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Quantitative evaluation of the shear threshold on *Carthamus tinctorius* L. cell growth with computational fluid dynamics in shaken flask bioreactors



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ABSTRACT

Quantitative evaluation of the shear threshold on *C. tinctorius* L. cell growth is vital for bioreactor system design and optimization of scaled-up industrial cultivation. The present research focused on investigating the effects of shear force on *C. tinctorius* L. cell growth by computational fluid dynamic (CFD) analysis in shaken flasks. The results revealed that specific cell growth rates were greatly inhibited as the shear force increased from 1.17 to 2.42 Pa. Recovery of viability and aggregation diameter to their normal levels could be implemented after a 4-day adaptation with large fluctuations in physiological state. With further correlation analysis on shear force and *C. tinctorius* L. growth rate, a threshold value was identified at an average and maximum shear stress of approximately 0.55 Pa (0.06 w/kg) and 4.00 Pa (0.93 w/kg) according to the influence on cell growth. Quantitative data on shear effects can facilitate the design of industrial processes and lead to more rational scale-up in industrial *C. tinctorius* L. cultivation.

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1. Introduction

Plant cell suspension cultivation has proven to be an effective method of producing many valuable bioproducts continuously and stably with independence from geographical or environmental variations and constraints [1]. *C. tinctorius* L. is one of the Chinese herbal medicines most commonly used to prevent and treat cardiac disease in clinical practice [2]. With the quickly increasing mar-

ket demand, suspension callus cultures have become one of the most commonly applied methods for *C. tinctorius* L. production. Like many other plant cell suspensions cultivated for active secondary metabolites, a separate two-stage culture strategy has been implemented for an industrial *C. tinctorius* L. cell culture process [3]. In the late stage, elicitors are added to induce biosynthesis of the secondary metabolites (Hydroxysafflor yellow A and kaempferide) [4]. To obtain the highest metabolite productivity, the most important and difficult problem encountered is to obtain high cell density and higher specific cell growth rates. However, in industrial *C. tinctorius* L. cell cultivation and scale-up in stirred tank bioreactors, fragile and slow-growing effects have always been a serious problem. Hydrodynamic shear stresses generated by the turbulent flow are thought to be the important inhibitory factor on growth [5–9]. However, hardly any reports could be found on *C. tinctorius* L. cell shear tolerance. Therefore, it is of great importance to quantitatively evaluate the shear threshold on *C. tinctorius* L. cell growth, which would be effectively applied for directing bioreactor system design and scale-up optimization for industrial *C. tinctorius* L. cells.

Previous studies into the shear sensitivity of plant cells have been carried out in well-defined shear devices, for example, couette type viscometers, capillary devices, and submerged jet apparatuses [10–13]. The definable shear devices had serious deficiencies such

Abbreviations: a, specific gas–liquid interfacial area (m²); c*, saturated dissolved oxygen (mmol/m³); c_o, concentration of oxygen in liquid (mmol/m³); d, largest inner diameter of shake flask (mm); d_o, shaking diameter (mm); k_la, mass transfer coefficient (h⁻¹); n, shaking frequency or rotation speed (rpm); D_L, diffusion coefficient of oxygen (cm²/s); OTR, oxygen transfer rate (mmol kg⁻¹ h⁻¹); OUR, oxygen uptake rate (mmol kg⁻¹ h⁻¹); Re, flask Reynolds number (–); V, shake flask nominal volume (ml); V_f, filling volume of shake flask (ml); ε, turbulent energy dissipation rate (m² s⁻³ or w/kg); μ, specific growth rate (h⁻¹); ρ, fluid density (kg m⁻³); ν, dynamic fluid viscosity (Pa s); τ_i, shear stress from fluctuating velocity gradient (Pa); τ_d, shear stress acting on opposite sides of the cell (Pa); ω, angular velocity (rad/s); η_k, kolmogorov length scale (m).

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as unsuitability for long-term cultivation and insufficient oxygen supply under low shear environments [14]. Furthermore, those studies mainly focused on short duration exposure effects with direct lethal or sub-lethal responses being considered [10–13]. In addition to the duration of exposure frequency, flow type (laminar or turbulent) also needs to be taken into consideration [15,16]. On the other hand, in commonly used bioreactors, most of the studies have concentrated on empirical correlations based on the use of the parameters of rotation speed or impeller tip speed to assess the effects of hydrodynamic stress [17–20]. Obviously, these parameters are always scale-dependent and difficult to evaluate quantitatively.

Compared with other culture systems, shaken flasks have unique advantages in shear sensitivity studies: (i) suitability for long-term cultivation; (ii) higher oxygen transfer rates under a low shear environment; (iii) the distribution of shear force is relatively uniform, with values of $\varepsilon_{\max}/\varepsilon_{\text{ave}}$ under 20; and (iv) it is possible to quantify hydrodynamic stress by CFD techniques, which have been widely used for shear environment investigations [21–23].

The threshold tolerance of shear force varies greatly by cell line, age, flow type and the frequency of exposure time [5]. The correlation of various lethal and sub-lethal biological activities of carrot cells with total energy dissipation was published by Dunlop et al. [12]. Loss of mitochondrial activity was found at energy dissipation higher than 1×10^{-3} w/kg. Furthermore, Takeda's studies indicated that ATP and NAD(P)H were reduced, while cytosolic calcium content increased at the level of 0.5–2.3 w/kg of energy dissipation rate in *C. tinctorius* L. cell culture [24,25]. Moreover, in Sieck's recent study with animal cells, the threshold value of the average energy dissipation rate is 0.4 w/kg, and higher levels would cause productivity loss and a transcriptomic stress response. In addition, a necrosis effect was discovered by Gregoriades and Tanzeglock's studies, observing an energy dissipation rate from 1.0 to 4.0 w/kg [26,27]. Similar results with a range from 0.59 to 1.19 Pa have been reported for CHO cells, while for HEK cells, it is between 1.19 and 1.67 Pa (under laminar and turbulent conditions) [28–32].

In this study, hydrodynamic stress was systematically analyzed with CFD technology in commonly used shaken flask bioreactors under various filled volume and rotation speed conditions. In comparison with only using energy dissipation rates to evaluate shear damage, a more extensive characterization of turbulence through eddy scale calculations was applied to determine the effect of shear damage, which may be better for evaluating the effect on large diameter plant aggregations. The sub-lethal effects caused by hydrodynamics on *C. tinctorius* L. cells in a fed-batch process were effectively evaluated. To develop an industrial plant cell bioreactor design and the maximum operating range of hydrodynamic stress, threshold values of hydrodynamic stress were determined.

2. Materials and methods

2.1. CFD model approach

Fluid flow was characterized by CFD with the commercial software ANSYS CFX (CFX 11.0). The simulations were performed as a two-phase flow using the volume of fluid method. The VOF model is a classical model that has been applied in many studies to simulate the movement of shaken bioreactors, and it has proved to be an effective method of flow evaluation in shake flasks [33]. The working liquid was modeled as a Newtonian fluid with the nominal properties of water (e.g., density, viscosity, surface tension) and air for the dispersed phase. The RNG k- ε turbulence model was used to describe the turbulent flow because of significant amounts of swirl in the movement of the shaken flask [34].

In this research, we paid more attention to details of the calculation process such as mesh independent solution and Free Surface model settings. Several refinement steps were used to obtain a grid-independent solution resulting in a final mesh size of 1.1×10^6 computational elements. Meanwhile, three interface compression levels were tested to mimic the air-water interface more realistically, and we use three different Volume Fraction Smoothing Types to obtain a gradient of a smoothed volume fraction field. These details on the CFD models are shown in the Supplementary Information. The solver ran over at least 5 s (approximately 10 cycles at 115 rpm), and average turbulence energy dissipation rates were monitored to ensure that the simulation would reach a quasi-steady state (Supplementary material).

The flask movement was modeled following a previous work's centrifugal force drive method [23]. Two forces that drive the movement of fluid in the flasks, gravity and centrifugal force, are formed as the flasks revolve. The centrifugal force is then translated into movement of the fluid in the flask with the formation of free surface. The cyclic centrifugal force equation is as follows:

$$F_y = \omega^2 r \cos(\omega t) \quad (1)$$

$$F_x = \omega^2 r \sin(\omega t) \quad (2)$$

where ω is the angular velocity (rad/s), r is the shaking diameter (50 mm), and t is the run duration (s).

2.2. Oxygen mass transfer modeling

Oxygen transfer coefficient always used for evaluating the efficiency of bioreactors is used as one of the scale-up factors. The interfacial mass transfer coefficient between the gas and the liquid phase is expressed in terms of the interfacial area, a , and transfer velocity, k_L . In a shaken flask, a can be calculated with Eq. (3):

$$a = \frac{A}{V} \quad (3)$$

where A is the gas-liquid interface area (m^2) and V is the liquid volume (m^3). The transfer velocity k_L is estimated using the eddy cell model proposed by Lamont and Scott [35] as follows (Eq. (4)):

$$k_L = K \sqrt{D_L} \left(\frac{\varepsilon}{\nu} \right)^{1/4} \quad (4)$$

where $K=0.4$ is the model constant. D_L represents the diffusion coefficient of oxygen at 25 °C, and ν is the liquid kinematic viscosity. As oxygen mass transfer occurs at the interface, the local averaged energy dissipation ε shows better agreement with reported experimental data than volumetric averaged energy dissipation. So here, ε is the face average energy dissipation rate at the gas-liquid interface.

2.3. Shear force analysis

Under these conditions, the stress to which cells were exposed was estimated using an approach previously developed by Soos et al. and Tanzeglock et al. [27,36]. Briefly, stress in the flow field depends on cell size relative to the turbulent eddy length scale (Kolmogorov length scale) $\eta_k = (\nu^3/\varepsilon)^{1/4}$, where ε is the local turbulent energy dissipation rate and ν represents the kinematic viscosity.

If cell size is smaller than this length scale ($d_{\text{cell}} < \eta_k$), any cell damage is controlled by the local hydrodynamics within an eddy. This type of shear force is described as τ_t (for more details, see Tanzeglock et al. [27,36]):

$$\tau_t = \frac{5}{2} \mu \sqrt{\frac{\varepsilon}{6\nu}} \approx \mu \sqrt{\frac{\varepsilon}{\nu}} \quad (5)$$

where μ is the dynamic viscosity of the liquid.

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