

Rheology and shear stress of *Centaurea calcitrapa* cell suspension cultures grown in bioreactor

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Abstract

Centaurea calcitrapa suspension cultures were grown either in Erlenmeyer flasks or in a mechanically stirred bioreactor. Its rheological behaviour, when fitted to the Oswald–de Waele model (power law), showed pseudoplastic characteristics in both cases. The flow behaviour index (n) decreased over the course of a growth cycle and the consistency index (K) increased, reached a value of $1.81 \text{ N s}^n \text{ m}^{-2}$ run on 2 l bioreactor. Bioreactor cultivation of *C. calcitrapa* cells at different agitation rates (30, 60, 100 and 250 rpm), highlighted the influence of shear forces on cell viability loss (90–34%) and phenol accumulation ($74\text{--}140 \mu\text{g l}^{-1}$), due to increased stirring speeds. Analysis of these results suggests that this cell line is shear-sensitive. An empirical exponential correlation was defined between apparent viscosity and biomass concentration, under the studied conditions, giving the possibility to estimate the prevailing broth regime and to optimize bioreactor design.

Introduction

Rheological studies of plant cell suspensions may help to resolve various technological problems related to O_2 supply, mixing and mass transfer. However, there are few reports that focus the rheology of plant cell cultures. A summary of relevant studies was published by Kieran *et al.* (1997) who indicated that the majority of suspension cultures exhibit non-Newtonian characteristics, mainly pseudoplastic flow behaviour. However, only a few authors have presented rheological measurements related to plant cell morphology (Wongsamuth & Doran 1997, Rodríguez-Monroy & Galindo 1999, Trejo-Tapia *et al.* 2001, Sánchez *et al.* 2002).

In the present work, rheological properties of *Centaurea calcitrapa* cell suspension cultures, grown in Erlenmeyer flasks and in a stirred

bioreactor are described, and correlated with morphological aspects and shear sensitivity of the cell culture. *Centaurea calcitrapa* is a star thistle, native to Portugal, which produces enzymes with milk clotting activity and may be a potential alternative to plant rennet (Raposo 1997). This is the first report, to the best of our knowledge, on *Centaurea calcitrapa* broth rheology, cell morphology and shear stress. A previous report (Jeffers *et al.* 2003) was published with the morphological characterization of *Centaurea calcitrapa* cell suspension, grown only in Erlenmeyer flasks.

Materials and methods

Cultivation conditions

Friable calli were obtained from hypocotyls sections and segments of *Centaurea calcitrapa* leaves

which were surface sterilized with 5% (w/v) sodium hypochlorite containing 0.05% Tween 80 for 10 min and inoculated in Schenk & Hildebrandt (SH) medium, supplemented with 1.5 mg naphthalenacetic acid l^{-1} and 0.15 mg kinetin l^{-1} , solidified with 0.8% agar. The cultures were maintained under 16/8 h photoperiod (1500 lux) at 24 °C.

Cell suspension cultures were obtained using friable calli, which were transferred to SH liquid medium, supplemented with 20 g glucose l^{-1} , 1.5 mg naphthalenacetic acid l^{-1} and 0.15 mg kinetin l^{-1} . Cultures were maintained in 500 ml Erlenmeyer flasks in the dark at 22 °C, at an orbital shaker (115 rpm). *C. calcitrapa* was sub-cultured weekly, using 20% (v/v) inoculum. Erlenmeyer experiments were performed in the same cultivation conditions, as stock cultures. All experiments were done in triplicate.

Bioreactor cultures

Bioreactor runs were performed in a 2 l mechanically stirred bioreactor, at different agitation speeds (30, 60, 100 and 250 rpm) and constant aeration rate (0.083 vvm) at 24 °C, with an inoculation ratio of 20% (v/v). The work volume of the bioreactor was 1.5 l. The bioreactor was equipped with a double pitched-blade turbine of 6.0 cm diameter. Each blade was 1.8 cm in width and 7.2 cm in length. All bioreactor experiments were performed twice.

Analytical methods

Biomass. Dry weight (DW) determinations were made after filtration of 10 ml samples. The cell material has been dried at 80 °C, until weight remained constant.

Phenols. Total phenol content was quantified in the supernatant by a direct spectrophotometric method, as described before (Lima-Costa *et al.* 1996).

Viability. Cell viability was evaluated by the Evans blue dye exclusion test, as described by Keßler & Furusaki (1997).

Rheological methodology

The rheological measurements were performed on samples collected periodically from the bioreactor

or from the Erlenmeyer flasks, at room temperature, using a UL adapter mounted on a Brookfield-type rotational viscometer. Rheological behaviour of the cell suspension was found to be well-described by Ostwald–de Waele (power law) model:

$$\tau = K\gamma^n \quad (1)$$

where τ is the shear stress ($N m^{-2}$), K is the consistency index ($N s^n m^{-2}$), γ is the shear rate (s^{-1}) and n is the flow behaviour index. Shear rates (γ) were in the range 6–61 s^{-1} . The shear stress (τ) and shear rate (γ) were calculated according to the following formulae

$$\tau(N m^{-2}) = 0.0783 \times \text{Dial reading} \quad (2)$$

$$\gamma(s^{-1}) = 1.224 \times \text{Rotational speed} \quad (3)$$

Measurements were made 10 s afterwards to avoid sedimentation effects.

Apparent Viscosity. Apparent viscosity (μ_{app}) of the suspension was determined using a Brookfield viscometer, by multiplication of the spindle characteristic factor by the dial reading (equation 4).

$$\mu_{app}(N s m^{-2}) = (0.064 / N) \times \text{Dial reading} \quad (4)$$

where N is the rotational speed of the impeller (rpm).

Hydrodynamic stress parameters

Impeller tip speed (v_i) was calculated according to the following formulae

$$v_i(m s^{-1}) = \pi N D_i \quad (5)$$

where N is the rotational speed of the impeller (s^{-1}) and D_i is the impeller diameter (m).

Specific power input (P/V) and **average energy dissipation rate** ($\bar{\epsilon}$) were calculated as described by Kieran *et al.* (2000).

Average shear rate (γ) was estimated for pitched-blade turbine using an equation developed by Metzner *et al.* (1961).

Maximum shear rate was evaluated by the impeller tip speed according to Midler & Finn (1966).

Results and discussion

Rheological properties and morphological aspects of cell suspensions

In order to contribute to a better characterization of cell suspension growth, the rheological properties of *Centaurea calcitrapa* cell suspensions cultivated in Erlenmeyer flasks and in a mechanically stirred bioreactor were correlated to cell aggregate size. Rheograms were constructed for different biomass concentrations grown in Erlenmeyer flasks (Figure 1). A power law model was fitted to the data for suspensions, from different stages of the 10-days batch cell suspension growth cycle, in order to estimate n and K (Figure 2). Flow behaviour index, n , decreases with increasing biomass concentration (in the range $1\text{--}10\text{ g l}^{-1}$) and reaches a final value of less than 0.2 for a biomass concentration of 10 g l^{-1} , suggesting a non-Newtonian fluid regime with a pseudoplastic behaviour.

Pseudoplastic behaviour was also apparent in suspensions cultivated in the 2 l stirred bioreactor (Figures 3 and 4). The flow behaviour index n decreased from 0.7 to 0.4, approximately, with increasing biomass concentration, over the first twelve days of growth. On the other hand, the consistency index, K , increased during the growth cycle, from an initial value of 0.1 N s m^{-2} to a value of 2 N s m^{-2} at the end of the growth. No measurable yield stress was observed for any of the *Centaurea calcitrapa* suspension cultures tested in this study.

Catharanthus roseus (Scragg *et al.* 1988), *Morinda citrifolia* (Kieran *et al.* 1997) and *Solanum chrysotrichum* (Trejo-Tapia *et al.* 2001) are some examples of plant cell suspensions reported that evidence a pseudoplastic rheological behaviour, as did *C. calcitrapa* culture. *Beta vulgaris* cell cultures (Rodríguez-Monroy & Galindo 1999), at low biomass concentration (4 g DW l^{-1}), showed Newtonian behaviour, but at high cell density (10 g DW l^{-1}) showed pseudoplastic behaviour.

It is possible that the differences observed between the corresponding profiles for *C. calcitrapa* cultures, grown in Erlenmeyer flasks (Figure 2) and in the stirred bioreactor (Figure 4), may be caused by differences in cell aggregation size of the cultures grown in different conditions. In shake flask assays the size of *C. calcitrapa* cell aggregates decreased slightly over the growth cycle, from $183\text{ }\mu\text{m}$ at initial phase to $171\text{ }\mu\text{m}$ at stationary phase (Jeffers *et al.* 2003). However, on the bioreactor run at 100 rpm agitation rate, a severe decrease of cell aggregates diameter was observed, from the lag phase ($174\text{ }\mu\text{m}$) and the end of the growth cycle ($83\text{ }\mu\text{m}$). This 48% reduction of cell aggregate size might influence the decrease of apparent viscosity measurements observed at day 15 (Table 1). This result is in agreement with results reported by Takeda *et al.* (1994) for *Carthamus tinctorius* cell aggregate size, which became smaller ($94\text{ }\mu\text{m}$) when cultivated in a stirred tank in comparison with a shake flask ($106\text{ }\mu\text{m}$). Thus the flow behaviour of plant cell broth was affected by the aggregate size, cell form and, particularly, by cell mass.

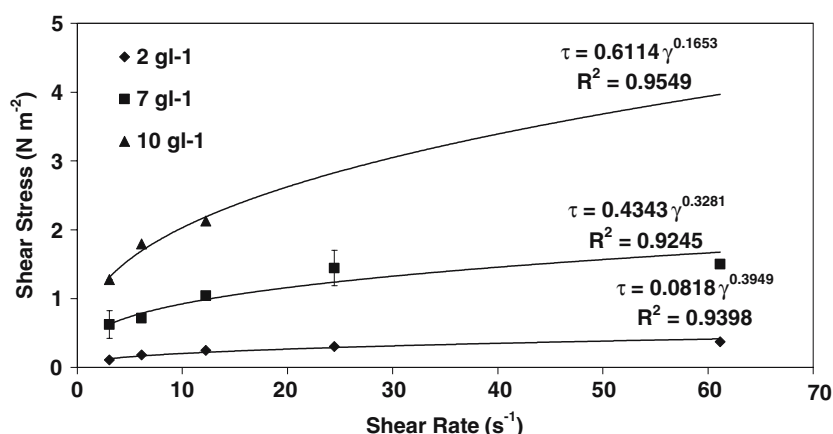


Fig. 1. Representative rheograms for *C. calcitrapa* cell suspensions at different biomass concentrations, grown in 500 ml Erlenmeyer flasks. Error bars indicate standard error.

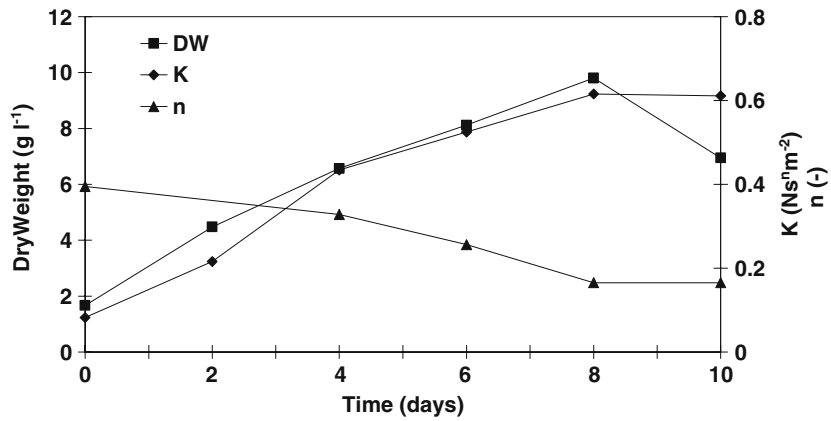


Fig. 2. Evolution of the flow behaviour and consistency indices for *C. calcitrappa* shake flask suspensions.

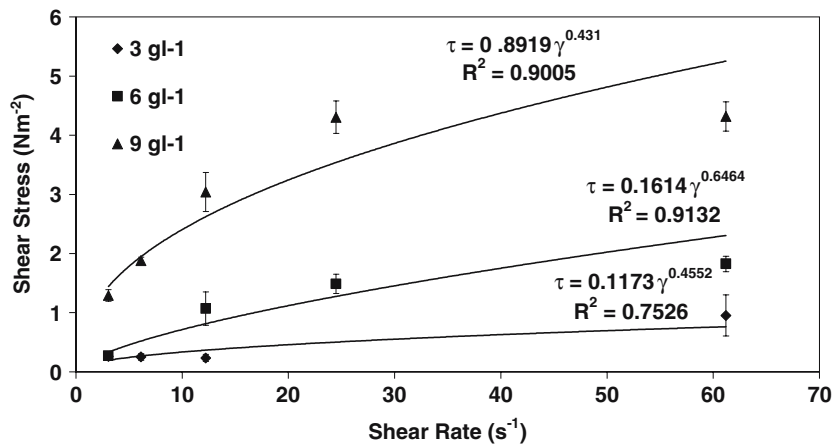


Fig. 3. Representative rheograms for *C. calcitrappa* cell suspension at different biomass concentrations, grown in a mechanically stirred bioreactor. Error bars indicate standard error.

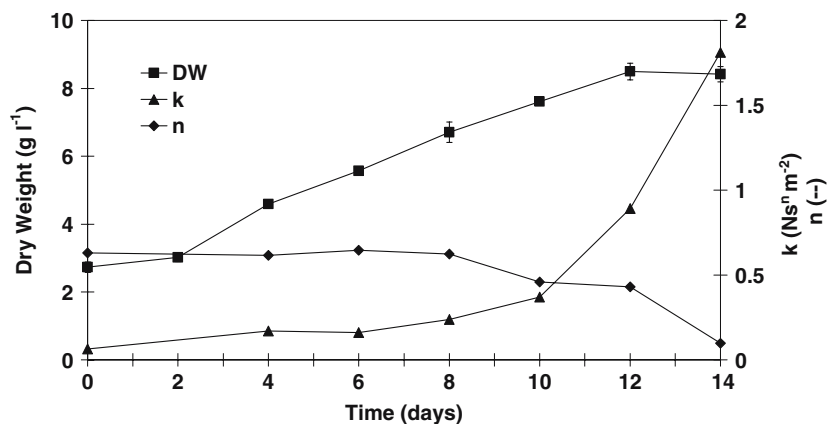


Fig. 4. Growth profile and evolution of the flow behaviour and consistency indices for *C. calcitrappa* cell suspensions cultivated in a 2 l mechanically agitated bioreactor. Error bars indicate standard error.

Table 1. Apparent viscosity (γ evaluated at 62 s^{-1}) and maximum phenol accumulation for *C. calcitrapa* cell suspensions cultivated in a stirred bioreactor, at different agitations: 30, 60, 100 and 250 rpm, at different days of culture.

Agitation (rpm)	Apparent Viscosity ($N \text{ s m}^{-2}$) $\times 10^3$ at day			Phenols _{max} ($\mu\text{g ml}^{-1}$)
	0	12	15	
30	4	18	20	74
60	10	71	42	65
100	8	42	35	106
250	5	50	44	140

Influence of agitation rate on apparent viscosity, cell viability, phenolic content and pH

To evaluate the influence of shear forces on cell survival and stress metabolism, *C. calcitrapa* cell culture runs in 2 l stirred bioreactor were conducted at different agitation conditions (30, 60, 100 and 250 rpm), while retaining the other established cultivation conditions. Cell viability of *C. calcitrapa* at 30 and 60 rpm presents high values in the range 80–90%, along the growth cycle (Figure 5). At 100 rpm, cell viability reached the value of 64%, in the stationary growth phase. However, at 250 rpm, cell viability has drastically dropped at the second day of culture, reaching the value of 34%, at the end of the growth cycle. Other reports suggest that plant cells could suffer damage imposed by the stress generated by high agitation in stirred tanks (Zhong et al. 1994, Rodríguez-Monroy & Galindo 1999).

An indirect metabolic indicator of cell exposure to shear environment is the release of phenolic compounds into the medium, as referred by Wongsamuth & Doran (1997). *C. calcitrapa* cultivation at 250 rpm showed an increase in phenolic compound accumulation (Table 1) when compared with the bioreactor cultivations performed at 30, 60 and 100 rpm. Similar phenol accumulation into the broth was observed with *Cynara cardunculus* cell suspension cultured in a bioreactor at 100 rpm (Lima-Costa et al. 1996), which may indicate secondary metabolism induction by environmental stress conditions.

The pH variation was not significant for cell culture runs at 30, 60 and 100 rpm. However, the run at 250 rpm presented an increasing pH, between 6 and 8. The high pH values may be related to the increase of shear stress, caused by the increased agitation rate, provoking cell damage with release of intracellular material.

Broth apparent viscosity values (Table 1), calculated at a shear rate of 61 s^{-1} , for *C. calcitrapa* cell suspensions cultivated at 30, 60, 100 and 250 rpm, showed an increase along the exponential growth phase. However, at the stationary phase, the apparent viscosity dropped, suggesting a increase on cell lysis. These results corroborate the cell viability results, which decreased in the end of the growth cycle. This can also be justified by a reduction in aggregate size at stationary phase, under exposure to shear conditions, which is also supported by visual evidence at 30 and 60 rpm runs.

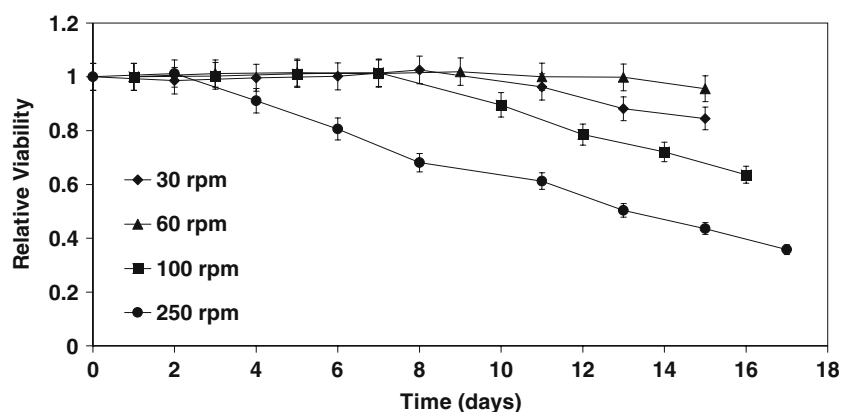


Fig. 5. Cell viability of *C. calcitrapa* cell suspensions cultivated in a 2 l mechanically agitated bioreactor at different agitation speeds: 30, 60, 100 and 250 rpm.

The viscosity of the filtrates was found to be similar to the value of water (approximately $1 \times 10^{-3} \text{ N s m}^{-2}$), suggesting that extracellular compounds did not contribute to the observed variations in broth viscosity.

Hydrodynamic stress parameters

To characterize the hydrodynamic environment of *C. calcitrapa* cell suspension in a 2 l stirred bioreactor, the stress parameters were determined at the start and at the end of the growth cycle (Table 2). For all the conditions assayed, the P/V and $\bar{\epsilon}$ increased during the growth cycle because the apparent viscosity increased, with a decrease of mixing turbulence.

The values of hydrodynamic stress parameters (P/V; $\bar{\epsilon}$) determined at 250 rpm were higher than for the other cultures, presenting values 11 fold bigger than at 100 rpm. Also, the γ value for 250 rpm was 42 s^{-1} and for 100 rpm was 17 s^{-1} . These results indicate that mixing conditions have a preponderant effect on *C. calcitrapa* cell suspension hydrodynamic behaviour.

The values of hydrodynamic stress parameters obtained in this work are in the range of other values reported in the bibliography. Pan *et al.* (2000) with *Taxus chinensis* cell suspensions cultivated in an airlift bioreactor attained a value of average shear rate (7.05 s^{-1}) much smaller than in the stirred tank reactor (55.0 s^{-1}). Scragg *et al.* (1988) with different cell suspensions grown in a stirred tank bioreactor, had average and maximum shear rate values higher than those

Table 2. Hydrodynamic stress parameters determined for *C. calcitrapa* cell suspensions cultivated in a stirred bioreactor, at different agitations: 30, 60, 100 and 250 rpm.

Agitation (rpm)		P/V (W m^{-3})	$\bar{\epsilon}$ (W Kg^{-1})	γ (s^{-1})	v_i (m s^{-1})
30	Start	0.04	4×10^{-5}	5	0.1
	End	0.07	7×10^{-5}		
60	Start	0.3	3×10^{-4}	10	0.2
	End	0.6	6×10^{-4}		
100	Start	1.2	1×10^{-3}	17	0.3
	End	2	2×10^{-3}		
250	Start	13	13×10^{-3}	42	0.8
	End	23	23×10^{-3}		

obtained in this work. In contrast *Catharantus roseus* cell suspension cultivated in 3 l bioreactor at 1000 rpm and an average shear rate 167 s^{-1} , present a decrease in fresh weight and dry weight during the first hour of exposure to the shear conditions. However, after 5 h of exposure to shear conditions, these cells did not show any difference in growth rate and final biomass level. Similar results were obtained with *Helianthus annuus* cells (Scragg *et al.*, 1988). *Picrasma quassiodes* cell cultures did not grow in a stirred tank bioreactor in similar conditions, and due to that was considered shear sensitive (Scragg *et al.*, 1988).

Centaurea calcitrapa suspended cells have shown sensitivity to hydrodynamic stress, particularly at 250 rpm stirring speed. These observations showed that sensitivity to shear depend on the cell line. Zhong *et al.* (1994) have shown that growth of *Perilla frutescens* cultivated in a stirred tank bioreactor with a marine impeller was conditioned by average shear rate and impeller tip speed. The results indicated that there was an optimum range of average shear rate ($20\text{--}30 \text{ s}^{-1}$) and an impeller tip speed of $0.5\text{--}0.8 \text{ m s}^{-1}$, which maximized specific growth rate, cell concentration and productivity.

Mathematical correlation for apparent viscosity and biomass

An empirical mathematical correlation was established between apparent viscosity and biomass concentration, during the growth cycle (Figure 6 and equation 6), clearly expressing the dependence of broth viscosity on biomass density. This correlation can be useful to estimate the apparent viscosity of cell culture broth grown in a 2 l mechanically stirred bioreactor, under similar experimental conditions and cell aggregate size.

$$\mu_{\text{app}} = 0.0027e^{0.369\text{DW}} \quad (6)$$

Knowledge of the apparent viscosity of a given suspension, under particular conditions of shear rate, is fundamental to the characterization of the prevailing flow regime, as well as the specification of optimal agitation equipment and conditions. This mathematical model has to be confirmed for other plant species.

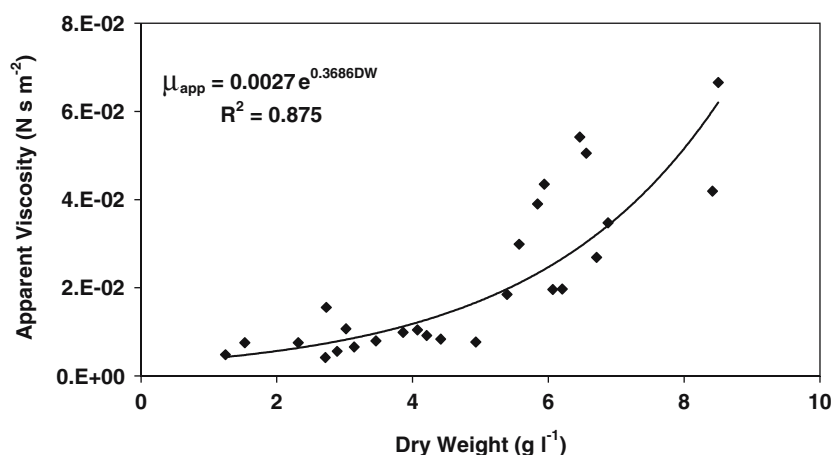


Fig. 6. Correlation between apparent viscosity and dry weight for *C. calcitrapa* cell suspensions grown in a mechanically stirred bioreactor.

Conclusions

The flow behaviour in a reactor can be affected by biomass density, apparent viscosity, cell volume and aggregate size. Non-Newtonian, pseudoplastic flow behaviour was determined for *C. calcitrapa* suspended cells, cultivated in a mechanically stirred bioreactor and in Erlenmeyer flasks. The results presented in this work also suggest that the viscosity of the broth is related to biomass concentration through an exponential correlation. All experimental observations indicate that the *C. calcitrapa* cell line is sensitive to shear forces under hydrodynamic stress environment (250 rpm). It is possible to conclude that every modification of initial operational stirring parameters can induce important rheological and physiological alterations, particularly with shear-sensitive cultures such as *C. calcitrapa*.

Further studies of other plant species with different aggregation and cell morphology are underway. The characterization of the rheology of plant cell suspensions related to cell morphology and cell viability is essential for the evaluation of the shear stress environment and may contribute crucially to optimal bioreactor design and to solving important technological problems.

References

Jeffers P, Raposo S, Lima-Costa ME, Connolly P, Glennon B, Kieran PM (2003) Focussed beam reflectance measurement

- (FBRM) monitoring of particle size and morphology in suspension cultures of *Morinda citrifolia* and *Centaurea calcitrapa*. *Biotechnol. Lett.* **25**: 2023–2028.
- Kefler M., Furusaki S (1997) Unsuitability of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) as a viability assay for plant cells in suspension. *J. Chem. Eng. Jpn.* **30**: 718–723.
- Kieran P, MacLoughlin P, Malone D (1997) Plant cell suspension cultures: some engineering considerations. *J. Biotechnol.* **59**: 39–52.
- Kieran P, Malone D, MacLoughlin P (2000) Effects of hydrodynamic and interfacial forces on plant cell suspension systems. *Adv. Biochem. Eng. Biotechnol.* **67**: 139–177.
- Lima-Costa ME, van Gulik W, ten Hoopen H, Pais MS, Cabral JM (1996) Protease and phenol production of *Cynara cardunculus* L. cell suspension in a chemostat. *Enzyme Microb. Technol.* **19**: 493–500.
- Metzner AB, Feehs RH, Ramos HL, Otto RE, Tuthill JD (1961) Agitation of viscous Newtonian and non-Newtonian fluids. *AIChE J.* **7**: 3–9.
- Midler M, Finn RK (1966) A model system for evaluating shear in design of stirred fermentors. *Biotechnol. Bioeng.* **8**: 71–76.
- Pan Z-W, Wang H-Q, Zhong J-J (2000) Scale-up study on suspension cultures of *Taxus chinensis* cells for production of taxane diterpene. *Enzyme Microb. Technol.* **27**: 714–723.
- Raposo S (1997) Proteinases aspárticas de células em suspensão de *Centaurea calcitrapa*: produção, purificação e caracterização. MsSc Thesis. Lisboa, Portugal: Faculdade de Ciências da Universidade de Lisboa.
- Rodríguez-Monroy M, Galindo E (1999) Broth rheology, growth and metabolite production of *Beta vulgaris* suspension culture: a comparative study between cultures grown in shake flasks and in a stirred tank. *Enzyme Microb. Technol.* **24**: 687–693.
- Sánchez M, Jiménez-Aparicio A, López G, Tapia G, Rodríguez-Monroy M (2002) Broth rheology of *Beta vulgaris* cultures growing in an air lift bioreactor. *Biochem Eng. J.* **12**: 37–41.
- Scragg AH, Allan EJ, Leckie F (1988) Effect of shear on the viability of plant cell suspensions. *Enzyme Microb. Technol.* **10**: 361–367.

- Takeda T, Seki M, Furusaki S (1994) Hydrodynamic damage of cultured cells of *Carthamus tinctorius* in a stirred tank reactor. *J. Chem. Eng. Jpn.* **27**: 466–471.
- Trejo-Tapia G, Jiménez-Aparicio A, Villarreal L, Rodríguez-Monroy M (2001) Broth rheology and morphological analysis of *Solanum chrysotrichum* cultivated in a stirred tank. *Biotechnol. Lett.* **23**: 1943–1946.
- Wongsamuth R, Doran P (1997) The filtration properties of *Atropa belladonna* plant cell suspension; Effects of hydrodynamic shear and elevated carbon dioxide levels on culture and filtration parameters. *J. Chem. Tech. Biotechnol.* **69**: 5–26.
- Zhong J-J, Fujiyama K, Seki T, Yoshida T (1994) A quantitative analysis of shear effects on cell suspension and cell culture of *Perilla frutescens* in bioreactors. *Biotechnol. Bioeng.* **44**: 649–654.