

Photobioreactor scale-up for a shear-sensitive dinoflagellate microalga

F. García Camacho^{a,*}, J.J. Gallardo Rodríguez^a, A. Sánchez Mirón^a, E.H. Belarbi^a,
Y. Chisti^b, E. Molina Grima^a

^a Department of Chemical Engineering, University of Almería, 04120 Almería, Spain

^b School of Engineering, Massey University, Private Bag 11 222, Palmerston North, New Zealand

ARTICLE INFO

Article history:

Received 21 October 2010

Received in revised form 6 December 2010

Accepted 3 January 2011

Keywords:

Dinoflagellates

Protoceratium reticulatum

Microalgae

Photobioreactors

Yessotoxins

Shear rate

ABSTRACT

Large scale culture of marine dinoflagellate microalgae has proved difficult owing to their extreme sensitivity to hydrodynamic forces in photobioreactors. This work discusses the scale up of *Protoceratium reticulatum* dinoflagellate culture from a successful 2 L stirred-tank photobioreactor operation to a 15 L stirred photobioreactor. Both bioreactors were equipped with internal spinfilters for cell retention. A semicontinuous perfusion culture in the 15 L photobioreactor proved to be more productive than fed-batch perfusion culture. Under the best operational conditions, the average cell productivity in the 15 L photobioreactor was 5228 cell mL⁻¹ day⁻¹, or nearly 3.7-fold greater than the best attainable value in static flask cultures, but similar to the results obtained in the 2 L stirred tank. At 9.16 µg L⁻¹ day⁻¹ the average volumetric productivity of yessotoxin in the scaled up operation in the semicontinuous perfusion mode was comparable to the results obtained in the small bioreactor. *P. reticulatum* has a cell damage threshold shear rate of as low as 0.1 s⁻¹. Notwithstanding this extreme shear sensitivity, the results suggest a good potential for mass scale culture of this dinoflagellate in suitably designed photobioreactors.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Dinoflagellates are one of the several divisions of microalgae. Dinoflagellates occur both in freshwater and in the seas. Although microalgae have an established history of commercial use and new uses are emerging [1], this is not the case for dinoflagellates. Dinoflagellates produce many structurally complex bioactive compounds of potential commercial interest [2], but development of marketable products from them has proved difficult. Small quantities of bioactives derived from dinoflagellates can be purchased, but generally at a prohibitively high price and in low purity. Unlike the other microalgae that are commonly grown in large-scale photobioreactors [3–6], many dinoflagellates appear to be overly sensitive to the myriad of hydrodynamic forces that are encountered in the turbulent environment of a typical photobioreactor.

Photosynthetic marine dinoflagellates respond to hydrodynamic shear forces in diverse ways [7]. Turbulence sensitivity has been studied for several dinoflagellate species, but invariably from an ecological perspective that may be relevant in the natural habitat, but not in photobioreactor culture. Small scale studies that are potentially relevant to photobioreactor culture have been reported using the highly sensitive dinoflagellate *Protoceratium reticulatum*. This microorganism is damaged by shear rate values as low as

0.1 s⁻¹ [8]. It is therefore a good model system for studies of photobioreactors that are intended for culturing highly shear sensitive microalgae. At operational scales of 2 L or less, the use of modified turbulence regimens and protective additives in the culture medium have been shown to reduce turbulence-associated damage to *P. reticulatum* [8–10]. How this successful operational capability at small scale might translate to a larger photobioreactor has not been previously addressed for dinoflagellates.

Scale-up of photobioreactors is complicated by a nonhomogeneous distribution of light within the culture as a consequence of the self-shading by cells. For microalgae that are physically robust, increased intensity of agitation can be used to limit the time that a cell spends uninterrupted in the relatively dark interior of the bioreactor. This strategy is not workable with highly fragile dinoflagellates. Therefore, photobioreactor design and scale up methods that have proved successful for many relatively robust microalgae [3] cannot be directly translated to culturing the fragile dinoflagellates. This work reports on scale up of *P. reticulatum* culture from a previously reported 2 L stirred-tank photobioreactor [7,9] to a 15 L photobioreactor.

2. Materials and methods

2.1. Species and culture medium

Nonaxenic monocultures of the red-tide dinoflagellate *Protoceratium reticulatum* (GG1AM) were used. This yessotoxins (YTXs)-producer was obtained from the Culture Collection of Harmful Microalgae of IEO, Vigo, Spain. Inocula were grown in filter sterilized (0.22 µm Millipore filter) L1 medium prepared in Mediterranean

* Corresponding author. Fax: +34 950015484.
E-mail address: fgarcia@ual.es (F.G. Camacho).

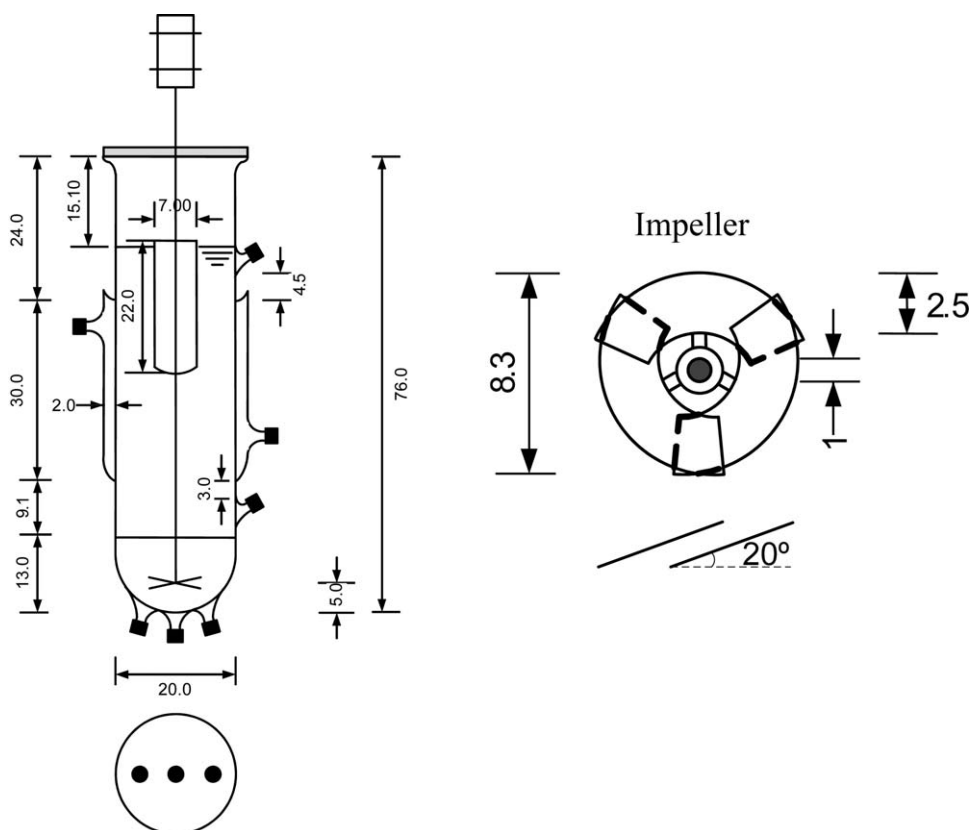


Fig. 1. The scaled-up photobioreactor. Dimensions in cm.

Sea water [11]. The alga was grown at 18 ± 1 °C under a 12:12 h light–dark cycle. During the photoperiod, the average irradiance at the surface of the cultures was $100 \mu\text{E m}^{-2} \text{s}^{-1}$. Four overhead Phillips TLD 36W/54 fluorescent lamps were used for illumination. Inocula were prepared in surface aerated 2 L Erlenmeyer flasks.

2.2. Photobioreactors

Optimal production of *P. reticulatum* biomass in a 2 L stirred tank photobioreactor (2L-STB) (Braun Biotech, Germany) has been previously reported [7,9]. The current study focussed on scaling up the previously reported successful process to 15 L in a stirred photobioreactor (15L-STB) equipped with an internal spinfilter for cell retention.

The smaller bioreactor (2L-STB) was non-baffled and had the following relevant dimensions: 0.13 m culture depth; 0.13 m vessel diameter; 0.055 m impeller diameter (a 3-bladed impeller; 20°-blade pitch; 0.013 m blade width; 0.055 m blade length); and 0.02 m clearance of the impeller from the bottom of the vessel. An internal nylon (10 μm mesh) spinfilter (diameter = 5.5 cm; height = 11.5 cm) was installed on the impeller shaft for perfusion culture runs. The temperature, pH and dissolved oxygen (DO) concentration were measured online (Biostat B-DCU control unit; Braun Biotech, Germany) and logged by a computer. The culture temperature was controlled at 18 ± 1 °C by a thermostated glass jacket that surrounded the bioreactor vessel.

The larger 15 L stirred photobioreactor (15L-STB) was custom built to a design that was based on experience with the 2 L bioreactor. The 15 L bioreactor vessel was made of 5 mm thick borosilicate glass and had an internal diameter of 0.193 m. The aspect ratio of the vessel was 4.5. The internal spinfilter was made of the same material as described above for the small reactor. The spinfilter had a diameter of 7 cm and a height of 22 cm. The gas-free liquid height was about 0.61 m. The working volume was 15 L. A 3-bladed marine propeller was used for mixing. The propeller was located 0.05 m above the bottom of the photobioreactor vessel. As direct sparging with air damages *P. reticulatum* [8], a perforated pipe air sparger was located within the spinfilter to prevent direct contact between the algal cells and air bubbles. The air flow rate was 4.5 L min^{-1} , or 0.3, v/v min. The complete geometric details of the culture vessel appear in Fig. 1.

The large photobioreactor was illuminated with up to 5 Phillips TLD 36W/54 fluorescent lamps placed around its periphery. The incident irradiance at the surface of the photobioreactor and in the center of the vessel was measured using a QSL-100 quantum scalar irradiance sensor (Biospherical Instruments, San Diego, USA). The incident irradiance (photosynthetically active) ranged from 242 to $766 \mu\text{E m}^{-2} \text{s}^{-1}$. During culture, the light level was progressively increased to prevent limitation of

growth as a consequence of the increasing cell concentration. The dissolved oxygen concentration (DO), measured as a percentage of the oxygen concentration in air-saturated seawater, was monitored continuously as an indicator of the photosynthetic activity. The monitoring electrode was calibrated before each run. The culture temperature was controlled at 19 ± 1 °C by circulating thermostated water through the glass jacket that surrounded a portion of the photobioreactor vessel. The pH was controlled at pH 8.4 by automatic injection of carbon dioxide within the spinfilter, as needed. The sparger used for injecting carbon dioxide was separate from the earlier mentioned air sparger. This prevented mixing of CO_2 with the air and enhanced the mass transfer of CO_2 .

Prior to each experimental run, the photobioreactor was thoroughly cleaned and any salt deposits were removed by an acid wash. For this, the bioreactor vessel was filled with tap water and concentrated hydrochloric acid (40 mL, 35%, w/w HCl) was added. The vessel was then sparged with air for at least 2 h. The bioreactor was then washed with tap water. The photobioreactor and associated pipework were sterilized by circulating filtered seawater (0.02 m^3) mixed with 10 mL of sodium hypochlorite (10% solution) for 2 h. The bioreactor was then rinsed with filter sterilized seawater until the pH of the rinse became pH 8.6. The reactor was then filled with the L1 medium made in filter sterilized Mediterranean sea water. The medium (15 L) was inoculated using 0.003 m^3 of an inoculum that was in the late exponential growth phase. The cell concentration in the freshly inoculated bioreactor was about $15,000 \text{ cells mL}^{-1}$.

The photobioreactor was operated as a perfusion culture (i.e. the cells remained in the bioreactor) in the fed-batch and semicontinuous modes in different experiments. In semicontinuous experiments, the volume of the culture medium removed was replenished with an equal volume of the fresh medium. When cell growth ceased, small volumes of highly concentrated aliquots of the L1 medium were added in order to increase the nutrient concentration in the culture medium to higher than the values in the normal L1 medium. The culture setup is shown in Fig. 2.

2.3. Hydrodynamic characterization of photobioreactors

The acid tracer method [12] was used to measure the mixing time (θ_m), a direct measure of the mixing capability of a reactor. Mixing time was defined as the time required to attain a 5% deviation from complete homogeneity from the instance of tracer addition. For tracer response measurements, the reactor was filled with seawater and the pH was reduced to 2 by adding hydrochloric acid (35%, w/v). The vessel was then bubbled with air for 20 min to remove any dissolved carbonates in the form of carbon dioxide. The stirrer speed was then set and the pH was raised to pH 4.5 by adding 12 M sodium hydroxide. The acid tracer (2 mL of 35% hydrochloric

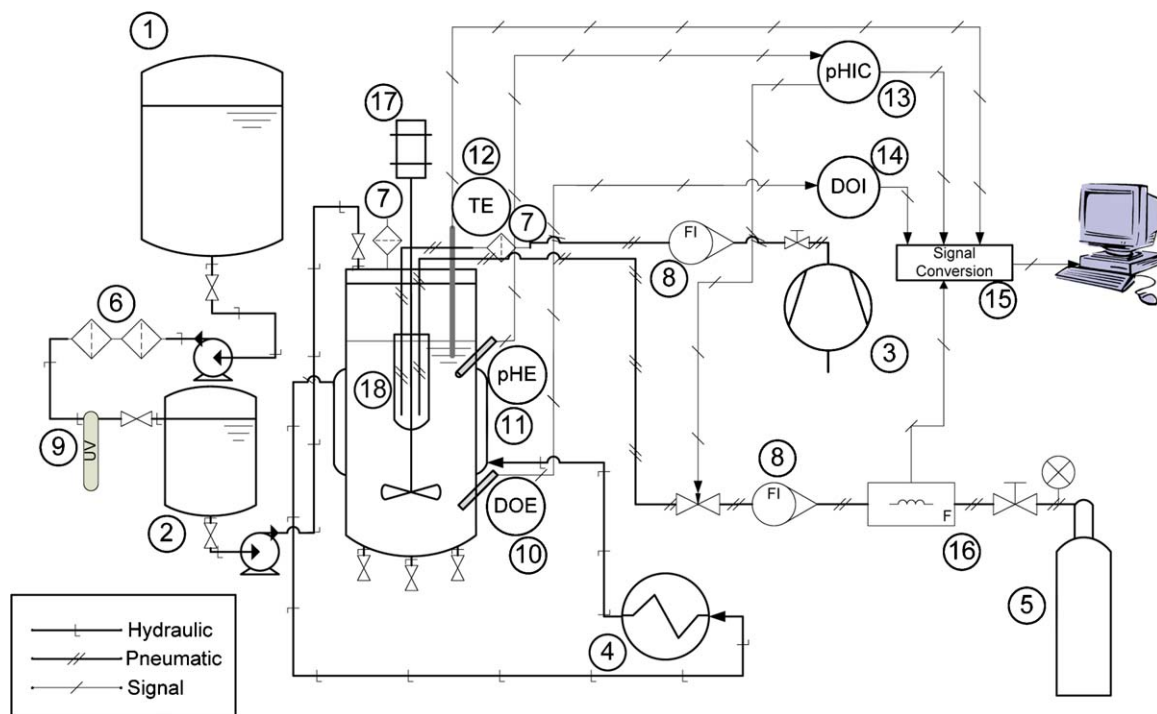


Fig. 2. Flow setup for the scaled-up photobioreactor: (1) seawater reservoir; (2) medium tank; (3) air compressor; (4) chiller for cooling water; (5) carbon dioxide cylinder; (6) water filters; (7) gas filters; (8) flow meter; (9) ultraviolet lamp; (10) dissolved oxygen electrode; (11) pH electrode; (12) temperature probe; (13) pH indicator and controller; (14) dissolved oxygen indicator; (15) data acquisition card; (16) carbon dioxide mass flow meter; (17) stirrer motor; (18) spinfilter.

acid) was now added instantaneously at the center of the surface of the dispersion. The pH did not go below 2.0 at any time during the measurements. The change in pH with time was monitored using a pH electrode. The dimensionless concentration C_T of the tracer (hydrogen ion) was calculated as follows:

$$C_T = \frac{[H^+]_{\text{instantaneous}} - [H^+]_{\text{initial}}}{[H^+]_{\text{final}} - [H^+]_{\text{initial}}} \quad (1)$$

All experiments were carried out in triplicate. The pH data were recorded digitally using an IBM PC compatible computer.

The average energy dissipation rate, ε_p , in the stirred bioreactor was calculated using the broadly applicable method of Ruszkowski [13] and Grenville et al. [14], as follows:

$$\varepsilon_p = \left(\frac{AT}{\theta_m D^{1/3}} \right)^3 \quad (2)$$

where A is a constant ($= 5.9$), D is the impeller diameter, T is the tank diameter, and θ_m is the mixing time. Eq. (2) implies that tanks having a given D/T ratio are equally efficient in the mixing process. That is, the relationship between ε_p and θ_m is independent of the type of stirrer.

The Kolmogorov's microscale of turbulence was estimated with the following equation [15]:

$$\lambda = \left(\frac{\mu_L}{\rho_L} \right)^{3/4} \varepsilon_p^{-1/4} \quad (3)$$

where μ_L is the broth viscosity.

The local maximum value of the energy dissipation rate (ε_{\max}) was estimated from the general equation of Liepe et al. [16] for the liquid volume in the vicinity of the stirrer [17]; thus:

$$\varepsilon_{\max} = 0.1 \left(\frac{u^3}{h_1} \right) \quad (4)$$

where h_1 is a characteristic length dimension, in this case the height of the impeller (see Fig. 1), and u is the relative liquid velocity, or the impeller tip velocity. The latter was calculated using the following equation:

$$u = \pi N_R D \quad (5)$$

where N_R is the rotational speed (rev s^{-1}) of the impeller.

The fluid circulation time (t_R) in the tank was estimated using the following equation:

$$t_R = \frac{V_L}{Q} \quad (6)$$

where Q is the volume flow rate generated by the impeller and V_L is the culture volume. The former was calculated using the following equation:

$$Q = N_Q N_R D^3 \quad (7)$$

where N_Q is the impeller Flow number. The Flow number was estimated as follows. First the average shear rate values were calculated for various agitation speeds using the following equations for unbaffled vessels [18]:

$$\gamma_p = \frac{112.8 N_R r_D^{-1.8} (r_T^{0.2} - r_D^{0.2}) (r_c / r_D)^{1.8}}{r_T^2 - r_D^2} \quad (8)$$

$$\frac{r_c}{r_D} = \frac{\text{Re}}{1000 + 1.6 \text{Re}} \quad (9)$$

where r_D , r_T , and r_c are the radii of the impeller, the bioreactor, and the forced vortex zone, respectively.

A plot of the average shear rate (γ_p) versus the rotational speed N_R had the characteristic constant slope k_f [19,20] as shown in Fig. 3 for the smaller bioreac-

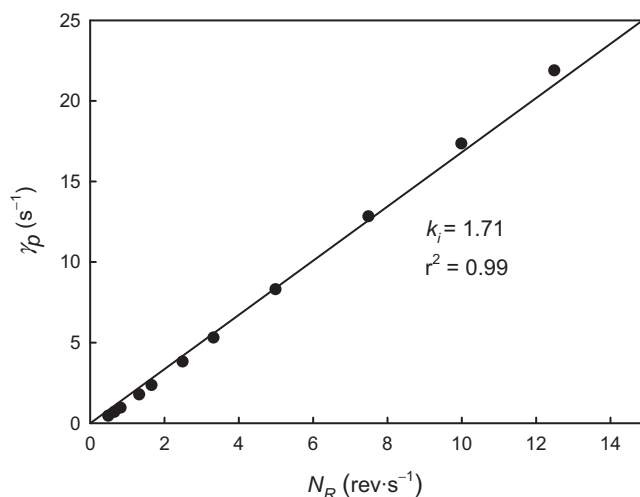


Fig. 3. Average shear rate γ_p in the small bioreactor (2L-STB) versus the impeller rotational speed (N_R).

tor 2L-STBs. The value of k_i for the impeller used was therefore 1.71. For different combinations of stirred tanks and axial-type impellers, the k_i has been previously shown to depend on the Flow number (N_Q) [21]. Using the experimentally determined value of k_i , a N_Q value of 0.24 could be read from the published correlation (Fig. 4 of ref. 21) [21].

2.4. Kinetic parameters

Growth was determined by cell counts. Broth samples (1 mL) were collected daily, fixed with Lugol's solution, and the cells were counted on a Sedgewick–Rafter counting slide. The cell-specific growth rate μ (days^{-1}) was calculated using the following equation:

$$\mu = \frac{\ln N_{i+1} - \ln N_i}{t_{i+1} - t_i} \quad (10)$$

where N_{i+1} and N_i are the cell concentrations (cells mL^{-1}) at times t_{i+1} and t_i (days), respectively.

2.5. Flow cytometric measurements

Flow cytometry was used for measuring the relative mean cell size and the concentration of reactive oxygen species (ROS) in the cells [15]. All flow cytometric measurements were carried out in a Coulter Epics XL-MCL (Beckman Coulter, Inc.) flow cytometer as described previously [15].

2.6. Determination of YTXs concentration

Yessotoxins (YTXs) were measured separately in the biomass as well as in the culture supernatant following the method of Paz et al. [22]. A HPLC system (Shimadzu AV10; Shimadzu Corporation, Kyoto, Japan) with a fluorescence detector (Shimadzu RF-10AX) and an autoinjector (Shimadzu SIL-10ADVP) was used.

3. Results and discussion

Scale-up of mechanically mixed photobioreactors for culturing dinoflagellates was the focus of this work. The scale up study was informed by prior experience of a successful culture in a 2 L photobioreactor, the 2L-STB [7,9], and the experimentally established limits on shear stress tolerance of *P. reticulatum* in shake flasks [8,15]. First, the fluid-dynamics in the 2L-STB were characterized under different operational regimens that had previously proved successful for *P. reticulatum* culture. This informed the design of the larger bioreactor 15L-STB and selection of the suitable operational regimens for evaluating the dinoflagellate culture in it.

3.1. Fluid-dynamics in 2L-STB

The 2 L stirred bioreactor (2L-STB) used an axial flow impeller and a baffles-free construction because such reactors have been successfully operated at quite large scales for growing fragile animal cells in suspension [17]. In bioreactors for animal cells, a relatively large pitched-bladed impeller is used as it can provide good mixing at a relatively low agitation speed. In keeping with this strategy, the impeller used was relatively large compared to the tank diameter, i.e. T/D ratio was 2.36 instead of a value of 3 that is typically used in stirred bioreactors intended for culturing microorganisms.

Mixing time in 2L-STB was characterized as an important index of the ability to attain homogeneity in the culture. The mixing time values are shown in Fig. 4 for various rotational speeds of the impeller. Consistent with a characteristic pattern, mixing improved rapidly, i.e. the mixing time declined, as the impeller speed increased up to a value of around 200 rpm. This was the transitional flow regime in which the bulk flow produced by the impeller had a significant influence on mixing time. Once the agitation speed exceeded about 200 rpm, the mixing time was barely affected by impeller speed (Fig. 4). This was the turbulent flow regime in which the eddies in the fluid had a greater influence on the mixing characteristics compared to the bulk flow produced by the impeller. In the transitional flow regime, the installation of the spinfilter actually improved the mixing somewhat compared

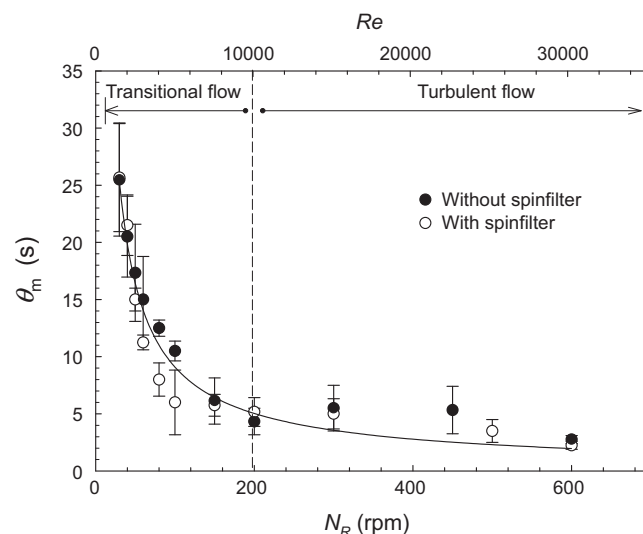


Fig. 4. Mixing time (θ_m) in the small bioreactor (2L-STB) versus the rotational speed (N_R) and the Reynolds number (Re) of the impeller. Data are shown with and without the spinfilter installed.

to operation without the spinfilter. This was due to turbulence produced by the spinfilter which rotated at the same speed as the impeller. In the turbulent regime, the spinfilter barely affected the mixing, as the turbulent eddies within the fluid were largely responsible for the observed mixing effect.

The average energy dissipation rate ε_p (Eq. (2)) and the maximum energy dissipation rate ε_{max} (Eq. (5)) in the reactor are shown in Fig. 5 for various values of the impeller speed. The ratio $\varepsilon_{max}/\varepsilon_p$ is also shown (Fig. 5) as an indicator of homogeneity of turbulence. The maximum energy dissipation rate, a measure of energy dissipation in the vicinity of the impeller, increased linearly with increasing agitation rate as expected. In contrast, $\varepsilon_{max}/\varepsilon_p$ increased exponentially with increasing agitation speed of the impeller (Fig. 5). This implied an increased spatial nonuniformity in the intensity of turbulence in the bioreactor. Notwithstanding this increased heterogeneity of turbulence, at the highest impeller speed, the $\varepsilon_{max}/\varepsilon_p$ value was well below 80. For comparison, $\varepsilon_{max}/\varepsilon_p$ values of nearly 100 are encountered in highly agitated bioreactors [23]. The presence of spinfilter considerably increased ε_p in the transitional flow region ($2500 \leq Re \leq 10,000$), to reduce the heterogeneity of turbulence (see Fig. 5).

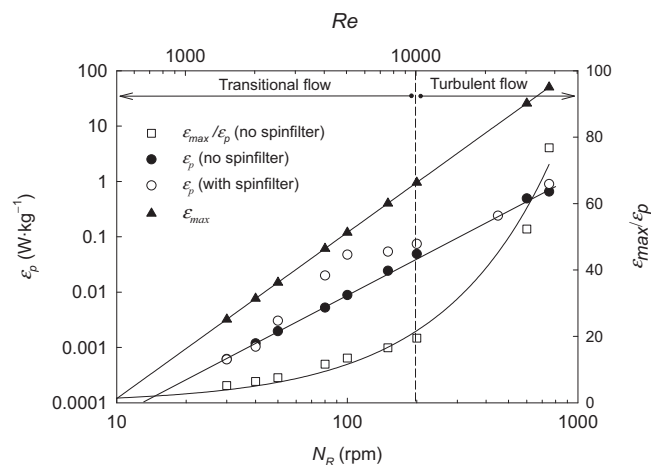


Fig. 5. Effects of impeller rotational speed (N_R) on the average energy dissipation rate (ε_p), the theoretical maximum energy dissipation rate (ε_{max}) and the homogeneity of turbulence (i.e. $\varepsilon_p/\varepsilon_{max}$). Data are for 2L-STB with and without the spinfilter.

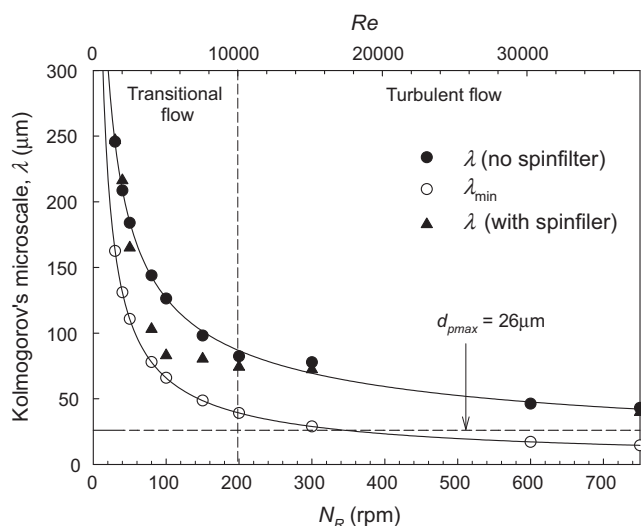


Fig. 6. Kolmogorov's microscale (λ) versus the impeller rotational speed (N_R) in the small reactor (2L-STB) with and without the spinfilter. The value of λ_{\min} was calculated using the maximum energy dissipation rate ε_{\max} . The maximum diameter measured for *P. reticulatum* is shown as $d_{p\max}$.

3.2. Shear stress in 2L-STB

Kolmogorov's microscale (λ) of turbulence is a measure of the size of the smallest fluid eddies in an isotropically turbulent field. Microeddies with a length scale that approaches the dimensions of the cells suspended in a fluid have the potential to interact with the cells and damage them [4]. The estimated (Eq. (4)) microeddy size (λ) at various agitation speeds in the 2 L bioreactor is shown in Fig. 6. The λ -values were estimated for the maximum energy dissipation rate that occurred in the vicinity of the impeller as well as for the average energy dissipation rate. Throughout the transitional flow regimen, the microscale of turbulence remained above the maximum measured cell size of 26 μm for *P. reticulatum* (Fig. 6); however, for ensuring that the microeddy length scale remained well above (e.g. 4-fold above) the dimensions of the cells, an allowable upper limit on the impeller agitation speed was approximately 50 rpm for the bioreactor with the spinfilter (Fig. 6). This upper limit was based on the maximum energy dissipation rate, as the cells had to unavoidably pass through the impeller zone on a periodic basis. The allowable upper limit on the microeddy size and, therefore the impeller speed, was consistent with experimental observations in shake flasks where a flow regimen with λ -values of <100 μm was found to damage *P. reticulatum* cells [8].

In shake flasks, the shear damage threshold for *P. reticulatum* corresponded to an average energy dissipation rate of approximately 8.8 mW kg^{-1} [8]. In the 2L-STB this level of average energy dissipation occurred at an impeller speed of 100 rpm (Fig. 5), but at this speed the value of the maximum energy dissipation rate was 120 mW kg^{-1} (Fig. 5), or 14-fold greater than the damaging threshold in the shake flasks. Furthermore, at an agitation speed of 100 rpm, the spinfilter also dissipated energy at an estimated level of 48 mW kg^{-1} , that was substantially above the damaging threshold in shake flasks [8].

The shear stress at the surface of a spinfilter could be conservatively estimated using the analysis of Bird et al. [24] for a Stormer viscometer. In a Stormer viscometer an inner cylinder of dimensionless radius f rotates with an angular speed in rads^{-1} ($N_{R\omega}$) inside a stationary outer cylinder of radius R . At the surface of the inner cylinder the shear stress τ_i can be estimated as follows:

$$\tau_i = 2\mu L N_{R\omega} \left(\frac{1}{1-f^2} \right) \quad (11)$$

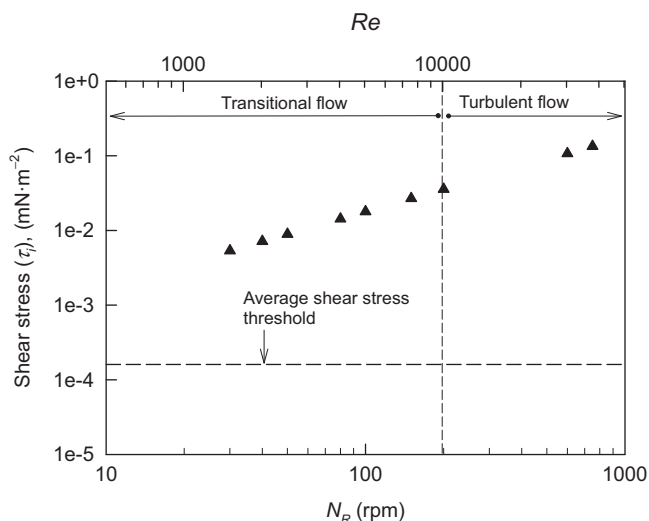


Fig. 7. Relationship between the impeller rotational speed (N_R) and the theoretical shear stress (τ_i) on the surface of the spinfilter installed in the small reactor (2L-STB).

For the bioreactor 2L-STB, the diameters of the spinfilter and tank were 5.5 and 13 cm, respectively; therefore the f -value was 0.42.

The shear stress at the surface of the spinfilter varied with the impeller agitation speed and the Reynolds number, as shown in Fig. 7. Clearly, the shear stress at the surface (Fig. 7) was much greater than the damaging shear stress threshold reported [8], but this was fully tolerated by the cells at least up to a rotational speed of 50 rpm [9]. This apparent discrepancy was explained by the cells having been kept out of the boundary layer of the spinfilter by the local hydrodynamic forces, as reported by others. For example, Figueredo-Cardero et al. [25] showed that the concentration of the cells near the surface of a rotating cylindrical filter mesh was low compared to the concentration in the bulk fluid. Therefore, for all practical purposes, the cells did not experience the shear stress associated with the spinfilter. As observed with certain cells [26], dinoflagellate cells could potentially be made to adapt to higher levels of shear stress than they would normally tolerate.

Based on the above considerations, a conservative impeller/spinfilter rotation speed value of 50 rpm appeared to be acceptable for a successful culture of *P. reticulatum* in the 2L-STB equipped with a spinfilter [7,9]. At this value of the rotation speed, the average energy dissipation (3.0 mW kg^{-1} including the spinfilter) was far below the damaging threshold and the maximum energy dissipation value was 14.9 mW kg^{-1} , or a little higher than the damaging threshold.

3.3. Scale-up considerations

Two scaling-up criteria were considered in designing the 15 L stirred photobioreactor (15L-STB). The first criterion related with the frequency of passage of the cells through the high-shear zone of the impeller. Frequency of the cell passage through the impeller zone is directly related to the circulation time (t_R) in a stirred reactor. Therefore, a possible basis for scale up of a shear sensitive culture is to attempt to keep the circulation times at the different scales the same. The circulation times and the volume flow rate generated by the impeller were calculated for the small bioreactor (2L-STB) and a hypothetical 15 L bioreactor using Eqs. (2)–(9). The calculated values are shown in Table 1. Two different impeller sizes were evaluated for the larger bioreactor. At the agitation rate of 50 rpm that had given good results in the small bioreactor, the circulation time was estimated to be 54 s (Table 1). For attaining approximately the same circulation time in the larger bioreactor at

Table 1

Circulation time (t_R) and impeller flow rate (Q) values in photobioreactors 2L-STB and 15L-STB for various combinations of the impeller diameter (D) and rotational speed (N_R).

N_R (rpm)	2L-STB		15L-STPB			
	$D = 5.5$ cm		$D = 8.1$ cm		$D = 11$ cm	
	Q ($L s^{-1}$)	t_R (s)	Q ($L s^{-1}$)	t_R (s)	Q ($L s^{-1}$)	t_R (s)
30	0.020	90	0.065	232	0.160	94
40	0.027	68	0.086	174	0.213	70
50	0.033	54	0.108	139	0.266	56
80	0.053	34	0.173	87	0.426	35
100	0.067	27	0.216	70	0.532	28
150	0.100	18	0.324	46	0.799	19

an agitation speed of 50 rpm, the reactor was predicted to require an impeller that had nearly twice the diameter (i.e. 11 cm) of the impeller used in the small bioreactor (Table 1).

The second scaling-up criterion frequently used with fragile cells consists of keeping the tip speed (u) of the impeller identical at the different scales [27]. This is because the tip speed is directly correlated with the maximum shear rate in a stirred bioreactor. For the small bioreactor (2L-STB) operated at an impeller rotational speed of 50 rpm, the tip speed was calculated to be 0.144 m s^{-1} . If this same tip speed was to be used in the larger bioreactor with an 11 cm diameter impeller, the rotational speed of the impeller would be too low for most motor-controller systems used in commercial stirred bioreactors. On the other hand, an 11 cm impeller rotating at 50 rpm would have a tip speed of 0.288 m s^{-1} which is probably too high for *P. reticulatum* as it would produce a relatively high maximum energy dissipation (ε_{\max}) value. Therefore, a decision was made to use a smaller impeller (diameter = 8.1 cm).

A further consideration in scale up was the need to minimize light limitations. Therefore, too large an increase in the diameter of the vessel as a consequence of scale up was unacceptable, as previously pointed out in relation to scale up of tubular photobioreactors [28]. In any vessel with a relatively large diameter, a high concentration of biomass, and the light being supplied solely through the outer surface, an optically dark zone would exist in the center. Turbulence is essential to prevent the cells from residing in the dark zone continuously for a long period. The size of the dark zone can be kept to a minimum by limiting the vessel diameter. Consequently, the diameter of the larger bioreactor was fixed at 20 cm and the volume was increased by increasing the aspect ratio 4.5-fold compared to that of the smaller bioreactor. In this taller bioreactor, the impeller-to-tank diameter ratio (D/T) was kept at nearly the same value as in the smaller bioreactor. This led to an impeller diameter of 8.14 cm in the larger vessel. An impeller of this size rotating at 50 rpm would have an acceptable tip speed of 0.2 m s^{-1} , but the circulation time would be nearly three times as long (Table 1) as in the small reactor. Of course, this higher circulation time would reduce the frequency of passage of the cells through the high-shear zone of the impeller. This was felt to be an acceptable compromise. Therefore, the larger bioreactor (15L-STB) was constructed with the dimensions shown in Fig. 1.

3.4. Evaluation of 15L-STB

Attempts were made to culture *P. reticulatum* in the large photobioreactor 15L-STB (Fig. 1) in a batch operation initially with an impeller speed of 75 rpm. Profiles of the cell concentration (N), dissolved oxygen (DO), the average cell diameter (d_p) and the reactive oxygen species (ROS) for this batch run are shown in Fig. 8a. The

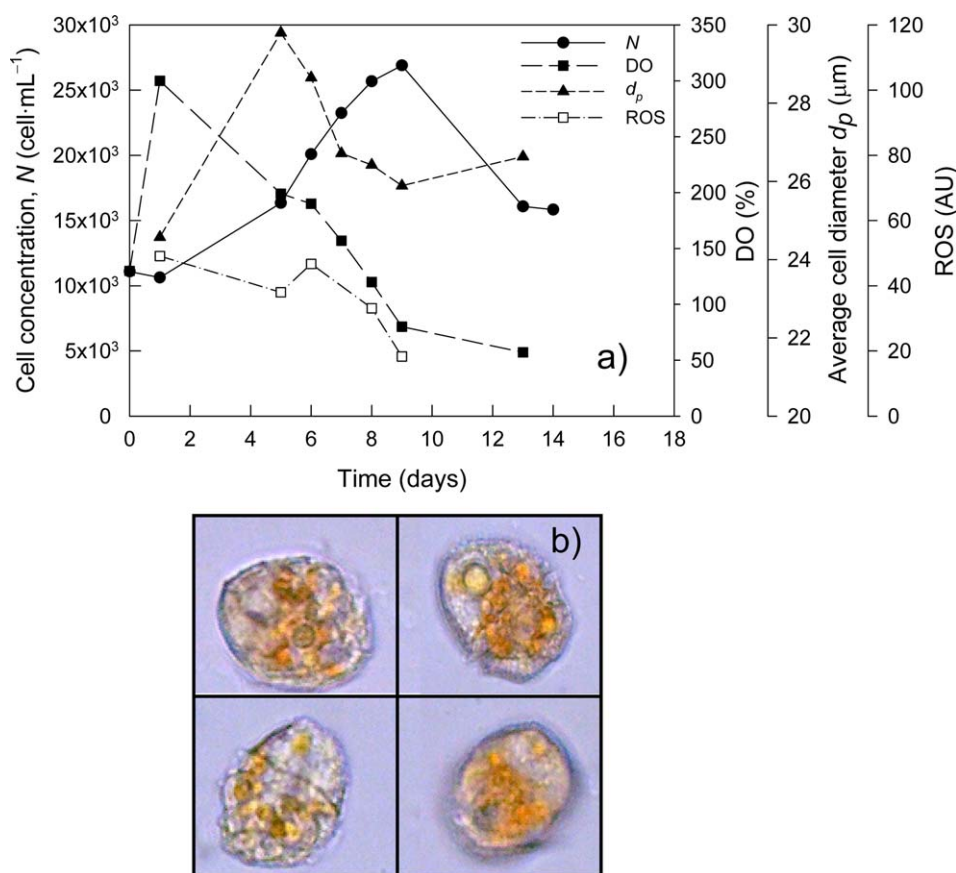


Fig. 8. (a) Temporal evolution of the cell concentration (N), the dissolved oxygen concentration (DO), the average cell diameter (d_p) and the reactive oxygen species (ROS) in the large photobioreactor (15L-STB) operated at the impeller speed of 75 rpm. (b) Swimming cells showing shrinkage of protoplasts.

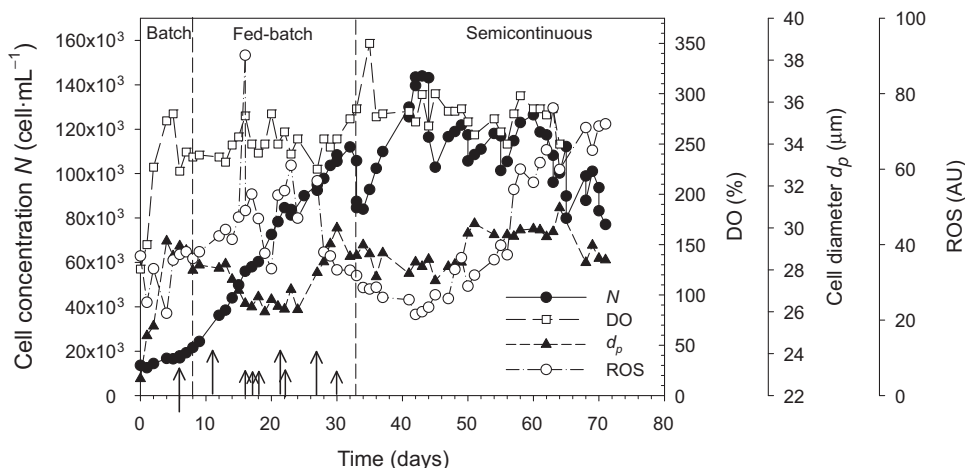


Fig. 9. Temporal evolution of the cell concentration (N), the dissolved oxygen concentration (DO), the average cell diameter (d_p) and the reactive oxygen species (ROS) in the large photobioreactor (15L-STB) operated at the impeller speed of 50 rpm. Long arrows indicate the instances of addition of NO_3^- and PO_4^{3-} . Short arrows indicate the instances of addition of ascorbic acid (see Table 2 for more details).

cell concentration peaked 8 days after inoculation and declined rapidly afterwards. The peak cell concentration was low at around $25,000 \text{ cell mL}^{-1}$. During the short initial growth phase, the average cell diameter increased sharply to attain a maximum value of $29.5 \mu\text{m}$. On day 5, the average cell diameter began to decline quite suddenly while the cells were still growing. The dissolved oxygen level rose initially because of photosynthesis, but after day 1 the level continually declined even though the cells grew until day 9 (Fig. 8a). This suggests that oxygen removal by aeration generally exceeded oxygen production by photosynthesis.

The rapid increase in cell size immediately after inoculation (Fig. 8a) suggests oxidative cell damage triggered by the inhibitory shear forces as the primary cause of the eventual cell death. Increase in cell size on exposure to inhibitory shear forces has been previously documented for *P. reticulatum* [15]. A declining concentration of dissolved oxygen despite an increasing cell number points to a progressive decline in the photosynthetic activity that too has been attributed to shear-induced oxidative damage [15]. Microscopic examination of the cells showed shrinkage of protoplast (Fig. 8b) as is associated with programmed cell death by oxidative stress [29,30].

In view of the poor results (Fig. 8a), a new batch run was conducted with a reduced impeller speed of 65 rpm. This reduced the damage to cells, but did not completely prevent it (data not shown). Therefore, the next run used an even lower impeller agitation speed of 50 rpm. This run began as a batch culture (Fig. 9) but was later switched to fed-batch operation and subsequently to a semicontinuous culture operation. In the batch phase, there was an initial increase in cell size, but oxygen continued to be produced (Fig. 9) well past the time at which peak production had occurred in the first batch run (Fig. 8a).

The exact operational strategy used in the fed-batch and semicontinuous phases of the run is summarized in Table 2. The specific instance of addition of the various nutrients to the culture are identified in Fig. 9 and further explained in Table 2.

Photosynthesis also produces reactive oxygen species (ROS). This explains the increasing level of ROS in an otherwise robustly growing population (Fig. 9). On day 16, ROS level reached nearly 90, the highest value observed for cultures of *P. reticulatum*. To prevent possible cell damage as a consequence of an increasing level of ROS, ascorbic acid (AA) was occasionally added to the culture to obtain an AA concentration of $100 \mu\text{M}$. Ascorbic acid is a scavenger of ROS and has previously proved useful in countering the harmful effects of ROS produced in response to damaging levels of

shear stress [15]. The increased bubbling within the spinfilter up to the full aeration rate from day 35 onwards was also beneficial as it contributed to reducing the DO level. This suggests that an excessive accumulation of DO may be another cause of oxidative stress on cells.

The average specific growth rate ($\mu = 0.06 \text{ days}^{-1}$) was similar in both the semicontinuous operation and the fed-batch operation, but the biomass productivity was higher in the semicontinuous culture. This was because the semicontinuous operation attained a higher cell concentration ($1.0\text{--}1.3 \times 10^5 \text{ cell mL}^{-1}$) (Fig. 9). The average cell productivity ($5228 \text{ cell mL}^{-1} \text{ day}^{-1}$) was nearly 3.7-fold greater than observed in static flasks but similar to the value attained in semicontinuous culture in the small bioreactor 2L-STB [7].

Production of yessotoxins (YTXs) by *P. reticulatum* in the various culture modes is shown in Fig. 10. During the first 20 days of operation, the YTXs contents of the cells decreased from 2 pg cell^{-1} to approximately 1.2 pg cell^{-1} , but remained fairly constant subsequently, i.e. during most of fed-batch and semicontinuous operation. This intracellular concentration of yessotoxins was only about 33% of the average level that has been reported for shake flask cultures [31]. This phenomenon was also observed in earlier studies with the small bioreactor 2L-STB and was attributed to the use of a nutrient-rich medium that does not generally promote the production of secondary metabolites such as yessotoxins

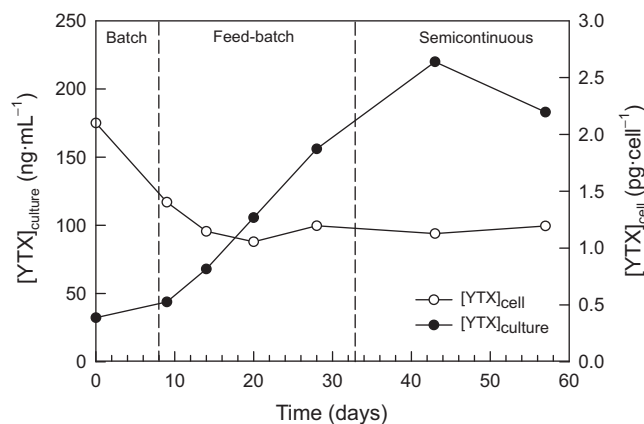


Fig. 10. Temporal evolution of yessotoxins (YTXs) concentration in the large photobioreactor (15L-STB) operated at the impeller speed of 50 rpm.

Table 2

The strategy used in the third attempt to culture *P. reticulatum* in the large bioreactor (15L-STB) with an impeller of 8.1 cm diameter rotated at 50 rpm. The specific instances of various additions to the culture are pointed out in Fig. 9.

Day	Culture mode	Incident irradiance ($\mu\text{E m}^{-2} \text{s}^{-1}$)	Aeration mode
0	Fed batch	241.7	60% surface aeration in headspace; 40% bubbled in spinfilter
5			
6	Fed-batch with perfusion (1 L/day of L1)		
13	Fed-batch with perfusion (1 L/day		
17	of L1 $\times 2$) ^a	300	20% surface aeration in headspace; 80% bubbled in spinfilter
23	Fed-batch with perfusion (1 L/day of L1 $\times 3$) ^a	766	
28	Fed-batch with perfusion (1 L/day of L1 $\times 3$) ^a ; (12 \times PO ₄ ⁻³ ; 4 \times NO ₃ ⁻) ^b		
33	Semicontinuous; removal of 15% of		
35	broth volume		100% bubbled in spinfilter
36	Fed-batch with perfusion (1 L/day of L1 $\times 3$) ^a ; (12 \times PO ₄ ⁻³ ; 4 \times NO ₃ ⁻) ^b		
44	Semicontinuous; removal of 10% of broth volume		
50	Semicontinuous; removal of 10% of broth volume		
55	Semicontinuous; removal of 10% of broth volume		
63	Semicontinuous; removal of 10% of broth volume		

^a L1 $\times n$ denotes the L1 medium with the nutrients concentrations of n -times the standard L1.

^b Nitrate and phosphate levels in the feed medium were 4-fold (4 \times) and 12-fold (12 \times), respectively, relative to corresponding levels in the standard L1 medium.

[7]. Nevertheless, the average volumetric productivity of yessotoxins was $9.16 \mu\text{g L}^{-1} \text{day}^{-1}$, or comparable to the results reported for the small bioreactor [7].

4. Concluding remarks

Principles for the successful scale-up of a stirred tank photobioreactor for growing the highly fragile dinoflagellate *P. reticulatum* were assessed. Successful scale-up from a 2 L operation to a 15 L photobioreactor was achieved by increasing the aspect ratio of the reactor to 4.5-fold of the value at the small scale. A relatively large axial flow impeller rotating at a slow speed was used for mixing. Scale up methods for photobioreactors remain largely undeveloped [32]. Although maintaining of geometric similarity is a widely used principle for scale up of stirred tanks, it cannot be directly translated to the case of stirred tank photobioreactors because an unacceptably large increase in culture depth would occur and this will adversely impact the productivity of a photosynthetic microorganism. In this study, the scaled up photobioreactor operated in the semicontinuous mode, had average volumetric productivities of the cells and yessotoxins that were comparable to the best results attained at the small scale.

Acknowledgements

This research was supported by the Spanish Ministry of Science and Innovation (CTQ2008-06754-C04-02/PPQ) and the General Secretariat of Universities, Research and Technology of Andalusian Government (TEP-5375).

Appendix A. Nomenclature

A	dimensionless constant in Eq. (2)
C_T	dimensionless concentration of tracer
D	impeller diameter (m)
DO	dissolved oxygen as percentage of air saturated seawater (%)
f	dimensionless radius of the spinfilter relative to the radius of the bioreactor vessel
$[H^+]$	concentration of tracer
h_1	characteristic length of the impeller (m)
k_i	slope of linear relation in Fig. 3
N	cell concentration (cell mL ⁻¹)
N_i	cell concentration at time t_i (cells mL ⁻¹)
N_Q	flow number

N_R	impeller rotational speed (rpm or rev s ⁻¹)
Q	impeller flow rate (m ³ s ⁻¹)
r_c	radius of the forced vortex zone (m)
r_D	radius of impeller (m)
r_T	radius of bioreactor (m)
2L-STB	2 L stirred-tank photobioreactor
15L-STB	15 L stirred-tank photobioreactor
t	time of culture (days)
t_R	recirculation time (s)
T	diameter of tank (m)
u	relative liquid velocity or impeller tip velocity (m s ⁻¹)
V_L	culture volume (m ³)
YTXs	yessotoxins

Greek symbols

ε_{max}	local maximum energy dissipation rate (W kg ⁻¹)
ε_p	Averaged energy dissipation rate (W kg ⁻¹)
ε_t	energy dissipation rate (W kg ⁻¹)
θ_m	mixing time (s)
λ	Kolmogorov's microscale (m)
μ	cell-specific growth rate (days ⁻¹)
μ_L	broth viscosity (Pa s)
ρ_L	broth density (kg m ⁻³)
τ_i	theoretical shear stress on spinfilter surface (mN m ⁻²)

References

- [1] Singh S, Kate BN, Banerjee UC. Bioactive compounds from cyanobacteria and microalgae: an overview. *Crit Rev Biotechnol* 2005;25:73–95.
- [2] García Camacho F, Gallardo Rodríguez J, Sánchez Mirón A, Cerón García MC, Belarbi EH, Chisti Y, Molina Grima E. Biotechnological significance of toxic marine dinoflagellates. *Biotechnol Adv* 2007;25:176–94.
- [3] Molina Grima E, Ación Fernández FG, García Camacho F, Camacho Rubio F, Chisti Y. Scale-up of tubular photobioreactors. *J Appl Phycol* 2000;12:355–68.
- [4] Molina Grima E, Fernández FGA, García Camacho F, Chisti Y. Photobioreactors: light regime, mass transfer, and scaleup. *J Biotechnol* 1999;70:231–47.
- [5] Chisti Y. Biodiesel from microalgae. *Biotechnol Adv* 2007;25:294–306.
- [6] Vunjak-Novakovic G, Kim Y, Wu X, Berzin I, Merchuk JC. Air-lift bioreactors for algal growth on flue gas: mathematical modeling and pilot-plant studies. *Ind Eng Chem Res* 2005;44:6154–63.
- [7] Gallardo Rodríguez JJ, Sánchez Mirón A, García Camacho F, Cerón García MC, Belarbi EH, Molina Grima E. Culture of dinoflagellates in a fed-batch and continuous stirred-tank photobioreactors: growth, oxidative stress and toxin production. *Process Biochem* 2010;45:660–6.
- [8] García Camacho F, Gallardo Rodríguez JJ, Sánchez Mirón A, Cerón García MC, Belarbi EH, Molina Grima E. Determination of shear stress thresholds in toxic dinoflagellates cultured in shaken flasks. Implications in bioprocess engineering. *Process Biochem* 2007;42:1506–15.
- [9] Gallardo Rodríguez JJ, Cerón García MC, García Camacho F, Sánchez Mirón A, Belarbi EH, Molina Grima E. New culture approaches for yessotoxin production from the dinoflagellate *Protoceratium reticulatum*. *Biotechnol Prog* 2007;23:339–50.

- [10] Gallardo Rodríguez JJ, Sánchez Mirón A, García Camacho F, Cerón García MC, Belarbi EH, Chisti Y, Molina Grima E. Carboxymethyl cellulose and Pluronic F68 protect the dinoflagellate *Protoceratium reticulatum* against shear-associated damage. *Bioproc Biosyst Eng* 2011;34:3–12.
- [11] Guillard RRL, Hargraves PE. *Stichochrysis immobilis* is a Diatom not a Chyrophyte. *Phycologia* 1993;32:234–6.
- [12] Chisti MY. *Airlift bioreactors*. N.Y.: Elsevier; 1989.
- [13] Ruszkowski S. A rational method for measuring blending performance, and comparison of different impeller types. In: *Proceedings of the 8th european conference on mixing*. 1994. p. 283–91.
- [14] Grenville R, Ruszkowski S, Garred E. Blending of miscible liquids in the turbulent and transitional regimes. In: *15th NAMF mixing conference*. 1995.
- [15] Rodríguez JJG, Mirón AS, Camacho FG, García MCC, Belarbi EH, Chisti Y, Grima EM. Causes of shear sensitivity of the toxic dinoflagellate *Protoceratium reticulatum*. *Biotechnol Prog* 2009;25:792–800.
- [16] Liepe F, Meusel W, Möckel HO, Platzer B, Weißgräber H. *Stoffvereinigung in Fluiden Phasen. Verfahrenstechnische Berechnungsmethoden* 1988.
- [17] Leckie F, Scragg AH, Cliffe KC. Effect of bioreactor design and agitator speed on the growth and alkaloid accumulation by cultures of *Catharanthus roseus*. *Enzyme Microb Technol* 1991;13:296–305.
- [18] Croughan MS, Hamel JF, Wang DIC. Hydrodynamic effects on animal cells grown in microcarrier cultures. *Biotechnol Bioeng* 2000;67:850–2.
- [19] Metzner AB, Otto RE. Agitation of non-Newtonian fluids. *AIChE J* 1957;3:3–10.
- [20] Nienow AW. Hydrodynamics of stirred bioreactors. *Appl Mech Rev* 1998;51:3–32.
- [21] Wu J, Graham LJ, Mehidi NN. Estimation of agitator flow shear rate. *AIChE J* 2006;52:2323–32.
- [22] Paz B, Riobó P, Luisa Fernández M, Fraga S, Franco JM. Production and release of yessotoxins by the dinoflagellates *Protoceratium reticulatum* and *Lingulodinium polyedrum* in culture. *Toxicon* 2004;44:251–8.
- [23] Zhou G, Kresta SM. Distribution of energy between convective and turbulent flow for three frequently used impellers. *Chem Eng Res Des* 1996;74:379–89.
- [24] Bird R, Stewart R, Lightfoot E. *Transport phenomena*. New York: John Wiley and Sons Inc.; 1960. p. 780.
- [25] Figueredo-Cardero A, Chico E, Castilho LR, Medronho RA. CFD simulation of an internal spin-filter: evidence of lateral migration and exchange flow through the mesh. *Cytotechnology* 2009;61:55–64.
- [26] Yim SS, Shamlou PA. The engineering effects of fluids flow on freely suspended biological macro-materials and macromolecules. *Adv Biochem Eng Biotechnol* 2000;67:83–122.
- [27] Stephenie W, Kabeir BM, Shuhaimi M, Rosfarizan M, Yazid AM. Influence of pH and impeller tip speed on the cultivation of *Bifidobacterium pseudocatenulatum* G4 in a milk-based medium. *Biotechnol Bioprocess Eng* 2007;12:475–83.
- [28] Sánchez Mirón A, Contreras Gómez A, García Camacho F, Molina Grima E, Chisti Y. Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. *J Biotechnol* 1999;70:249–70.
- [29] Vardi A, Berman-Frank I, Rozenberg T, Hadas O, Kaplan A, Levine A. Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO₂ limitation and oxidative stress. *Curr Biol* 1999;9:1061–4.
- [30] Franklin DJ, Berges JA. Mortality in cultures of the dinoflagellate *Amphidinium carterae* during culture senescence and darkness. *Proc R Soc B* 2004;271:2099–107.
- [31] Gallardo Rodríguez JJ, Sánchez Mirón A, Cerón García MC, Belarbi EH, García Camacho F, Chisti Y, Molina Grima E. Macronutrients requirements of the dinoflagellate *Protoceratium reticulatum*. *Harmful Algae* 2008.
- [32] Merchuk JC, Garcia-Camacho F. *Bioreactors, air-lift reactors*. In: *Flickinger MC, editor. Encyclopedia of industrial biotechnology, bioprocess bioseparation, and cell technology*, vol. 2. Wiley; 2010. p. 851–912.