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

Víctor D. Busto[a], Ariel Calabró-López[a], Julián Rodríguez-Talou[a], Ana M. Giulietti[a,*], José C. Merchuk[b,c]

[a] Microbiología Industrial y Biotecnología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina
[b] Department of Chemical Engineering, Ben-Gurion University, PO Box 653, Beer Sheva 84105, Israel
[c] Department of Pharmaceutical Engineering, Jerusalem College of Engineering, Israel

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ABSTRACT

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.

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

* Corresponding author. Tel.: +54 11 49648269; fax: +54 11 49648200x8377.
E-mail address: agiule@ffyb.uba.ar (A.M. Giulietti).

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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

is not a satisfactory comparison criterion for comparison of bioreactors and shaken flasks.

1.1.2. Characteristic eddy length

Another criterion that could be selected is the critical eddy length, *l*, following Kolmogoroff's theory of isotropic turbulence [48]. It is assumed that the primaries eddies, generated usually mechanically or pneumatically, reach instability and break down transferring their kinetic energy to smaller eddies, and those to still smaller ones, and so on. During this process the original direction of the primary eddies is lost, and the smaller eddies have no preferential direction (isotropic flow). Finally, a range is reached where the eddy Reynolds number is very small, and most of the energy is dissipated. The critical eddy length characterizes the transition from inertia range to dissipation range. It is widely accepted that the potential damages to a suspended particle depends on the ratio between its size and *l*. In our previous work in a bioreactor at 450 rpm, a value of $l = 3 \times 10^{-5}$ [m] was calculated using standard correlations for stirred vessels. Similar calculations were performed for the case of shaken Erlenmeyer flasks, using the correlations presented lately for the evaluation of power dissipation in this system [31–33,38,47]. Performing the corresponding calculations, it becomes evident that the maximal turbulence that could be provided by the available shaker at its maximal agitation rate, 360 rpm, would not be able to produce a small enough *l*. The calculated values of the critical eddy length were higher than that in the bioreactor by one order of magnitude. The correlations provided by Büchs et al. [32] indicate that higher flask diameters would lead to higher power consumption (and consequently to lower *l*) for the same rotation speed. However, higher flask diameters destabilize the desirable flow configuration in the flask, leading to the operating conditions defined by Büchs et al. as "out-of-phase" [49]. In a 1 L flask the calculation of the Phase Number (*Ph*) proposed by those authors (see Appendix A) led to a *Ph* value lower by one order of magnitude from the minimum criteria established, and a test showed as expected from the theory that most of the liquid remained on the bottom of the flask and only a small part of it circulated on the side walls. In view of that, it was decided to modify the flasks adding indents in their sides that would act as baffles, increasing thus the turbulence. The exact value of *l* in the baffled flasks used in the present experiments could not be predicted, since no correlations for energy dissipation in such system is available. The information available [50] refers to larger baffled flasks (300 and 500 cc *vs*. the 100 cc flasks used here). This makes the use of the critical eddy length not suitable as a criterion for comparison of bioreactors and shaken flasks.

1.1.3. Mass transfer coefficient as indicator

The advantages and disadvantages of baffled flasks have been reported in the literature [50–54]. The main disadvantage reported is the higher standard deviation obtained in the experimental results, which stems probably from the lack of standardization in the fabrication procedure, since the baffles are added usually by hand and the reproducibility of the geometry obtained is not very high. On the other hand, it can be expected that the turbulence added by the baffles will be much higher than that expected from a jump in flask diameter or agitation speed. Mc Daniel and Bailey, for example, report that baffles increase the mass transfer rates of one order of magnitude [51]. The study by Peter et al. also reports an increase of one order of magnitude, this time in the actual volumetric power consumption [50].

In the present work, the mass transfer coefficient was measured both in the bioreactor at 450 rpm and in the shaken Erlenmeyer flasks during absorption of oxygen into water. The results can be seen in Table 1. The mass transfer coefficient in the baffled flasks at the higher agitation rate is, as expected from the previous published data mentioned before, much higher than in the unbaffled flasks. In fact, the k_La was quite close to that obtained in the stirred tank bioreactor. However, this cannot be considered by itself more than a general indication. The shear effects we are focusing here take place at the liquid–cell interface. Oxygen absorption is a phenomenon taking place at the gas–liquid interface, which is different in both of the experimental systems used here: it is essentially the bubbles superficial area in the stirred tank reactor, and only the surface of the agitated liquid in the Erlenmeyer.

Table 1
Volumetric oxygen transfer coefficients (k_La) in unbaffled and baffled Erlenmeyer flasks, and in a stirred tank bioreactor system at different shaking rates. nd: not determined.

Bioreactor design	k_La (h^{-1})		
	Shaker speed (rpm)		
	100	360	450
Unbaffled flasks (100 mL)	31.9	49.7	nd
Baffled flasks (100 mL)	33.4	112	nd
Stirred tank bioreactor (1.5 L)	nd	nd	91.1

1.1.4. Nishikawa's global method

Nishikawa et al. [39] faced the problem of correlating the experimental values of the heat transfer coefficient (*h*) in a bubble column with the gas superficial velocity J_G in the case of non-Newtonian liquids. In the case of Newtonian liquids, *h* decreases linearly with the viscosity. But in the case of non-Newtonian liquids, the viscosity (or pseudo-viscosity) is a function of J_G. The method used by Nishikawa et al. was as follows: He made measurements of *h* directly without entering in the analysis of fluid dynamics, for each of a series of gas superficial velocities. He then found the viscosity corresponding to the same heat transfer coefficient *h* in Newtonian liquids. This was called the equivalent of global viscosity of the non-Newtonian system studied, and from the corresponding flow curve he could find an equivalent or global value of the shear rate, which characterized the both the liquid rheology and the flow in the bubble column.

In the present case, the procedure was parallel to that described above. While the basic aim was the identification of the fluid dynamic condition in the flask that mimics that of the bioreactor, the variable selected as base of the comparison was the amount of AQs produced. It was therefore assumed that the fluid dynamics in the shaken system producing AQs at a rate similar to that observed in the bioreactor is similar to the fluid dynamics in the bioreactor.

2. Materials and methods

2.1. Cell suspension cultures

R. tinctorum cells were generously provided by Dr Rob Verpoorte (Leiden University, The Netherlands) and were maintained subculturing every 10 days in B5 medium [55] containing 20 g/L sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/L 1-naphthaleneacetic acid (NAA), 0.5 mg/L indoleacetic acid (IAA), and 0.2 mg/L kinetin.

During the preparation of the cells for the experiments the subculturing was carried out every 7 days, using a 3-fold dilution of cells. Cultures were grown in 250 mL Erlenmeyer flasks at 25 ± 2 °C on an orbital shaker at 100 rpm with a 16 h photoperiod using cool white fluorescent tubes at a light intensity of approximately 90 μmol m^{-2} s^{-1} (PAR).

2.2. Shear stress application assays

The experimental procedure used in the experiments was the following: A 7 days-old culture of *R. tinctorum* suspension cultures

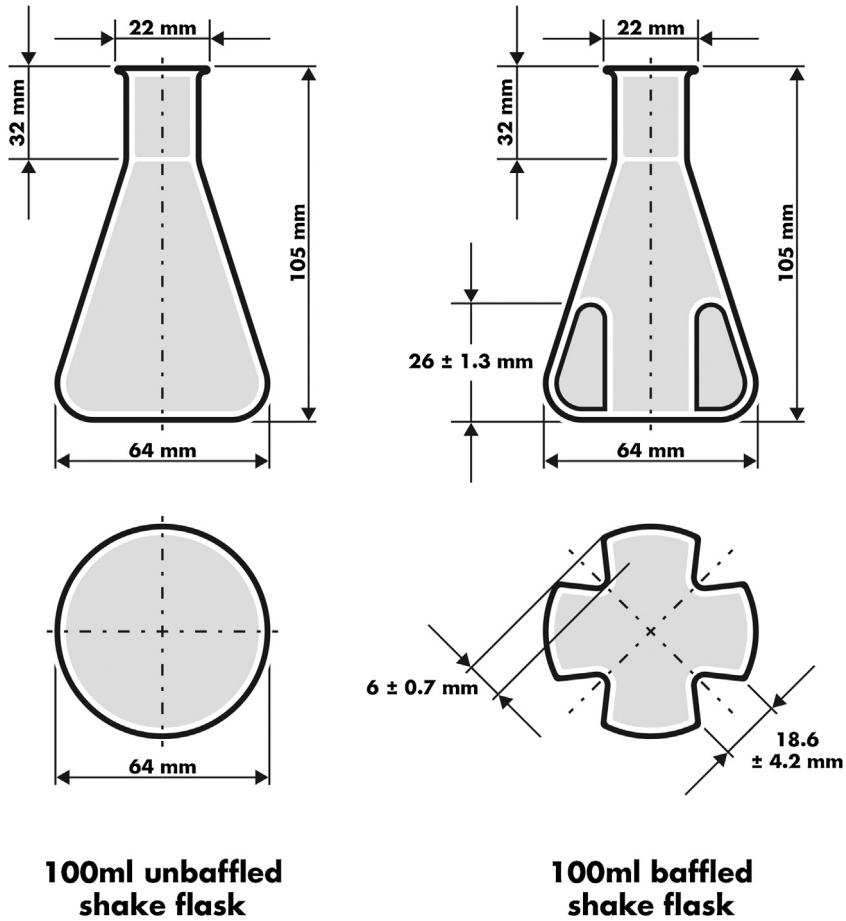

Fig. 1. Schematic drawing of unbaffled and baffled shake flasks used in this work.

in sufficient amount to give an initial concentration of approximately 85 g/L fresh weigh (FW) was inoculated in 25 mL of B5 medium (as described previously) contained in unbaffled and in four-baffled 100 mL Erlenmeyer flasks. The baffled flasks where obtained modifying manually standard 100 mL Erlenmeyer flasks. The flasks were heated at high temperatures, and four indents were made regularly on their flanks at each 90°. The height of the indent was approximately 26 mm. A detailed scheme of the Erlenmeyer flasks used is shown in Fig. 1. The inoculated shake flasks were agitated on an orbital shaker at 360 rpm with shaken diameter of 12 mm during 14 days. At the same time, inoculated baffled and unbaffled shake flasks agitated at 100 rpm were used as control cultures. All the cultures were grown at 25 ± 2 °C in presence or in absence of light with a 16 h photoperiod using cool white fluorescent tubes at a light intensity of approximately 90 μmol m^{-2} s^{-1} (PAR).

The stressed cultures and the controls were harvested at 7 and 14 days after inoculation. Each sample was tested for biomass concentration (fresh weight basis, FW), AQs content in the cells, pH in the medium and cell death. The following ratios were calculated from the experimental results, for the analysis of efficiency of growth and AQs production:Growth index (GI):

$$\mathrm{GI} = \frac{X_{\max} - X_{in}}{X_{in}} \quad (1)$$

Specific productivity (q_p):

$$q_p = \frac{p}{x} \cdot t \quad (2)$$

Volumetric productivity (Q_p):

$$Q_p = \frac{p}{v} \cdot t \quad (3)$$

2.3. Analytical techniques

The biomass quantification method was the same as described previously [56]. Anthraquinones concentration was estimated according to Schulte et al. [14] and cell death by the Evans Blue dye uptake test, as described by Smith et al. [57].

2.4. Mass transfer coefficients

Volumetric oxygen transfer coefficients (k_La) in both types of flasks were determined by the sulfite method according to Értola et al. [58] and Garcia-Ochoa and Gomez [59]. k_La values in the stirred tank bioreactor were estimated by the dynamic gassing out method, as described by Garcia-Ochoa and Gomez [59].

2.5. Statistical analysis

The results presented were the means of three independent experiments. Sample variability was given as the standard deviation of the mean. Significance of treatment effects was determined by using variance analysis (STATISTIX 8.0 software).

2.6. Chemicals

All the media components were purchased from Sigma Chemical Co. (St. Louis, MO) and Evans Blue dye from Merck (Darmstadt).

3. Results and discussion

3.1. Time course of growth, pH and cell death

Fig. 2 shows the biomass concentrations obtained under all cultivation conditions, after 7 and 14 days. The first point that becomes evident in the figure is that almost no growth takes place during the second week of cultivation. This is similar to the results obtained in our previous work [17]. The second feature on the graph is that two groups of experimental results can be detected on the first view: one conformed by the data obtained in unbaffled shaken flasks, which render considerably higher biomass concentrations (Fig. 2B), and the second one including all the results of baffled flasks, with lower biomass concentrations (Fig. 2A).

Within Fig. 2A it is seen that the biomass concentrations obtained at 7 and 14 days of culture at 360 rpm were lower than those attained in the same flasks agitated at 100 rpm at the same times. These biomass decreases are about 28% comparing to control conditions after 14 days of culture. A similar effect was observed at 7 and 14 days of culture in unbaffled Erlenmeyer flasks agitated at 360 rpm, where the biomass concentrations decreased compared to those obtained in the same flasks agitated at 100 rpm (Fig. 2B). However, this reduction was not statistically significant. In addition, these biomass decreases were observed either in presence or absence of light in both types of flasks. Therefore, no clear effect of light was detected in this case.

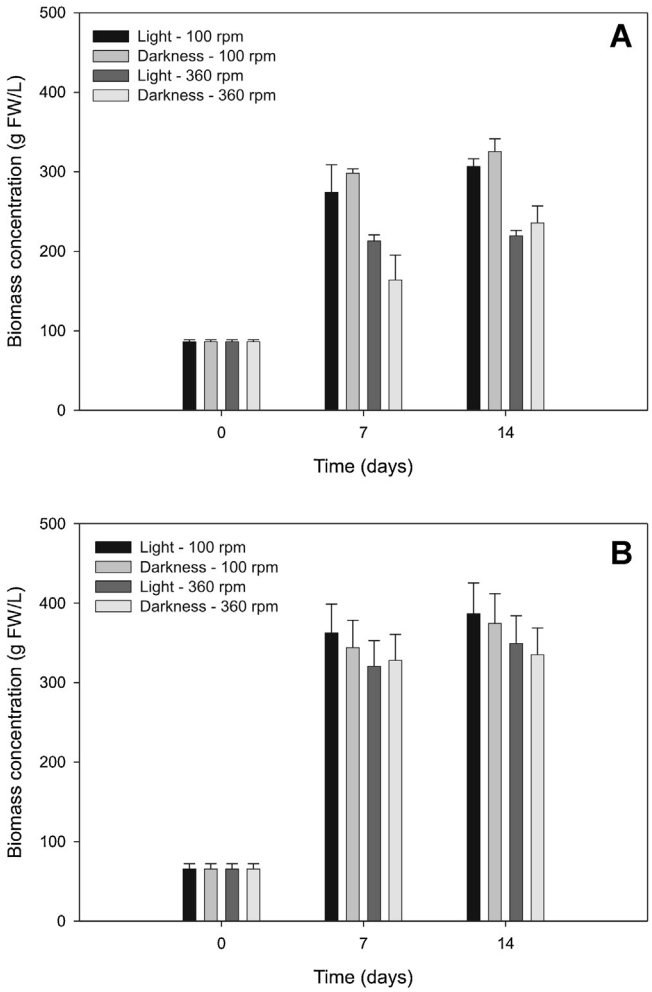

Fig. 2. Time course of growth of *R. tinctorum* suspension cultures grown in baffled (A) and unbaffled Erlenmeyer flasks (B) agitated at 100 and 360 rpm, and in presence or absence of light. All values are the mean ± standard deviation (SD) of three independent experiments.

Table 2
Comparative values of growth in *R. tinctorum* suspension cultures growing in baffled and unbaffled Erlenmeyer flasks under shear stress and light/darkness conditions.

Treatments	Baffled flasks		Unbaffled flasks	
	FW (g/L)	GI	FW (g/L)	GI
Light 100 rpm	306.7 ± 9.6	2.56 ± 0.11	386.5 ± 37.6	4.89 ± 0.39
Darkness 100 rpm	325.3 ± 16.1	2.78 ± 0.18	374.4 ± 34.4	4.71 ± 0.28
Light 360 rpm	219.5 ± 6.7	1.55 ± 0.08	349 ± 31.9	4.32 ± 0.30
Darkness 360 rpm	235.6 ± 21.5	1.73 ± 0.25	335.1 ± 32.5	4.11 ± 0.16

Growth index values (GI) of baffled shake flasks cultures agitated at 100 rpm were approximately 65% and 60% higher than those registered for cultures agitated at 360 rpm in presence and absence of light, respectively (Table 2). These GI stem from the higher biomass concentrations (FW basis) obtained. Besides, GI values were about 14% higher when cultures were grown in unbaffled shake flasks agitated at 100 rpm compared to those cultivated at 360 rpm in the same flasks, both in presence and in absence of light (Table 2).

Although decreases of biomass concentrations and GI values were observed at 360 rpm in both culture systems, the most important effects were registered in baffled Erlenmeyer flasks, as shown in Table 2. This shows that hydrodynamic conditions generated by baffles did affect significantly the biomass production. Nevertheless, presence or absence of light did not have an effect on it.

These results are in line with those reported by Tanaka [60], who worked with two different plant cell lines. This author observed decreases of specific growth rates when *Cudrania tricuspidata* suspension cultures were cultivated in shake flasks at high agitation rates. Moreover, biomass concentrations of *Catharanthus roseus* suspension cultures decreased when turbulence was increased by baffles addition in the Erlenmeyer flasks.

Additionally, biomass concentration decreases have been reported in *Beta vulgaris*, *Uncaria tomentosa* and *Solanum chrysotrichum* suspension cultures growing under shear stress in stirred tank bioreactors [61–63]. Similarly, this behavior was previously registered for *R. tinctorum* growing in a mechanically stirred bioreactor operating at 450 rpm [17].

The values of pH in the culture medium shown in Fig. 3A for baffled flasks agitated at 100 rpm in both light and darkness conditions are similar both at 7 and 14 days. pH values in baffled flasks agitated at 360 rpm resulted higher than those observed in the control at 7 days of culture. At 14 days of culture, similar results were observed in illuminated Erlenmeyer flasks at 360 rpm, whereas pH values in Erlenmeyer flasks kept in darkness at the same agitation rate were not so different from those of control line.

On the other hand, all the pH values registered for unbaffled Erlenmeyer flasks were similar at 7 days of culture, while pH values observed after 14 days of culture at 360 rpm were slightly higher than those observed in the control cultures both in presence and in absence of light (Fig. 3B).

However, all these changes are small if compared to the big jump in pH from the initial conditions to date 7. The pH values for all the tested conditions and in both types of flasks were quite higher than the original pH value, showing an alkalinization of the culture medium. This alkalinization along the cultures is usually a common behavior of several plant cell lines. However, this response becomes more important when cells are subjected to shear stress. pH increases have been reported by *Taxus cuspidata* [64,65] and *Centaurea calcitrapa* [66] suspension cultures growing under shear

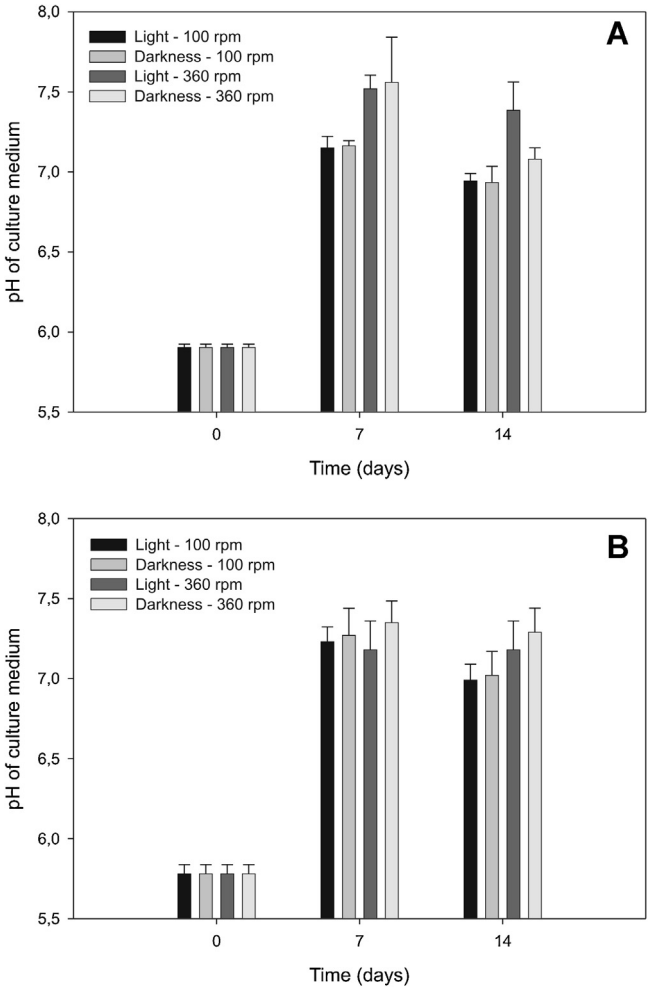

Fig. 3. pH profile of *R. tinctorum* suspension cultures grown in baffled (A) and unbaffled Erlenmeyer flasks (B) in presence or absence of light. All values are the mean ± standard deviation (SD) of three independent experiments.

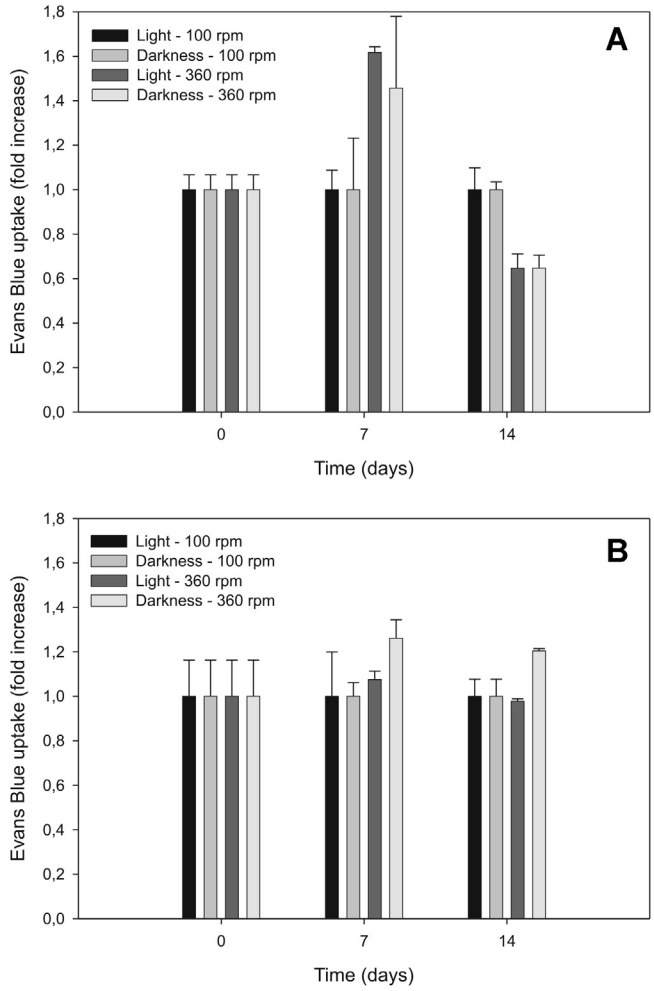

Fig. 4. Cell death indicated as Evans Blue uptake of *R. tinctorum* suspension cultures grown in baffled (A) and unbaffled (B) Erlenmeyer flasks in presence or absence of light. All values are the mean ± standard deviation (SD) of three independent experiments.

stress. Experiments run in our laboratory (not shown here) show that the alkalinization process is very strongest during the first stage of the culture. It is highly probable, therefore, that the pH increased more dramatically during the first days and subsided later. This will be considered in depth in our future work, where the shear effects will be analyzed from a biochemical point of view.

Concerning the cell death, an increase of approximately 60% and 40% of Evans Blue uptake was observed in cells cultivated at 360 rpm in baffled Erlenmeyer flasks at 7 days of culture in presence and absence of light, respectively (Fig. 4A). This cell viability loss is probably associated with the damage produced by the shear stress imposed. However, this reduction in viability was not as significant as that registered in a stirred tank bioreactor operating at 450 rpm where Evans Blue uptake rates reached about 6-fold the initial value [17]. After 14 days of culture, a reversion of viability loss was observed in cultures at 360 rpm, and cell death became lower than that of control cultures. These results suggest a culture adaptation to the cultivation environment, which means a reversion of the negative effects of turbulence on both viability and biomass concentration. This phenomenon was also observed on regrowth ability and viability studies by *R. tinctorum* cells cultured in a bioreactor [17]. However, it is important to note that Evans Blue dye exclusion test measures membrane integrity alone. Thus, turbulence-related membrane damage may only be temporary and is not necessarily indicative of viability loss [67].

Besides, cell viability did not show changes under light conditions in cells cultivated at 360 and 100 rpm in unbaffled Erlenmeyer flasks (Fig. 4B). However, a reduction of about 26% and 20% was observed in cultures agitated at 360 rpm under darkness conditions at 7 and 14 days, respectively (Fig. 4B).

In contrast, *Podophyllum hexandrum* cell suspension cultures cultivated in unbaffled Erlenmeyer flasks at high agitation rates (up to 300 rpm) were susceptible to shear stress showing high cell death rates. In that case, an inversely proportional relationship between cell viability of *P. hexandrum* and agitation rate was demonstrated [8]. The present results indicate the membrane damage under hydrodynamic stress is species-dependent.

3.2. AQs production: shear and light effects

The effect of shear stress on AQs production by *R. tinctorum* suspension cultures in baffled Erlenmeyer flasks is shown in Fig. 5A and B, where an increase in the synthesis of secondary metabolites is presented both in *per cell* and in volumetric terms (AQs produced in the liquid culture). At 7 days of culture, AQs content (μmol/g FW) of lighted suspension cultures at 360 rpm was 1.3 times higher than that of control cultures. However, a superior AQs production was obtained in the cells kept in the darkness agitated at 360 rpm, whose AQs content was 1.82 times higher than that in control cells at the same time, with a specific (q_p) and volumetric (Q_p)

Table 3
Comparative values of AQs production parameters in *R. tinctorum* suspension cultures growing in baffled and unbaffled Erlenmeyer flasks under shear stress and light/darkness conditions.

Treatments	Baffled flasks		Unbaffled flasks	
	q_p (μmol/g FW d)	Q_p (μmol/L d)	q_p (μmol/g FW d)	Q_p (μmol/L d)
Light 100 rpm	0.145 ± 0.013	39.73 ± 3.60	0.161 ± 0.001	58.45 ± 0.28
Darkness 100 rpm	0.141 ± 0.011	42.04 ± 3.24	0.213 ± 0.039	73.51 ± 13.63
Light 360 rpm	0.330 ± 0.011	70.35 ± 2.32	0.245 ± 0.015	78.51 ± 4.96
Darkness 360 rpm	0.396 ± 0.077	65.01 ± 12.73	0.378 ± 0.023	124.17 ± 7.54

productivity of 0.396 μmol/g FW d and 65.01 μmol/L d, respectively. This q_p value implies an AQs enhancement of 181% comparing to control conditions (Table 3). Moreover, the maximum AQs accumulation was achieved when suspension cultures were cultivated under shear stress and darkness conditions in baffled flasks during 14 days. This maximum value of AQs, which is about 2.2 times higher than that obtained in control conditions, is similar to that achieved in *R. tinctorum* suspension cultures growing in a stirred tank bioreactor operating at 450 rpm, at 10 days of culture [17]. In that case AQs content, which was 2.3 times higher than that in the control cultures, was 2.7 μmol/g FW (specific production) and 681.3 μmol/L (volumetric production).

The AQs production obtained in unbaffled Erlenmeyer flasks is shown in Fig. 6A and B, where a AQs increase of 52% can be observed after 7 days of culture at 360 rpm in presence of light compared to the same flasks agitated at 100 rpm. The absence of light enhanced even more AQs production by 77% at 360 rpm compared to control cultures agitated at 100 rpm. Production parameters (q_p and Q_p) were increased under darkness conditions by 78% and 69%, respectively (Table 3). These Q_p values resulted higher than those attained using baffled flasks due to a superior biomass concentration obtained in this type of flasks.

It has to be noted that when *R. tinctorum* cells are subjected to shear stress at 360 rpm in both, unbaffled and baffled flasks, AQs productions per unit biomass attained at 14 days of culture are not so different to those observed at 7 days of culture, considering standard deviation (SD) of the experiments (Figs. 5 and 6). It is apparent therefore that the optimal length of a run is between 7 and 14 days, probably closer to one week.

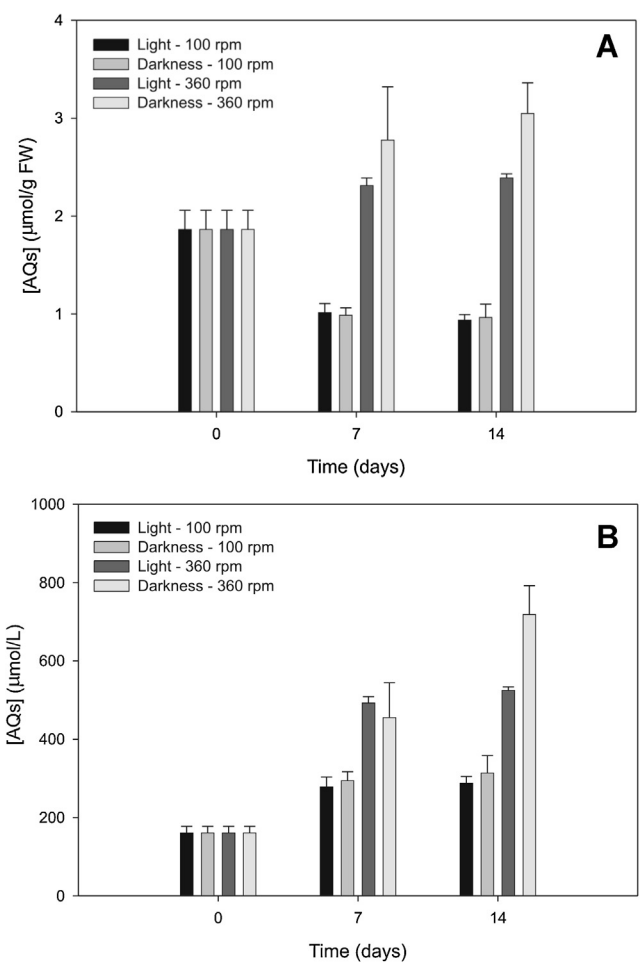

Fig. 5. Time course of AQs content of *R. tinctorum* suspension cultures grown in baffled Erlenmeyer flasks agitated at 100 and 360 rpm, and in presence or absence of light. Specific production (μmol/g FW) (A) and volumetric production (μmol/L) (B). All values are the mean ± standard deviation (SD) of three independent experiments.

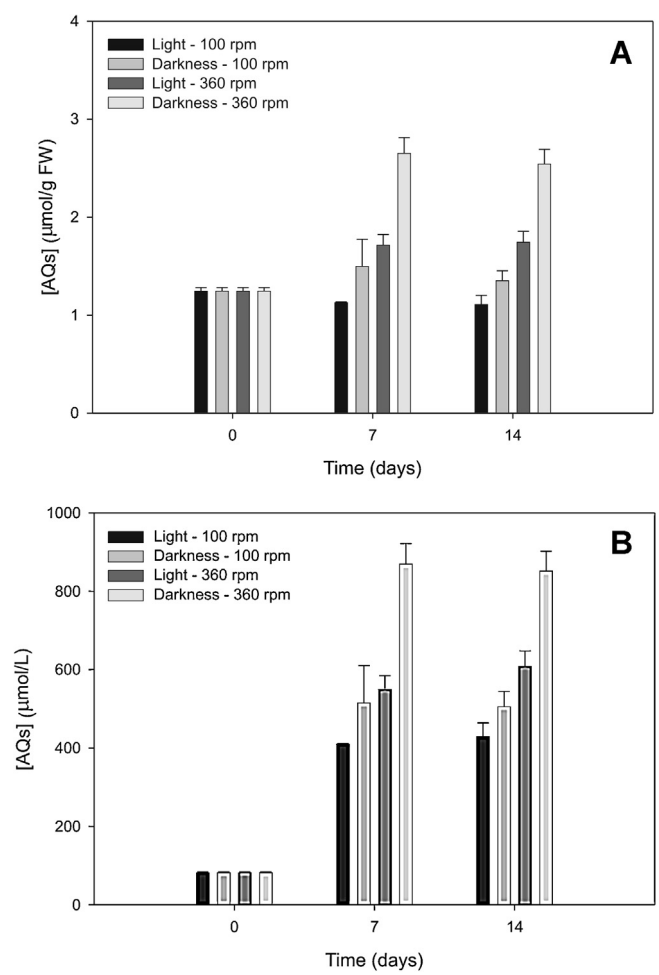

Fig. 6. Time course of AQs content of *R. tinctorum* suspension cultures grown in unbaffled Erlenmeyer flasks agitated at 100 and 360 rpm, and in presence or absence of light. Specific production (μmol/g FW) (A) and volumetric production (μmol/L) (B). All values are the mean ± standard deviation (SD) of three independent experiments.

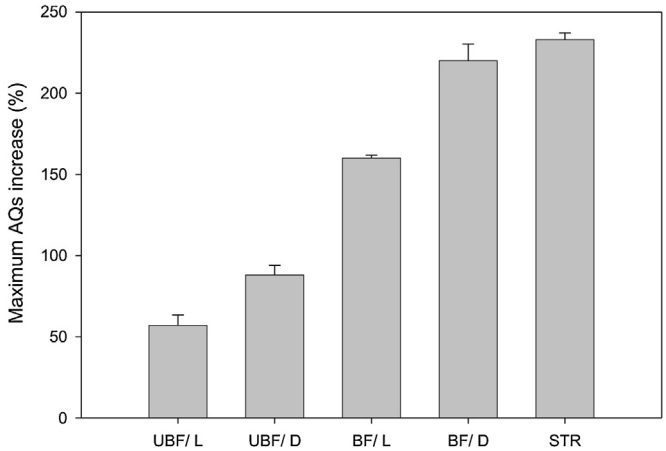

Fig. 7. AQs increases obtained in *R. tinctorum* suspension cultures grown in unbaffled (UBF) and baffled Erlenmeyer flasks (BF) at 360 rpm under light (L)/darkness (D) conditions, and in a stirred tank bioreactor system (STR) operating at 450 rpm [17]. The reference value is the AQs production in the control, flasks shaken at 100 rpm (baffled and unbaffled flasks gave the same results at this conditions). All values are the mean ± standard deviation (SD) of three independent experiments.

A similar shear stress response was also reported in the technical literature for several secondary metabolites produced by other plant suspension cultures. *U. tomentosa* suspension cultures increased its monoterpenoid oxindole alkaloids content (MOA) when growing in a bioreactor at 400 rpm [68]. L-DOPA production by *Stizolobium hassjoo* was enhanced under shear stress generated by different impeller types in a stirred bioreactor [69,70].

The maximum AQs production achieved in unbaffled and baffled flasks under light/darkness conditions and in a bioreactor system [17] are shown in Fig. 7. In this figure the AQs production (μmol/g FW) is presented as the percentage of improvement over that corresponding to the control, the flasks shaken at 100 rpm. It can be seen that the effect of light is similar in both types of flasks, and indeed the darkness promotes AQs generation in any case. But in absolute values, the rate of AQs synthesis is much lower in sheared unbaffled flasks. Under darkness conditions, which are the most favorable, the amount of AQs produced in baffled flasks is 2.2 times higher, and reaches approximately the same productivity of the bioreactor at 450 rpm [17].

In several cultured plant cells, the formation of secondary products has been reported to be significantly affected by light. The diverse effects depend on plant species, type of light used, light intensity and duration. Some authors have observed an increased secondary metabolites production due to light irradiation [71,72]. Nevertheless, most of reports point toward an inhibition of the synthesis and a decreased accumulation of secondary products. *L. erythrorhizon* dark-grown cell cultures were reported to accumulate an elevated level of acetylshikonin [73]. Cell growth and podophyllotoxin production by *P. hexandrum* suspension cultures were relatively higher in the dark [8]. Suspension cultures of *Rubia cordifolia*, another AQs producer, were affected by monochromatic blue and red light on AQs formation and cell growth [74].

In our case, the presence of light did not affect AQs production in control conditions, but a significant difference (approximately 40%) was observed in cultures agitated at 360 rpm in baffled flasks with and without light irradiation at 7 days of culture. This difference was incremented up to 48% when *R. tinctorum* suspension cultures were cultivated in unbaffled Erlenmeyer flasks at the same time. In addition, these differences observed in cell suspensions cultured with and without light irradiation do not change significantly at 14 days of culture in both, unbaffled and baffled Erlenmeyer flasks. Since it was found that the maximum AQs production was achieved in absence of light we could assume that darkness plays an important role on AQs synthesis, and its effects were increased at higher shear rates.

The similar AQs production is a strong indication that baffled Erlenmeyer flasks agitated at 360 rpm in absence of light is a convenient culture system to study shear stress-induced AQs production in *R. tinctorum* cell suspension cultures. The aim of the present work is establishing the possibility of scale-down of processes induced by hydrodynamic stress, and we started evaluating the critical eddy length l in a bioreactor agitated at 450 rpm [17]. Calculations and experiments showed that this level of agitation was not attainable in conventional Erlenmeyer flasks agitated at the maximal speed that we could get in our laboratory shaker, 360 rpm. We resorted then to improving the turbulence in the flasks by adding baffles in the flasks, which lead to the hoped rise in AQs production. Neither data nor correlation on the power consumption in such system is available, and therefore no calculation on the actual value of l in the flasks could be performed. However, the actual level of AQs obtained (same order as in the bioreactor at 450 rpm) can be seen as clear indication that the cells in the baffled flasks were subjected to similar hydrodynamic stress, following the methodology of Nishikawa et al. described above. This information is valid only as an approximation for other researchers, because of the lack in repeatability in the fabrication of the Erlenmeyer flasks. Nevertheless, we consider this an extremely important proof of principle. Potentially, this method may provide a base for the calculation of the conditions leading to hydrodynamic stress in shaken flasks similar to that generated in a standard stirred bioreactor.

4. Conclusions

Suspension cultures of *R. tinctorum* were grown under shear stress generated using unbaffled and four-baffled shake flasks agitated at 360 rpm. The biomass concentration and AQs production obtained using baffled flasks were similar to those achieved in *R. tinctorum* suspension cultures growing in a stirred tank bioreactor operating at 450 rpm [17]. The negative effects of shear stress on viability and biomass concentration were reverted at14 days of culture. Cultures grown in darkness produced more AQs, and this effect was increased at higher turbulence.

The shear stress generated by four-baffled shake flasks at 360 rpm elicited mechanically the AQs synthesis in *R. tinctorum* suspension cultures in a satisfactory degree. The amount of AQs obtained in the baffled flasks was very similar to that obtained in the stirred bioreactor at 450 rpm. Following Nishikawa's method, it is concluded that the hydrodynamic stress on the suspended cells was similar as well. The present results may not be directly used by others because the repeatability in the geometry of hand-fabricated baffled flasks is not very reliable, but they strongly indicate the potential of the method. It can be concluded that this down-scaled and simple culture system is a suitable and valid instrument for the study of shear stress-induced AQs production in *R. tinctorum* cell suspension cultures. Generalization of the technique will be reached once standardization of baffled flasks is attained.

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Appendix A

The calculation of the power consumption in unbaffled shake flasks was done following Büchs [33]. The modified Newton number was defined as:

$$Ne' = \frac{P}{\rho n^3 d^4 V_L^{1/3}} \tag{A.1}$$

where

$$P = M(2\pi n) \tag{A.2}$$

The modified Newton number is correlated as:

$$\mathrm{Ne}' = 70Re^{-1} + 25Re^{-0.6} + 1.5Re^{-0.2} \tag{A.3}$$

And the Reynolds number for the shake flask is defined by:

$$Re = \frac{\rho n d^2}{\eta} \tag{A.4}$$

The "out of phase phenomenon" and the dimensionless group that identifies it, Ph, where defined in reference [49].

$$\mathrm{Ph} = \frac{d_0}{d}\left(1 - 3\log\left(\frac{\rho n d^2}{\eta}\frac{\pi}{2}\left(1 - \sqrt{1 - \frac{4}{\pi}\left(\frac{V_L^{1/3}}{d}\right)^2}\right)^2\right)\right) \tag{A.5}$$

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