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Kinetics of Cell Growth and Cyclosporin A Production by *Tolypocladium inflatum* when Scaling Up from Shake Flask to Bioreactor

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The kinetics of cell growth and Cyclosporin A (Cyc A) production by *Tolypocladium inflatum* were studied in shake flasks and bioreactors under controlled and uncontrolled pH conditions. In the case of the shake flask, the production time was extended to 226 h and the maximal antibiotic concentration was 76 mg/l. When scaling up the cultivation process to a bioreactor level, the production time was reduced to only 70 h with a significant increase in both the cell growth and the antibiotic production. The maximal dry cell weights in the case of the controlled pH and uncontrolled pH cultures in the bioreactor were 22.4 g/l and 14.2 g/l, respectively. The corresponding maximal dry cell weight values did not exceed 7.25 g/l with the shake flask cultures. The maximal values for Cyc A production were 144.72 and 131.4 mg/l for the controlled and uncontrolled pH cultures, respectively. It is also worth noting that a significant reduction was observed in both the dry cell mass and the antibiotic concentration after the Cyc A production phase, whereas the highest rate of antibiotic degradation was observed in the stirred tank bioreactor with an uncontrolled pH. Morphological characterization of the micromorphological cell growth (mycelial/pellet forms) was also performed during cultivation in the bioreactor.

Keywords: Cyclosporin A, *Tolypocladium inflatum*, antibiotic production, growth morphology

Cyclosporine is a lipophilic cyclic undecapeptide antibiotic derived from the fungus *Tolypocladium inflatum*. This antibiotic binds to the cell cytosolic receptor cyclophilin D, and then the complex cyclosporine-cyclophilin D binds to the calmodulin-stimulated protein phosphatase calcineurin, thereby inhibiting calcineurin's serine-threonine phosphatase activity [21]. Cyclosporine is also a powerful immunosuppressive drug with no appreciable effect on the bone marrow. It was initially discovered in 1970 at the Sandoz research laboratories in Basel, Switzeland, from two strains of fungi imperfecti, *Cyclindocarpon lucidum* and *Tolypocladium inflatum*. The first human kidney transplant patients were treated with cyclosporine in 1978, and now it is widely used post-allogenic organ transplantation to reduce the activity of the patient's immune system and hence the risk of organ rejection. It has also been studied with skin, heart, kidney, lung, pancreas, bone marrow, and small intestine transplants. Cyclosporine is a cyclic nonribosomal peptide of 11 amino acids produced by the fungus *Tolypocladium inflatum* initially isolated from a Norwegian soil sample. Although the international nonproprietary name is now *Ciclosporin*, it is still referred to as *Cyclosporine* in most scientific journals and medical publications. Beside its use as an immunosuppressant drug, Cyc A is also useful for antifungal, antiparasitic, and antiinflammatory activity [24]. Therefore, the annual sales of cyclosporine were around $1 billion in 2005 [20].

The production of Cyc A is mainly carried out by submerged fermentation using either free or immobilized cells [3, 5, 12, 20]. Moreover, various attempts have been made to produce Cyc A using solid-state fermentation [18, 23]. Like most antibiotic metabolites, the production levels of Cyc A are dependent on several regulating factors, such as the medium composition and additives [1, 3, 10, 12], in addition to typical process parameters, such as temperature, pH, and partial oxygen pressure. However, it would appear that no information has yet been published on the influence of the kinetics of cell lysis and antibiotic degradation on the scaling-up process of Cyc A production.

Accordingly, the present study examined the production of cyclosporine in a shake flask and bioreactor. The cultivations were undertaken in a complex medium containing mixed carbon sources (glucose, sucrose, and starch). The kinetics of cell growth and antibiotic production in the shake flask and bioreactor were then studied with a controlled and

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uncontrolled pH. Moreover, the influence of scaling up from a shake flask to a bioreactor on the kinetics of cell degradation and antibiotic decomposition was studied. In addition, changes in the growth morphology and its relation to antibiotic production were also investigated in the bioreactor.

MATERIALS AND METHODS

Microorganism

The *Tolypocladium inflatum* (DSMZ 915) was delivered as a lyophilized culture from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The spores were obtained from a sporulation agar (ISP2 medium), and spore suspensions prepared after 10 days of cultivation and maintained in 50% glycerol at −80°C. The viability of the deep-frozen cultures was periodically controlled by plating them onto an ISP2 agar (malt extract 10 g/l, yeast extract 4 g/l, glucose 4 g/l, agar 20 g/l, pH 7.2), and then counting the colonies formed to confirm the spore viability.

Cultivation Conditions

The inoculum for the shake flask and bioreactor was prepared by placing the spore suspension in a 250-ml Erlenmeyer flask containing 50 ml of a DSMZ 90 medium (malt extract 30 g/l, Bactopeptone 3 g/l, pH 5.7), incubating on a rotary shaker at 27°C and 200 rpm for 48 h, and inoculating the production medium at a 5% v/v ratio. The cyclosporin A production step was then performed using a fermentation medium (containing glucose 10 g/l, sucrose 20 g/l, starch 20 g/l, yeast extract 10 g/l, $(NH_4)_2SO_4$ 10 g/l, KH_2PO_4 1 g/l, $MnSO_4 \cdot 7H_2O$ 1 g/l, pH 5.9). The antibiotic production was performed in both a shake flask and a bioreactor. The shake flask cultivations were performed in a 250-ml Erlenmeyer flask with a working volume of 50 ml. The inoculated flasks were shaken at 200 rpm on a rotary shaker (New Brunswick, NJ, U.S.A.) with eccentricity of 2.0 cm, and cultivated at 27°C. The bioreactor cultivations were carried out in a 2-l stirred tank bioreactor (Bioflow 110; New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.) with a working volume of 1.5 l. The stirrer was equipped with two 6-bladed rushton turbine impellers ($d_{i(\text{impeller diameter})}$=52 mm; $d_{t(\text{tank diameter})}$=125 mm, d_i/d_t=0.416), and the agitation adjusted to 100 rpm throughout the cultivation. Aeration was performed using filtered sterile air at a rate of 0.5 l/min, and the pH adjusted to 5.9 throughout the cell cultivation by the addition of 0.1 mol/l NaOH. Any foam was suppressed by the addition of the antifoam reagent Struktul (Th. Goldschmidt AG, Essen, Germany), and the dissolved oxygen concentrations analyzed using a polarographic electrode (Ingold, Mittler-Toledo, Switzerland).

Sample Preparation and Determination of Dry Cell Weight

Samples, in the form of 2 flasks of 50 ml of broth, or 10 ml of broth in the case of the bioreactor cultures, were taken at different times during the cell cultivation in preweighed centrifugation tubes (Falcon, U.S.A.) and centrifuged at 3,000 rpm for 20 min. A small fraction of the supernatant was frozen at −20°C for glucose and carbohydrate analyses, whereas the remaining centrifuged cells were washed twice using distilled water, followed by centrifugation. The centrifugal tubes were then dried to a constant weight at 80°C to determine the dry cell weight.

Glucose Determination

The glucose was determined enzymatically using the glucose oxidase-glucose peroxidase method (Diamond Diagnostics, Egypt). The intensity of the developed color was determined at 500 nm using a spectrophotometer (Pharmacia Biotech, Cambridge, England).

Total Carbohydrates Determination

The total carbohydrates were determined spectrophotometrically according to the method of Dubios *et al.* [6]. The samples were heated with sulfuric acid to hydrolyze the polysaccharides and hydrate the monosaccharides to form furfural from pentoses and hydroxymethylfurfural from hexoses. The solutions of furfural and hydroxyfurfural were then treated with a phenol reagent to produce a colored compound, and measured spectrophotometrically using a Novaspec II spectrophotometer (Pharmacia Biotech., Cambridge, England).

Extraction of Cyclosporin A from Culture

The cyclosporin A was extracted from the culture broth according to the method of Agathos *et al.* [1] with certain modifications as follows: 50 ml of ethyl acetate was added to the fermentation flask, which was then shaken at 27°C, 200 rpm for 20 h. Thereafter, the samples were centrifuged at 4,000 rpm for 30 min to separate the organic and aqueous layers. The ethyl acetate layer was removed and filtered using a bacterial filter.

Quantification of Cyclosporin A

The cyclosporin A was quantified using HPLC according to the modified method of Kreuzig [11], where an A hyper clone 5 μ C8 (250×40 mm) column was used with a mobile phase composed of acetonitrile:water:phosphoric acid (700:300:0.1) pumped at a flow rate of 2 ml/min (pump model 168 solvent module; Beckman, U.S.A.) at 60°C. Samples (20 μl) containing 0–100 mg/l Cyc A were used, and the product detected based on the UV absorbance (variable wavelength photos array detector model 168; Beckman, U.S.A.) at 214 nm. The method was calibrated using a standard Cyc A solution obtained from Sigma, U.S.A.

Morphological Characterization

The samples were examined immediately after being withdrawn from the bioreactor, and the morphology of the bioparticles was tracked over the cultivation period using an image analysis system. A colored digital camera (Camedia C-4040 zoom; Olympus, Japan) connected to a phase contrast microscope (BX40; Olympus, Japan) and image analyzer software (Analysis 3.1; Soft Image System GmbH, Münster, Germany) were used for the morphological characterization. The average diameter of the bioparticles was measured manually (by touching two opposite points using a mouse). Data represented are an average of 50–80 randomly selected bioparticles.

RESULTS

Cell Growth and Cyclosporin A Production in Shake Flask Cultures

The cell growth and Cyc A production kinetics in the shake flask during cultivation for 10 days are represented in Fig. 1. As shown, the growth curve could be divided

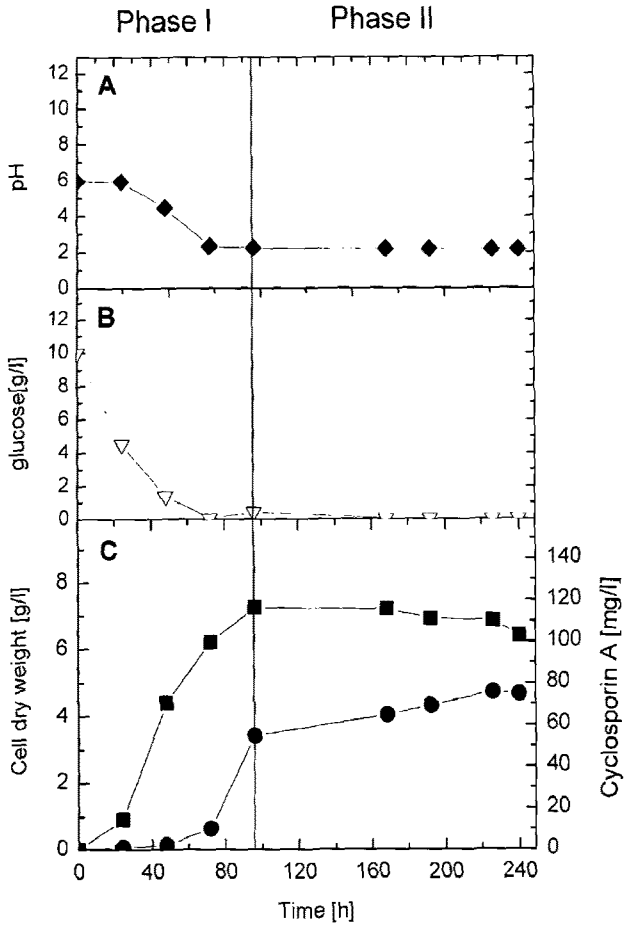

Fig. 1. Kinetics of cell growth and Cyc A production during batch cultivation of *Tolypocladium inflatum* cells in shake flasks. ■, Cell dry weight; ●, Cyclosporin; ▽, glucose; ◆, pH.

into two phases. During the first phase, the first 96 h, the cells grew exponentially at a rate of 0.088 g/l/h and specific growth rate of 0.028 l/h, reaching a maximal value of 7.25 g/l. During this phase, the glucose was consumed gradually at a volumetric rate of 0.138 g/l/h, and a complete glucose limitation was observed after 72 h. However, the carbon sources were not completely limited at this time, as high concentrations of other carbon sources (sucrose and starch) still existed in the culture medium. It is also worth noting that, during the growth phase, the pH dropped gradually from 5.9 to 2.33 after 72 h, and then remained more or less constant for the rest of the cultivation period up to the 4th day. During the second phase, from 96 h to 240 h, the dry cell weight decreased gradually at a very low rate of about 0.0059 g/l/h, reaching 6.4 g/l at the end of the cultivation period.

The production of Cyc A started after 48 h and increased gradually during the first phase with a volumetric production rate [Q_p] of 1.1 mg/l/h and specific production rate [q_p] of about 0.08 mg/g/h, reaching a volumetric antibiotic production of about 55 mg/l after 96 h. During the second phase, the rate of Cyc A production was lower at a rate of 0.160 mg/l/h, reaching about 76 mg/l after 226 h, and then remained more or less constant for the rest of the cultivation period, which would suggest that the biosynthesis of Cyc A was not terminated by glucose limitation, but due to the presence of other carbon sources (sucrose and starch) in the cultivation medium. The specific antibiotic production [$Y_{P/X}$], representing the mg amount of antibiotic produced per g glucose consumed, calculated before the cell lysis phase, was about 7.58 mg/g.

Cell Growth and Cyclosporin Production in Bioreactor Cultures

The cell growth and Cyc A production kinetics were also studied during the batch cultivation of *Tolypocladium inflatum* in a laboratory-scale bioreactor using the same medium composition and inoculum size used in the shake flask experiment. Two sets of experiments were performed using a stirred tank bioreactor with a 1.5-l working volume under controlled and uncontrolled pH conditions.

Cultivation of Cells in Bioreactor Under Uncontrolled pH Conditions

The concentration profiles of the dry cell weight, glucose, total carbohydrates, and Cyc A during cultivation are shown in Fig. 2. The results indicate that the cultivation process of the cells in the stirred tank bioreactor could also be divided into two phases, as in the shake flask experiments. During the first phase, the first 60 h of cultivation, the cells grew exponentially at a rate of 0.284 g/l/h and specific growth rate of 0.033 l/h. The maximal cell dry mass after 60 h reached about 14.2 g/l. During this phase, the glucose was gradually consumed at a rate of about 0.156 g/l/h, and reached the limitation level at the end of phase I. The total carbohydrate concentration curves revealed a concomitant consumption of the other carbohydrates in the culture medium (sucrose and starch) and reached about 20 g/l (about 60% of the initial concentration remained unconsumed in the culture). However, during the growth phase, the pH dropped from 5.9 to 2.33 after only 48 h, and then decreased gradually at a very low rate, reaching 2.14 by the end of the cultivation period. During the growth phase, the DO decreased and reached a minimal value of about 26% saturation after 60 h, and then increased gradually thereafter as a function of the cell-growth termination, reaching about 90% saturation by the end of the cultivation process. The production of Cyc A started immediately after the cell inoculation and increased gradually at a volumetric production rate [Q_p] of about 1.9 mg/l/h and a specific production rate [q_p] of about 0.123 mg/g. The maximal volumetric production of 131.4 mg/l was achieved after 75 h of cultivation (about 15 h after termination of the cell-growth phase).

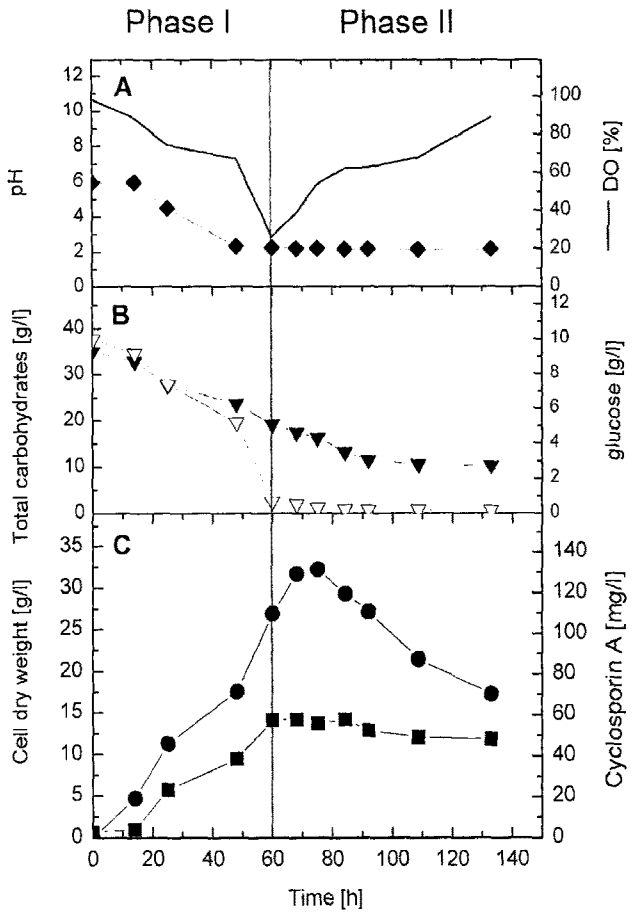

Fig. 2. Kinetics of cell growth and Cyc A production during batch cultivation in a bioreactor under noncontrolled pH.
■, Cell dry weight; ●, Cyclosporin; ▽, glucose; ▼, total carbohydrates; ◆, pH.

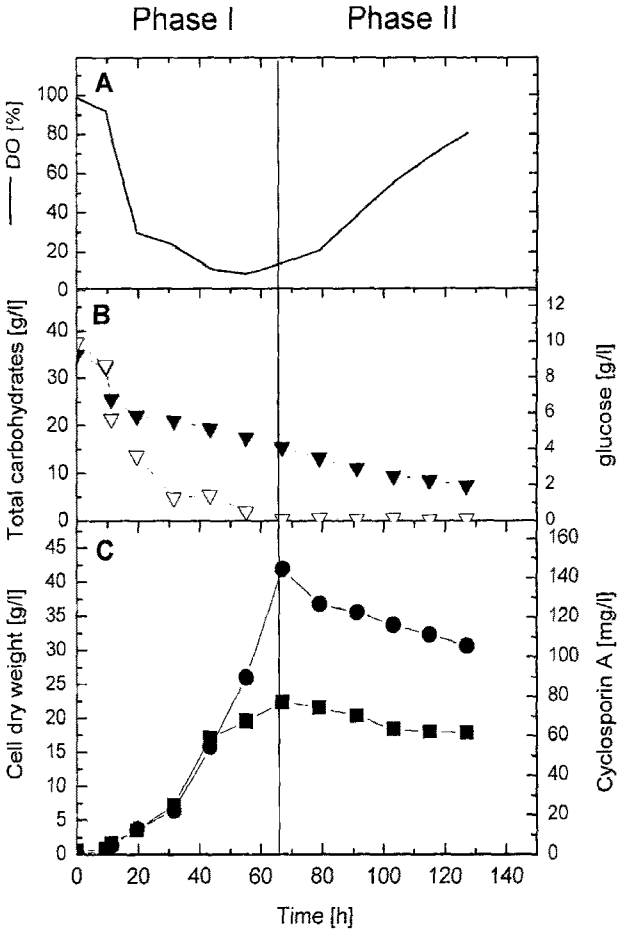

Fig. 3. Kinetics of cell growth and Cyc A production during batch cultivation in a bioreactor under controlled pH.
■, (CDW) Cell dry weight; ●, Cyclosporin; ▽, glucose; ▼, total carbohydrates.

The second phase of cultivation was characterized by a decrease in the biomass at a very low rate of about 0.0180 g/l/h and significant decrease in the antibiotic concentration at a high decomposition rate of about 1.054 mg/l/h. At the end of the cultivation period, after 133 h, the cell mass and antibiotic concentrations were 11.8 g/l and 70.4 mg/l, respectively. However, during the second phase, the carbohydrates (sucrose and starch) were gradually consumed, and no carbon source limitation was observed in the culture until the end of the cultivation period (about 10 g/l of total carbohydrates remained unconsumed).

Cultivation of Cells in Bioreactor Under Controlled pH Conditions

Fig. 3 presents the kinetic data for the cell growth, C-source consumption, and Cyc A production during batch cultivation in the stirred tank bioreactor with a controlled pH of 5.9. Similar to the other cultivations in the shake flask and bioreactor, the cultivation could be divided into two phases. During the growth phase, the cells grew exponentially at a higher rate than the other cultures at about 0.375 g/l/h, while the specific growth rate was 0.045 1/h and the dry cell mass reached a maximal value of 22.4 g/l after 67 h. During the growth phase, the glucose was consumed at a rate of 0.17 g/l/h, and almost completely consumed after 67 h. The carbohydrates at that time were about 15.4 g/l. The Cyc A production started after 10 h and increased at a volumetric production rate [Q_p] of about 2.48 mg/l/h, reaching a maximal volumetric production of 144.7 mg/l after 67 h. During the growth phase, the DO decreased gradually, reaching a minimal value of about 8% saturation after 43 h, and then increased gradually thereafter. During the second phase of cultivation (67–127 h), the cell mass decreased at a very low rate of about 0.07 g/l/h, and reached 17.9 g/l by the end of the cultivation period. The concentration of total carbohydrates also decreased to about 7.5 g/l by the end of the cultivation period (about 21% of the initial concentration). Concomitant to the decrease in the cell mass, a significant decrease in the antibiotic concentration was also observed at a rate of 0.652 mg/l/h, and became about 105 mg/l by the end of the cultivation period.

Morphological Characterization During Submerged Cultivation of *T. inflatum* in Stirred Tank Bioreactor

Figs. 4-1 to 4-8 show the change in morphology during the cell cultivation. The inoculum, which was mainly in small micropellet aggregates imbedded in a mycelial network, was obtained from a 48 h-old shake flask culture. During the first hours of cultivation, the growth was mainly in the micropellet structure in the mycelial network. After 15 h, the microbial growth turned to a pelleted form with a hairy surface. As the cultivation time increased, the pellet increased in both diameter and density. After 40 h of cultivation, many hyphal aggregates were observed and shown to be connected together with a mycelial network. The number and diameter of the small hyphal aggregates increased with time, along with a significant reduction in the mycelial network. After 100 h, the large pellets underwent a process of lysis and degradation, and most of the large pellets were deformed by the end of the cultivation period. The data in Table 1 summarize the different phases of the growth morphology during the cultivation process.

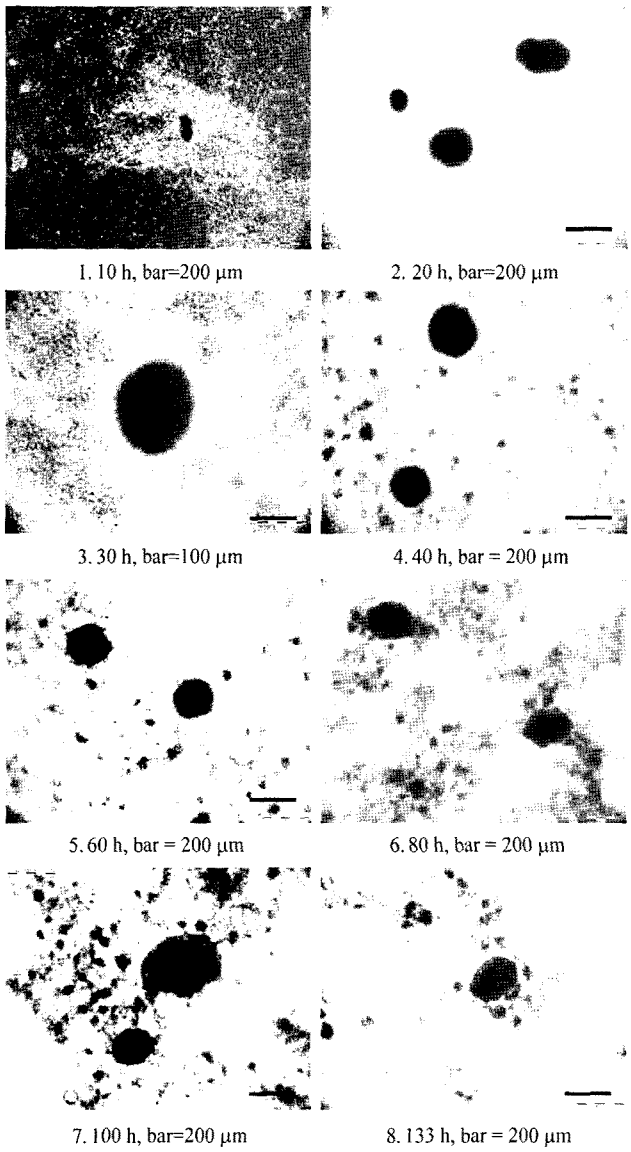

Fig. 4. Morphological characterization of the submerged growth of *T. inflatum* during batch cultivation in a stirred tank bioreactor.

Discussion

The data revealed many significant differences in the cell growth and Cyc A production kinetics, and the differences between the cultivation parameters are summarized in Table 2. The bioreactor cultures were characterized by a higher cell mass of about 100% compared with the shake flask cultures under the same cultivation conditions (uncontrolled pH). When controlling the pH during cultivation, the cell mass increased about 3.1-fold and 1.6-fold when compared with that for the shake flask cultures and uncontrolled bioreactor cultures, respectively. The specific growth rate [μ] for the controlled bioreactor culture was also higher than that for the uncontrolled bioreactor and shake flask cultures. The increased growth in the bioreactor cultures was due to a higher aeration and agitation, reflecting better mixing between the air/liquid phase and increased oxygen availability in the culture [4, 14]. For growth, aerobic microorganisms require good oxygen availability in a culture to eliminate oxygen as a growth-limiting factor. Conversely, the high shear in the bioreactor cultures resulted in cell decomposition, which decreased the cell mass after the growth phase (in unlimited nutrient conditions). However, this observation was also supported when calculating the value of the cell decomposition rate (-dx/dt) during the second phase. The cell decomposition rate was a minimal value of 0.0059 g/l/h. However, the cell decomposition was higher in the pH-controlled culture. Since no C-limitation was observed in any of the cultures, the cell degradation was mainly due to the higher shear stress in the bioreactor compared with that in the shake flasks, and may also have been due to the protease enzyme effect after cell termination. This is also supported by the higher cell autolysis under controlled pH conditions (5.9), which is more suitable for protease enzyme activity than a pH of less than 3, as in the case of the uncontrolled pH cultures in the shake flask and bioreactor.

Various studies have already noted the role of shear stress and nonspecific proteases in the cell degradation of filamentous microorganisms. For example, the cell autolysis of *Penicillium chrysogenum* and production of proteases were accelerated by shear stress in a bioreactor [8, 17], whereas the presence of proteases in culture media was found to be responsible for the degradation of produced antibiotics [2, 28]. It has also been reported that the susceptibility of *Saccharomyces erythraea*, an erythromycin-producing actinomycetes, to mechanical damage is higher in a nutrient-limited culture [9]. Moreover, the presence of

Table 1. Different phases of growth during cell growth in bioreactor under controlled pH conditions.

Time (h)	Morphological characteristics
10	Growth was mainly in a filamentous form, with small micropellet structure mainly of irregular form imbedded in mycelial network. Small number of mycelial aggregates.
15	The first arise of small microbial pellet form. The microbial pellets turned to a more regular spherical form. The size of pellets varied and ranged from 50 μm upto 200 μm.
20	Pellet form was predominant and the density of pellet increased with time. The outer pellet surface was mainly in a hairy form. The size of pellets ranged between 75 and 200 μm.
30	Pellet density increased significantly. Pellet diameter ranged between 75 and 200 μm.
40	Small hyphal aggregates were observed imbedded in mycelial network with a dense core (micropellet structure) with diameter not exceeding 50 μm. Pellet size ranged between 75 and 200 μm
60	The number of micropellets increased significantly. Pellet size ranged between 75 and 250 μm
80	The size of micropellet increased with significant reduction of mycelial network. Pellet size ranged between 75 and 250 μm
100	The size of micropellet increased with continuous reduction in mycelial network fraction. The large pellets (larger than 200 μm) start to be deformed significantly.
115	The dispersed disintegrated mycelial network fraction was dominant. Pellet size ranged between 75 and 150 μm. The larger pellets were completely deformed (broken in irregular way).
133	Culture was mainly in the form of a disintegrated mycelial network. Pellet structure was rare and mainly deformed.

a high antibiotic concentration in a culture medium can accelerate cell autolysis and play a regulatory role in the autolysis of the producer strain [26]. Thus, high shear stress, protease production, and antibiotic production are all factors causing higher cell lysis in a bioreactor culture under controlled pH conditions.

Table 2. Kinetic parameters of cell growth and Cyc A production by *Tolypocladium inflatum* during shake flask and bioreactor cultivations in batch cultures under pH-uncontrolled and -controlled conditions.

Parameter	Type of cultivation vessel		
	Shake flask	Bioreactor	
	Uncontrolled pH	Uncontrolled pH	Controlled pH
	Cell growth and Cyclosporin production phase (Phase I)		
X_{max} [g/l]	7.25	14.2	22.4
dx/dt [g/l/h]	0.088	0.284	0.375
μ [1/h]	0.028	0.033	0.045
P_{max} [mg/l]	76	131.4	144.72
Q_P [g/l/h]	1.10	1.90	2.48
$Y_{P/X}$ [mg/g]	7.58	9.26	6.46
q_p [mg/g/h]	0.08	0.123	0.096
	Cell lysis and Cyclosporin degradation phase (Phase II)		
-dx/dt [g/l/h]	0.0059	0.0180	0.070
$-Q_P$ [g/l/h]	-	1.054	0.652

Abbreviations: X_{max}, maximal cell dry weight; dx/dt, cell growth rate; -dx/dt, cell degradation rate; μ, specific cell growth rate; P_{max}, maximal volumetric Cyc A production; Q_P, volumetric Cyc A production rate; $-Q_P$, volumetric Cyc A degradation rate; q_p, specific Cyc A production rate. Yield coefficients: $Y_{P/X}$, [mg] Cyc A produced/[g] cell dry weight.

The maximal volumetric production of Cyc A was higher in the bioreactor cultures than in the shake flask cultures by about 73% under non-pH-controlled conditions. When controlling the media pH during the cultivation process, the antibiotic production was about 90% higher than the maximal concentration in the shake flask cultures. In addition to the higher total volumetric production in the bioreactor, the rate of production [Q_p] almost doubled when transferring the process from the shake flask to the bioreactor with a controlled pH. This also resulted in a significant reduction in the production time from 233 h to only 70 h. However, the increased Cyc A production in the case of the controlled pH culture resulted from a higher cell mass rather than an increase in cell productivity.

As bioreactor cultivations are characterized by a short production time, the antibiotic decomposition was highly significant after reaching its maximal value. The rate of Cyc A degradation [$-Q_p$] was higher in the pH-uncontrolled culture, at about 1.054 mg/l/h. This rate was lower in the pH-controlled culture by about 38%. The decrease in the Cyc A concentration after reaching its maximal value resulted from a combined effect of a low pH and other factors affecting antibiotic degradation. The decrease in Cyc A concentration during the stationary phase may also have been due to cultivation parameters other than the pH, such as the aeration and agitation. The high cell mass concentration in the bioreactor culture, which was about 96% and 209% higher than that in the shake flask cultures with an uncontrolled and controlled pH, respectively, may also have accelerated the antibiotic decomposition through a self-resistance mechanism. Antibiotic self-resistance by the producing strain after cell growth has already been reported for many antibiotics [25]. However, the production of growth-protecting substances by growing cells simultaneously to product formation is

also known to be an important mechanism by which antibiotic-producing species avoid cell death [15, 16].

The effect of growth morphology on metabolite production by filamentous microorganisms has already been reviewed by many authors [7, 19]. In general, the cultivation of cells in the form of a pellet not exceeding 400 μm in diameter is the best morphological feature for the maximal production of different antibiotics [27, 29]. In the present study, the pellet diameter did not exceed 250 μm, ensuring better oxygen transfer in the pellet and better oxygenation inside the biopellet to reach maximal antibiotic productivity.

In conclusion, this comparative study of results obtained from shake flask and bioreactor cultures revealed that the rate of cell growth, antibiotic production, and antibiotic degradation in the bioreactor were all higher than those observed in the shake flask. Moreover, the pH-controlled culture was characterized by a higher cell mass, higher volumetric production, and less specific production compared with the uncontrolled pH culture. These differences were due to differences in the aeration, agitation, and mixing characteristics between the shake flask and bioreactor cultures, and also significantly decreased the production time.

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