plots between τ and γ showed all broths to be pseudoplastic in nature, and their viscosity-time profiles confirmed their thixotropic nature [27]. Thus, the rheological profiles suggested that viscosity could be a significant factor during fermentation but would lose its importance during downstream processing.

Power consumption

The aeration and agitation requirements of any large-scale fermentation facility could be correlated to their power consumption [22, 30]. Therefore, it was possible to obtain a preliminary estimate of power consumption for a large-scale *Trichoderma* conidia production, based on this study. In this study, the aeration and agitation requirements were transformed into rpm. h and lpm. h, respectively, in order to correlate them with power requirements. The areas under the curves of the aeration and agitation profiles in Figs. 1a, 2a and 3a were calculated and are presented in Table 2. The information from this interpretation is very useful when compared at the three DO levels. For example, an increment of 14 to 80% in agitation and 47 to 350% in aeration power was observed, when ≥ 30% DO process was compared with ≥ 40 and ≤ 80% DO. However, it can be concluded from previous discussions that maintaining a DO level > > 30% would be energy and cost intensive. Furthermore, this approximation would be improved if a pilot facility was studied for this purpose.

BCA efficacy and enzyme activity

The samples of fermentation broths taken at different times were subjected to insecticidal and fungicidal

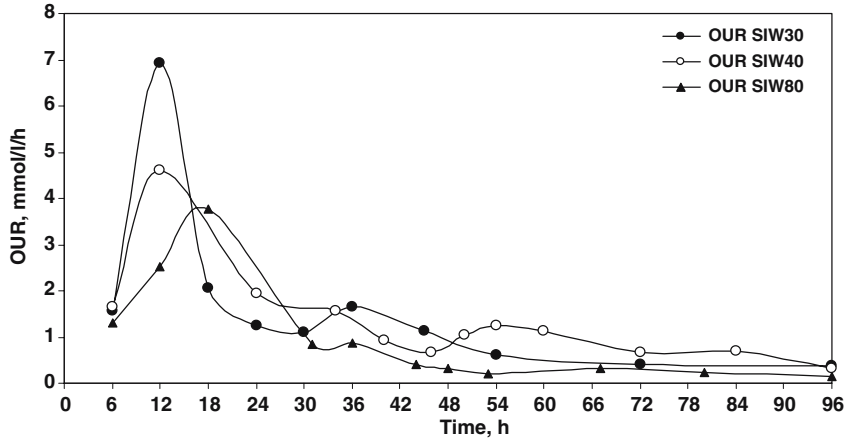

Fig. 5 OUR profile of *T. viride* in SIW at ≥30, 40 and ≤80% DO

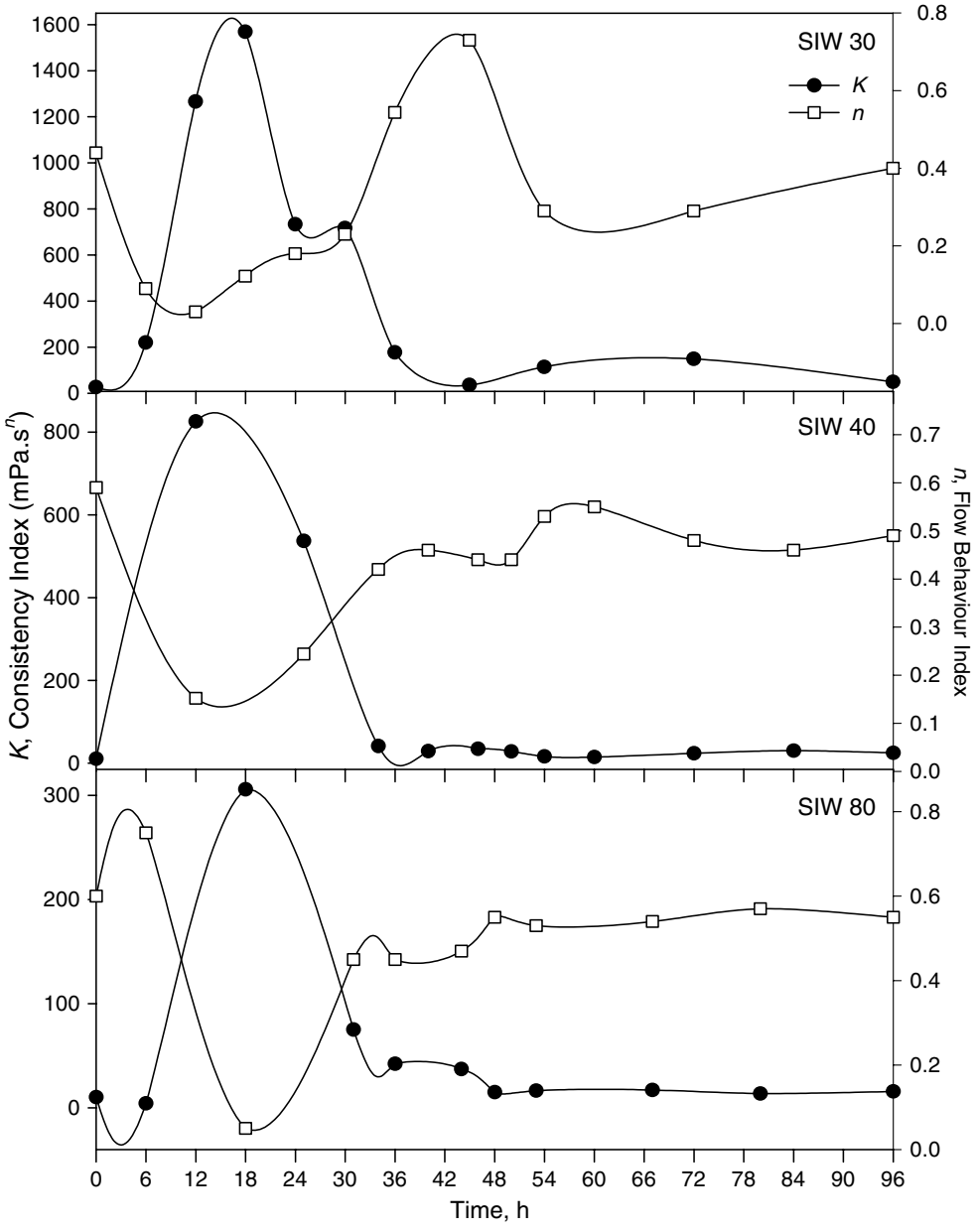

Fig. 6 Rheological profile of *T. viride* in SIW at ≥30, 40 and ≤80% DO

bioassays. Figure 7 presents the entomotoxicity (Tx), inhibition index (*I*), amylase activity and protease activity profiles at all DO levels. A common feature observed in the two bioassays was that BCA efficacy decreased with increasing level of DO (entomotoxicity: 12467 to 10058 SBU/μl; inhibition index: 0.733 to 0.568). The near maximum Tx and *I* increase could be attained at approximately 66 h in all cases, which was also concurrent with the protease activity. However, the increase in Tx and *I* was not totally dependent on conidia production (Figs. 1b, 2b, and 3b). A time lag was observed between conidia formation and Tx and *I* increase. Moreover, physiological characteristics of conidia (function of incubation time) might have influenced Tx and *I* by increasing virulence, i.e., biological efficacy [18]. As the final CFU concentration was almost similar in all cases, the adverse effect on BCA efficacy at higher DO could be due to a decreased concentration of metabolites (lytic enzymes and antibiotics) [7, 8, 34]. The lytic enzymes and

Table 2 Power requirements in terms of rpm.h and lpm.h for fermentation

% DO	rpm. h	lpm. h
80	63303	348.13
40	40139	133.26
30	35585	90.48

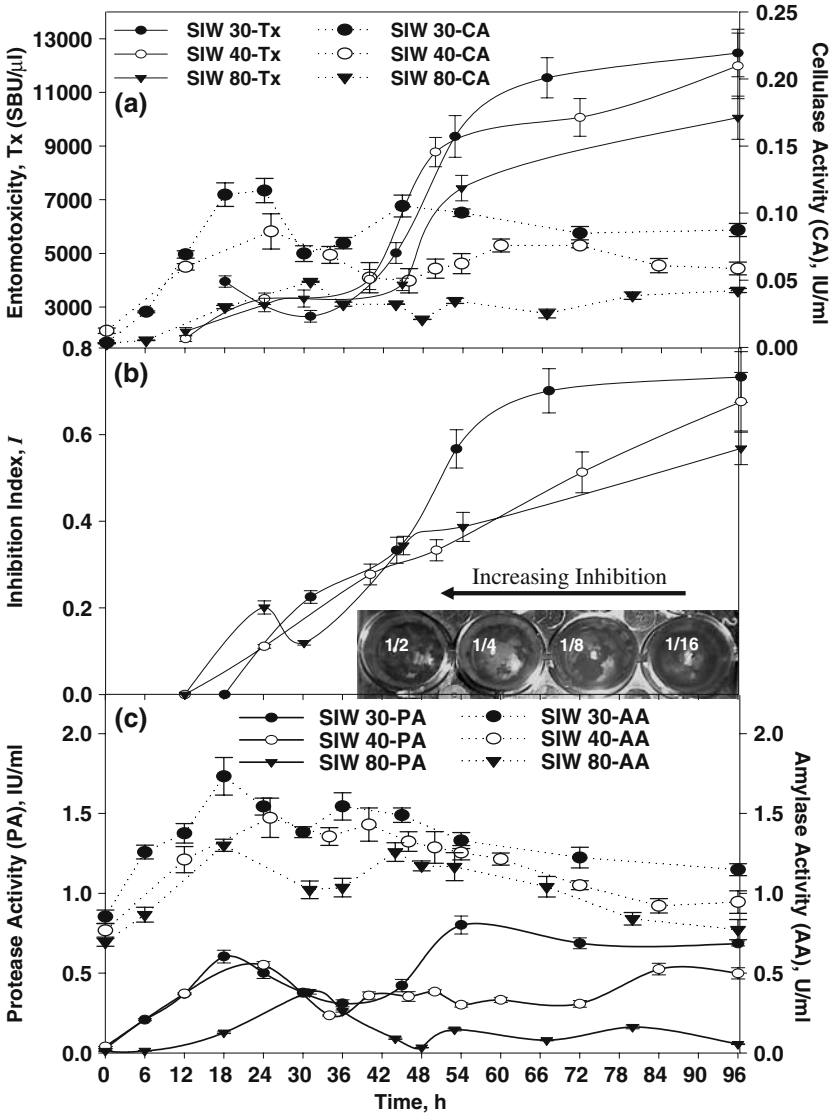

Fig. 7 BCA activity of *Trichoderma viride*; **a** entomotoxicity, cellulase activity and **b** inhibition index profile (*Inset* appearance of fungal bioassay wells), and **c** amylase and protease activities with incubation time

antibiotics have been considered as major synergistic factors along with conidia in different bioassays [16, 24]. Moreover, many researchers have suggested that a wide range of microbes contain proteins and polysaccharides (similar to starch, e.g., glycogen) in their cell wall. This enables proteases and amylases produced by *Trichoderma* spp. to antagonize pathogens by degrading their cell wall [32, 34].

Protease activity profiles (Fig. 7c) of the fermentation broths showed a decrease in activity after 18, 24 and 31 h, respectively, for ≥30, ≥40 and ≤80% DO. The decreasing DO profile continued until ≈36–48 h, followed by an increase or no net change until the end of fermentation. This could be due to the adverse effects of agitation on mycelial biomass and consecutive lower enzyme production, as reported in previous studies also [19]. It was noteworthy that many enzymes showed activity in the presence of certain co-enzymes and/or co-factors (inducing agents). Therefore, it was also possible that adverse growth conditions due to agitation (breakage of mycelial mass) might have affected the production of inducing agents. Subsequently, it resulted in a decrease in overall enzyme activity instead of the higher enzyme concentration that could be present due to mycelial lysis. Similarly, amylase and cellulase activity was adversely affected by intense agitation. The decrease in metabolites could be due to lesser mycelial growth at higher agitation conditions, as reported in various studies [9, 13].

The two types of bioassays carried out proved the dual efficacy of *Trichoderma viride*, which would be advantageous from an application point of view. Thus, single application of *Trichoderma* formulation on

spruce trees (commercially valuable) would control two spruce pests: (a) an insect pathogen, the spruce budworm, and (b) a fungal pathogen, *Cylindrocladium floridanum*. This would result in pathogen control via *Trichoderma viride* formulation, which would be performant and cost effective.

Conclusions

This study investigated the kinetic behaviour of fermentation and rheological parameters. Furthermore, power consumption was estimated in correlation to agitation and aeration requirements. Dissolved oxygen concentration at ≥30% was the most suitable option for *Trichoderma* conidia production (≥10^8 CFU/ml) and biocontrol efficacy (higher entomotoxicity and inhibition index). Higher dissolved oxygen concentrations (≥40 and ≤80%) were ineffective in increasing conidia as well as BCA activity. The amounts of total reducing sugar and soluble COD consumed were similar, irrespective of dissolved oxygen levels, justifying 96 h as optimal batch time. Maximum viscosity increased (54, 188 and 416 mPa s, respectively, for ≤80% (31 h), (40% (12 h) and ≥30% (18 h)) owing to less shear on the mycelial mass. Rheological analyses (evaluation of K and n) showed the pseudoplastic and thixotropic (time-dependent) nature of *Trichoderma*-fermented wastewater broths. The bioassay proved the dual efficacy (against phytopathogens, *Cylindrocladium floridanum* and spruce budworm larvae) of *Trichoderma viride* fermented starch industry wastewater. Thus, a plausible alternative to costly synthetic medium with simultaneous value-addition of a waste was explored. This batch study will be a critical step for modeling a pilot scale strategy and, finally, large-scale fermentation of BCAs.

Acknowledgments The authors are sincerely thankful to Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, Canada Research Chair) for financial support. The views and opinions expressed in this article are those of the authors.

References

1. Aiba S, Humphrey AE, Millis NF (1973) Aeration and agitation. In: Biochemical engineering. 2nd edn. Academic, New York
2. APHA-AWWA-WPCF (1998) In: Clesceri LS, Greenberg AE, Eaton AD (eds) Standard methods for examination of water and wastewaters, 20th edn. American Public Health Association, Washington, DC
3. Bailey JE, Ollis DF (1986) Biochemical engineering fundamentals, 2nd edn. McGraw-Hill, New York
4. Beegle CC (1990) Bioassay methods for quantification of Bacillus thuringiensis δ-endotoxin: analytical chemistry of bacillus thuringiensis. In: Hickle LA, Fitch WL (eds) Analytical chemistry of *Bacillus thuringiensis*, American Chemical Society, pp 14–21
5. Bhargava S, Nandakumar MP, Roy A, Wenger KS, Marten MR (2003) Pulsed feeding during fed-batch fungal fermentation leads to reduced viscosity without detrimentally affecting protein expression. Biotechnol Bioeng 81(3):341–347. DOI: 10.1002/bit.10481
6. Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY (2005). Starch industry wastewater-based stable *Bacillus thuringiensis* liquid formulations. J Econ Entomol 98(6):1890-1898
7. Butt TM (2000) Fungal biological control agents. In: Pesticide. Outlook, October 2000. The Royal Society of Chemistry, pp 186–191
8. Campos L, Felix CR (1995) Purification and characterization of a glucoamylase from *Humicola grisea.* Appl Environ Microbiol 61(1):2436–2438
9. Chet I (1987) *Trichoderma*—Applications, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. In: Chet I (ed) Innovative approaches to plant disease control. Wiley, New York, pp 137–160
10. Cooney JM, Lauren DR (1998) *Trichoderma*/pathogen interactions: measurement of antagonistic chemicals produced at the antagonist/pathogen interface during tubular bioassay. Lett Appl Microbiol 27:283–286
11. Dinham B (2005) Agrochemical markets soar—pest pressures or corporate design? Pestic News 68:9–11
12. Felse PA, Panda T (1999) Self-directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. Process Biochem 34:563–566
13. Harmann GE, Björjmann T (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: Harmann GE, Kubicek CK (eds) *Trichoderma* and *Gliocladium* vol 2., Taylor and Francis Ltd., London, pp 229–265
14. Humphrey A (1998) Shake flask to fermentor: what have we learned? Biotechnol Prog 14:3–7
15. Jenkins NE, Heviefo G, Langewald J, Cherry AJ, Lomer CJ (1998) Development of mass production technology for aerial conidia for use as mycopesticides. Biocontrol News Inform 19(1):1N–31N
16. Kanauchi M, Bamforth CW (2001) Growth of *Trichoderma viride* on crude cell wall preparations from barley. J Agric Food Chem 49:883–887
17. Kunitz M (1947) Crystalline soybean trypsin inhibitor. J Gen Physiol 30:291–310
18. Lewis JA, Papavizas GC (1983) Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. Soil Biol Biochem 15(3):351–357
19. Lejeune R, Baron GV (1995) Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation. Appl Microbiol Biotechnol 43:249–58
20. McIntyre M, Breum J, Arnau J, Nielsen J (2002) Growth physiology and dimorphism of *Mucor circinelloides* (syn. *racemosus*) during submerged batch cultivation. Appl Microbiol Biotechnol 58:495–502
21. MENV (2004) Guide sur la valorisation des matières résiduelles fertilisantes: Critères de références et normes réglementaires. Direction du milieu rural, environnement Québec, Canada, p 138
22. Michell BJ, Miller SA (1962) Power requirements of gas–liquid agitated systems. AIChEJ. 8:262–266

23. Miller GL (1959) Use of dinitrosalycylic acid reagent for determination of reducing sugars. Anal Chem 32:426–428
24. Mischke S (1997) A quantitative bioassay for extracellular metabolites that antagonize growth of filamentous fungi, and its use with biocontrol fungi. Mycopathologia 137:45–52
25. Olsvik ES, Kristiansen B (1992) Influence of oxygen tension, biomass concentration, and specific growth rate on the rheological properties of a filamentous fermentation broth. Biotechnol Bioeng 40:1293–1299
26. Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv 22:189–259. DOI:10.1016/j.biotechadv.2003.09.005
27. Perry RH, Green DW (1997) Perry's chemical engineers' handbook, 7th edn. McGraw-Hill, New York
28. Pickett JA, Butt TM, Doughty KJ, Wallsgrove RM, Williams IH (1995) Minimising pesticide input in oilseed rape by exploiting natural regulatory processes. Plenary lecture. In: Proceedings of the GCIRC 9th International Rapeseed Congress, Cambridge, 4–7 July 1995, 2:565–571
29. Punja ZK, Utkhede RS (2003) Using fungi and yeasts to manage vegetable crop diseases. Trends Biotechnol 21(9):400–407. DOI 10.1016/S0167-7799(03)00193-8
30. Rushton JH, Costich EW, Everett HJ (1950) Power characteristics of mixing impellers. Chem Eng Progr 46:395–404
31. Verma M, Brar SK, Tyagi RD, Valéro JR, Surampalli RY (2005) Wastewater sludge as a potential raw material for antagonistic fungus (*Trichoderma* sp.): role of pre-treatment and solids concentration. Water Res 39(15): 3587–3596. DOI 10.1016/j.watres.2005.07.001
32. Wang SL, Yen YH, Tsiao WJ, Chang WT, Wang CL (2002) Production of antimicrobial compounds by *Monascus purpureus* CCRC31499 using shrimp and crab shell powder as a carbon source. Enzyme Microb Technol 31:337–344
33. Weber J, Agblevor FA (2005) Microbubble fermentation of *Trichoderma reesei* for cellulase production. Process Biochem 40:669–676. DOI 10.1016/j.procbio.2004.01.047
34. Zaldívar M, Velásquez JC, Contreras I, Pérez LM (2001) *Trichoderma aureoviride* 7–121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. Electron J Biotechnol 4(3):160–168
35. Zheng Z, Shetty K (1998) Cranberry processing waste for solid state fungal inoculant production. Process Biochem 33(3):323–329
36. Zlokarnik M (1998) Problems in the application of dimensional analysis and scale-up of mixing operations. Chem Eng Sci 53(17):3023–3030