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Anthraquinones production in *Rubia tinctorum* cell suspension cultures: Down scale of shear effects

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A B S T R A C T

The effect of turbulence on suspended cells is one of the most complex problems in the scale-up of cell cultures. In the present paper, a direct comparison of the effects of turbulence on suspension cultures of *Rubia tinctorum* in a standard bioreactor and in shake flask cultures was done. A procedure derived from the well known global method proposed by Nishikawa et al. (1977) [39] was applied. Standard flasks and four-baffled shake flasks were used. The effect of turbulence and light irradiation on cell viability, biomass, and anthraquinones (AQs) production was evaluated. The biomass concentration and AQs production obtained using baffled shake flasks agitated at 360 rpm were similar to that achieved in *R. tinctorum* suspension cultures growing in a stirred tank bioreactor operating at 450 rpm, previously published (Busto et al., 2008 [17]). The effect of light on AQs production was found to be very significant, and a difference of up to 48% was found in cells with and without illumination after 7 days of culture. It is concluded that this down-scaled and simple flask culture system is a suitable and valid small scale instrument for the study of intracellular mechanisms of turbulence-induced AQs production in *R. tinctorum* suspension cultures.

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1. Introduction

Elicitation of plant cell cultures is a well-known and effective strategy to enhance the production of many commercially important plant-derived compounds [1,2].

These cultures can be elicited by molecules that stimulate secondary metabolism, which are called "elicitors". Depending on their origin, they are classified as biotic or abiotic [3]. Biotic elicitors are organisms, whole cells, cell components or cell-free chemicals of biological origin. Some traditional biotic elicitors are chitosan, arachidonic acid and pathogen cell walls. Abiotic elicitors are chemicals or physical stimuli, such as UV light or ultrasound [4–6]. These are an interesting alternative since after some time biotic elicitors may lose their ability to facilitate the biosynthesis of the secondary metabolites or can negatively affect the physiological behavior of the cells or tissue cultures. Furthermore, biotic elicitors and chemical abiotic elicitors are substances that are added to the tissue or cell cultures, and must be removed at the end of the process, increasing the cost of purification of the final products [7]. This step is not necessary when physical stimuli, such as UV light, ultrasound or shear stress are used as abiotic elicitors.

Secondary products can be significantly affected by light irradiation in plant cell cultures. Light intensity and duration can induce the production of enzymes required for secondary metabolism. Thus, the presence or absence of light may play an important role in product synthesis and can contribute to its accumulation in the cells [8], which is a point of considerable interest.

Anthraquinones (AQs) are secondary metabolites which are used as dyes in textile and food industries. In addition, there have been reports on therapeutic properties such as antifungal, antiseptic, antioxidant, antileishmanial, and antimalarial activity, as well as on their use for treatment in Alzheimer disease [9–12]. A number of AQs isolated from Rubiaceous species have exhibited strong antitumor activity [13]. AQs have been obtained using *in vitro* cultures from *Rubiaceae* plant species [14], *Rubia tinctorum* suspension cultures among them [15,16].

R. tinctorum cell suspension cultures are a convenient experimental model to investigate the intracellular mechanisms that are activated by mechanical elicitation, because the characteristics of the culture and the simplicity of AQs concentration measurement.

In a previous work [17], we have reported that AQs production in *R. tinctorum* cell suspension cultures in a 1.5 L stirred tank bioreactor (BIOFLO III, New Brunswick Scientific, U.S.A., agitated

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Nomenclature

d	largest inner diameter of the flask (mm)
d_0	shaking diameter (orbital shaker) (mm)
GI	growth index, Eq. (1)
M	net torque (N m)
n	shaking frequency (min^{-1})
Ne′	modified Newton number, Eq. (A.1) and Eq. (A.3)
p	amount of product (μmol)
P	power, Eq. (A.2) (W)
Ph	Phase number, Eq. (A.5)
q_p	specific productivity, Eq. (2) (μmol/g FW d)
Q_p	volumetric productivity, Eq. (3) (μmol/L d)
Re	flask Reynolds number, Eq. (A.4)
t	time of culture (d)
v	culture volume (L)
V_L	filling volume of the shaking flask (mL)
x	amount of biomass (g)
X_{in}	initial biomass concentration (g/L)
X_{max}	maximum biomass concentration (g/L)

Greek symbols

ρ	liquid density (kg m^{-3})
η	dynamic viscosity of the liquid (m Pa s)

with a 55 mm Rushton type six-bladed disk turbine impeller. The power input per unit mass reached 11,700 [cm^2/s^3]) operating at 450 rpm, an increase of 233% above the control cultures (flasks shaken at a speed of 100 rpm, see calculation methods in Appendix A). The detailed description of the bioreactor and all the original experimental data can be found elsewhere [17]. This increase was attributed to the effect of the shear stress generated in the bioreactor.

The search of optimal operation conditions in the bioreactor and the study of the mechanisms involved in shear stress elicitation would require a careful experimental design and a large number of experimental units working in parallel. This is complex and extremely costly if such units are full-fledged bioreactors. As an alternative, a series of shake-flask cultures can be easily set-up, harvested and analyzed in large numbers with relative simplicity, greatly facilitating process optimization through the use of statistically designed experiments [18]. Small-scale cultivation has the advantages of parallelization and cost reduction, provided that the results obtained can be transferred to the later large-scale process [19].

Shaken flasks have been used as simple bioreactors for basic and applied studies carried out on bacteria [20,21], yeast, fungi [22,23], animal cells [24,25], insect cells [26] and also on plant cells [27–30]. In addition, the variety of tasks for which shaken bioreactors are applied is considerable, including elucidation of metabolic pathways [31]. But in spite of its widespread use, no much information was available till recently on the liquid flow and shear rate in shake flask cultures. During the last decade, several papers have been published extending the basic studies on the relation between power input and power dissipation in reactors to the case to shake-flasks [31–38].

Those studies open the possibility of quantification of the flow-related forces acting on suspended cells in shaken flasks, and would therefore make possible the comparison, from the point of view of shear effects, of cultures growing in those flasks and in bioreactor cultures. In the present case however, the available information is not sufficient, as will be discussed further on. We decided therefore to apply a procedure derived from the widely recognized global method proposed by Nishikawa et al. [39] for heat transfer in non-Newtonian heterogeneous reactors.

Ideally, if conditions were found in a shaker such that the liquid dynamics mimics the liquid dynamics in a bioreactor of given geometry and operated at certain agitation rate, it could be expected that the behavior of a culture would be the same in both equipments. This would enable the conduction of multiple experiments in flasks leading to an optimal condition that can be then translated to the process in the bioreactor.

The general aim of this work was to establish a down-scaled and simple culture system using shaken flasks to study shear stress-induced AQs production in *R. tinctorum* cell suspension cultures. The specific objective was to obtain fluid dynamics similar to those prevailing in the bioreactor experiments reported previously [17], in Erlenmeyer flasks.

1.1. Shear stress effects

The fact that hydrodynamic forces influence the behavior of cells in suspension has been recognized long ago one of the first reviews on the matter was published previously [40]. But the recognition and even the analysis of the phenomenon was based on the acceptance of recognized facts with no mechanistic explanation. Only lately deeper studies have been published revealing some of the patterns by which mechanical forces acting around the cell are transduced into biochemical signals that trigger specific events inside the cell. Terms as channel proteins, mechanosensitive channels or ion channel gating are now not uncommon in the literature [41–46]. In the present paper, however, we are interested in the overall performance of the system, represented by AQs production, rather than in the intracellular mechanisms involved. Therefore we are following the classical path of comparing global effects and global results. The results indicate a potential of the method for the study of intracellular phenomenon.

In order to expose the flask cultures to a shear stress similar to that reached in a stirred tank bioreactor operating at 450 rpm as in our previously published paper [17], a hydrodynamic criterion has to be chosen to compare those two environments. There are several possible ways to do this comparison:

1.1.1. Maximum energy dissipation rate

One of them could be to choose the maximum local energy dissipation rate as comparison criterion. That was proposed as the engineering parameter for characterization of hydro mechanical stress controlling the droplet dispersion, pellet breakage, or cell rupture [31,47]. The drawback of such a choice is that hydrodynamic forces acting on a drop may either break it or not: there is nothing between those two extremes. Hydrodynamic forces tend to break the drops, and they are opposed by forces related to physical properties, mainly surface tension and to drop radius: the smaller the drop the stronger the forces opposing hydrodynamic forces. There is a drop size where those opposing forces equilibrate, and this is the maximal stable drop size. If the size of a drop is such that the maximal hydrodynamic forces are below the conservative surface forces, no effect of hydrodynamic forces is detected on the drop. So, the maximum local energy dissipation rate will correlate well with the maximal stable drop, and also with the disruption of cells in a suspension.

But lyses or morphological changes are not the only effects observed in living cells. One of the first reviews on sub-lethal effects of fluid dynamic shear on cells was published in 1991 [40]. Since then multiple sources, several of them mentioned in the introduction section, reinforced the notion that hydrodynamic forces can affect cell functions even before reaching cell-disruption levels. Since this is precisely the type of interaction we are considering here, we conclude that the maximum local energy dissipation rate

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