Down-Scale of Hydrodynamic Shear Effects on Suspended Cells

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ABSTRACT

The effect of shear forces on suspended cells is one of the most complex problems in the scale-up of cell cultures. In the present paper, recent advances in the understanding of power consumption during agitation of Erlenmeyer flasks in rotary shakers are applied to direct comparison of the effects of shear forces on suspension cultures of *Rubia tinctorum* cells in a standard bioreactor and in shake flask cultures. The criterion selected as indicative of the shear stress acting on the cells was the critical eddy length, *l*, following Kolmogoroff's theory of isotropic turbulence. Standard flasks and four-baffled shake flasks were used. The effect of shear stress 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 previously in *R. tinctorum* suspension cultures growing in a stirred tank bioreactor operating at 450 rpm. It can be concluded that this down-scaled and simple flask culture system is a suitable and valid small scale instrument to study intracellular mechanisms of shear stress-induced AQs production in *R. tinctorum* suspension cultures, including intracellular phenomena, and can be eventually extended to other systems.

INTRODUCTION

Elicitation of plant cell cultures is a well-known and effective strategy to enhance the production of many commercially important plants 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 biotic elicitors can lose their capacity over time to elicit the biosynthesis of the secondary metabolites or can negatively affect the physiological properties of the cells or tissue cultures. In addition, biotic elicitors and chemical abiotic elicitors must be added to the tissue or cell cultures stimulating secondary metabolites production, and the desired compounds have to be separated from the elicitor, increasing the cost of the final products *(7)*. However, 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 plays an important role in product synthesis and can contribute with its accumulation into the cells *(8)*.

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

In a previous work, we have reported that shear stress generated in a stirred tank bioreactor operating at 450 rpm increased AQs production up to 233% in *R. tinctorum* cell suspension cultures *(17)*. Due to cultural properties and easy AQs determination, *R. tinctorum* cell suspension cultures represent a convenient

experimental model to investigate the intracellular mechanisms that are activated by mechanical elicitation.

The search of optimal operation conditions and the study of the mechanisms involved in shear stress elicitation 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 in small-scale studies can be transferred to the later large-scale process *(19)*.

Shaked flasks have been used as simple bioreactors for basic and applied studies carried out by bacteria *(20, 21)*, yeast, fungi *(22, 23)*, animal cells *(24, 25)*, insect cells *(26)* and also plant cells *(27 - 29)*. In addition, the variety of tasks for which shaking bioreactors is applied is considerable, including elucidation of metabolic pathways *(30)*. 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 *(30 - 32)*. Those studies open the possibility of quantification of the flow-related forces acting on suspended cells in a shaked flask, and therefore the comparison of cultures growing in those flasks to bioreactor cultures, from the point of view of shear effects.

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 rpm, 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 process in the bioreactor

The aim of this work was to design a down-scaled and simple culture system using shaked flasks to study shear stress-induced AQs production in *R. tinctorum* cell suspension cultures, aimed at obtaining in the Erlenmeyer flasks results similar to those found on bioreactor experiments conducted previously *(17)*.

MATERIALS AND METHODS

Cell suspension cultures. *R. tinctorum* cells were a gift from Dr Rob Verpoorte (Leiden University, The Netherlands) and were maintained subculturing every 10 days in B5 medium *(33)* 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.

In 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).

Shear stress application assays. In order to subject the flask culture to a similar shear stress as that reached using a stirred tank bioreactor operating at 450 rpm *(17)*, a hydrodynamic criterion had to be chosen to compare those two environments. The criterion selected was the critical eddy length, *l*, following Kolmogoroff's *(34)* theory of isotropic turbulence. It is assumed that the primaries eddies, generated usually mechanically or pneumatically, reaches 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 stirred bioreactor, a value of $l = 3*10^{-5}$ [m] was calculated using standard correlations for stirred vessels. Similar calculations were performed for the case of shaked Erlenmeyer flasks, using the correlations presented lately for the evaluation of power dissipation in this system *(30 - 32, 35)*. 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. Preliminary experiments run at speeds of agitation up to360 rpm (to be shown latter on Figure 5) confirmed those calculations, since the effect in the measured AQ concentration was significantly lower than that registered in the bioreactor operating at 450 rpm *(17)*. The correlations provided by Büchs *et al. (31)* 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 de-stabilize the desirable flow configuration in the flask, leading to the operating conditions defined by Büchs *et al.* "out-of-phase" *(36)*. The calculation of the Phase Number (*Ph*) proposed by the authors led to a *Ph* value lower by one order of magnitude from the minimum criteria established by the authors, and a test

in a 1 liter flask showed as predicted 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 advantages and disadvantages of baffled flasks have been reported in the literature *(37 – 40)*. 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 is much higher that that expected from a jump in flask diameter or agitation speed. McDaniels and Bailey *(37)*, for example, report that baffles increase the mass transfer rates of one order of magnitude. The study by Peter *et al. (40)* also reports an increase of one order of magnitude, this time in the actual volumetric power consumption. 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, but it was expected that the same order of magnitude as in the bioreactor *(17)* would be approached. The effect of such increase in energy dissipation would be detected as an increase in the production of AQs.

The experimental procedure used in the experiments was the following: A 7 days-old culture of *R. tinctorum* suspension cultures 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 100 mL four-baffled Erlenmeyer flasks. The inoculated baffled shake flasks were agitated on an orbital shaker at 360 rpm during 14 days. At the same time, inoculated baffled shake flasks agitated at 100 rpm were used as control cultures. All the cultures were grown at 25 ± 2 °C in presence or 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 viability. The following ratios were calculated from the experimental results, for the analysis of efficiency of growth and AQs production:

Growth index (GI): [Maximum cell density – Initial cell density] / [Initial cell density]

Volumetric productivity (Qp): [Amount of product] / [(Culture volume) (Culture time)] (μmol/L.d)

Analytical techniques. The biomass quantification method was the same as described previously *(41)*. Anthraquinone analysis was estimated according to Schulte *et al. (14)* and cell viability by the Evans Blue dye exclusion test, as described by Smith *et al. (42)*.

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 were determined by using variance analysis (STATISTIX 8.0 software).

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

RESULTS AND DISCUSSION

Time course of growth, pH and cell viability. Figure 1 shows the time course of *R. tinctorum* suspension cultures grown in control and shearing conditions, both in presence and in absence of light. It can be seen that the biomass concentration obtained after 14 days of culture in baffled Erlenmeyer flasks agitated at 360 rpm was about 28% lower than that attained in the same flasks agitated at 100 rpm at the same time. This decrease was observed either in presence or absence of light. Growth index values (GI) of 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. These GI stem from the higher biomass concentrations (FW basis) obtained (Table I).

Table I. Comparative values of growth and AQs production parameters in *R. tinctorum* suspension cultures growing under shear stress and light/darkness conditions.

Treatments	FW (g/L)	[AQs] (μmol/g FW)	[AQs] (μmol/L)	GI	Q_p (μmol/L.d)
Light 100 rpm	306.7 ± 9.6	0.94 ± 0.05	287.7 ± 17	2.56 ± 0.11	20.55 ± 1.21
Darkness 100 rpm	325.3 ± 16.1	0.96 ± 0.13	313.7 ± 44.3	2.78 ± 0.18	22.41 ± 3.17
Light 360 rpm	219.5 ± 6.7	2.39 ± 0.04	524.6 ± 9.1	1.55 ± 0.08	37.47 ± 0.64
Darkness 360 rpm	235.6 ± 21.5	3.05 ± 0.31	718.4 ± 73.6	1.73 ± 0.25	51.31 ± 5.25

These results are in line with those reported by other authors, where a decrease of biomass concentration was found in *Beta vulgaris*, *Uncaria tomentosa* and *Solanum chrysotrichum* growing under shear stress in stirred tank bioreactors *(43 - 45)*. In addition, this behavior was previously registered for *R.*

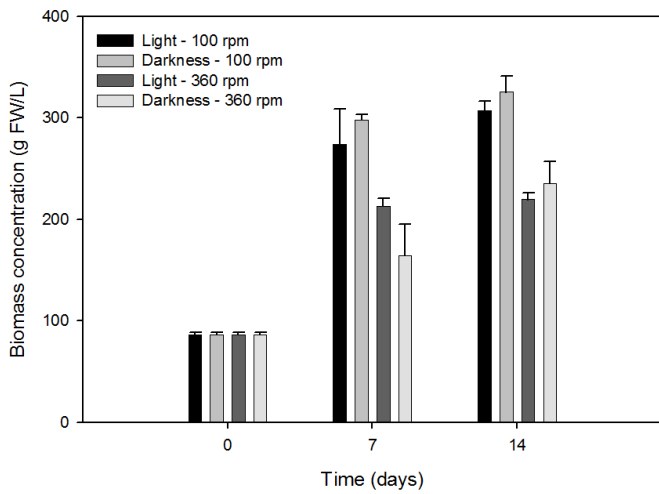*tinctorum* growing in a mechanically stirred bioreactor operating at 450 rpm *(17)*.

Figure 1. Time course of growth of *R. tinctorum* suspension cultures grown in baffled Erlenmeyer flasks 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.

The values of pH in the culture medium shown in Figure 2 for baffled flasks agitated at 100 rpm in both light (A) and darkness conditions (B), are similar during the time tested. 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 lighted Erlenmeyer flasks, whereas pH values in Erlenmeyer flasks kept in darkness were not so different from those of control line. It has to be noted that all the pH values 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, it becomes more important as a response when cells are subjected to shear stress. pH increases have been reported by *Taxus cuspidata (46, 47)* and *Centaurea calcitrapa (48)* suspension cultures growing under shear 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.
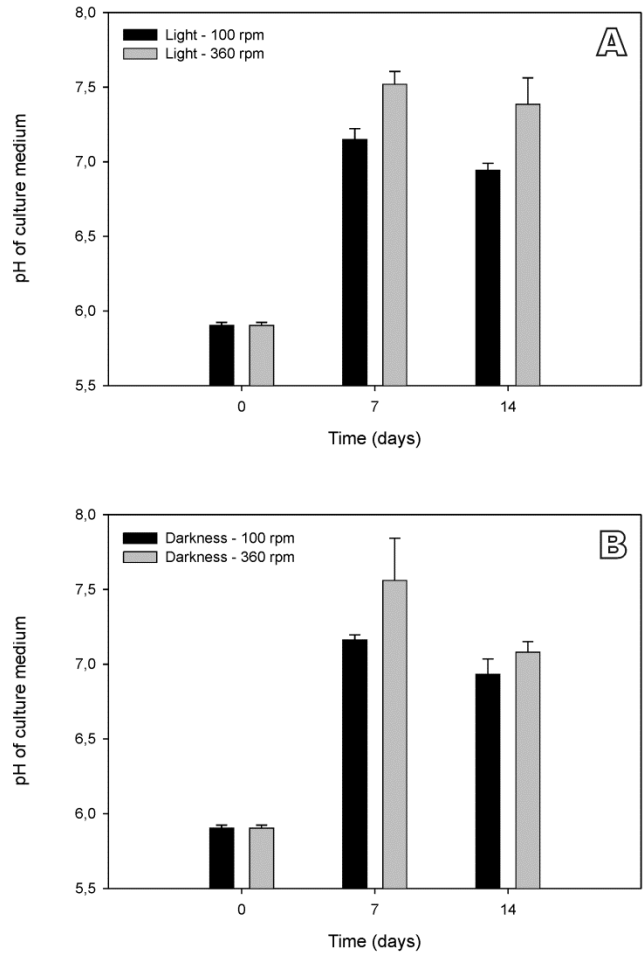

Figure 2. pH profile of *R. tinctorum* suspension cultures grown in baffled Erlenmeyer flasks in presence (A) or absence of light (B). All values are the mean ± standard deviation (SD) of three independent experiments.

Concerning the cell viability, a reduction of approximately 60% and 40% was observed in stressed cells at 7 days of culture in presence and absence of light, respectively (Figure 3, A and B). This cell viability loss is probably associated with the damage produced by the shear stress imposed. However, this reduction 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 at day 3 *(17)*. After 14 days of culture, a reversion of viability loss was observed in stressed cultures, 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 shear stress 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, shear-related membrane damage may

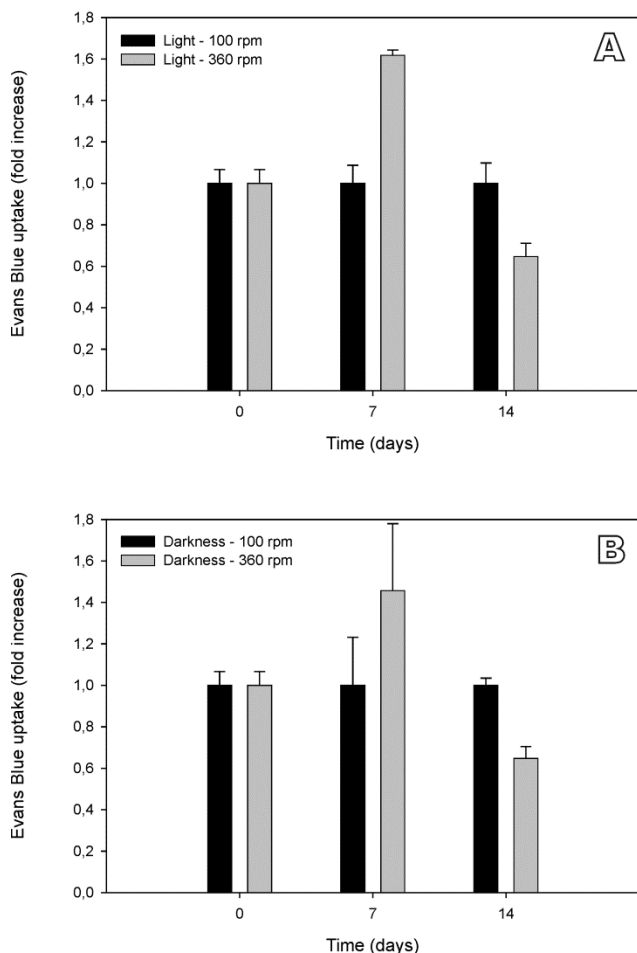only be temporary and can not necessarily be indicative of viability loss *(49)*.

Figure 3. Cell death indicated as Evans Blue uptake of *R. tinctorum* suspension cultures grown in baffled Erlenmeyer flasks in presence (A) or absence of light (B). All values are the mean ± standard deviation (SD) of three independent experiments.

AQs production and light effects. The effect of shear stress on AQs production is shown in Figure 4 (A and B), where an increase in the synthesis of secondary metabolites is observed. A similar shear stress response was also reported for several secondary metabolites produced by other plant suspension cultures. *Uncaria tomentosa* suspension cultures increased its monoterpenoid oxindole alkaloids content (MOA) growing in a bioreactor at 400 rpm *(50)*. L-DOPA production was enhanced under shear stress generated by different impeller types in a stirred bioreactor by *Stizolobium hassjoo (51, 52)*. At 14 days of culture, AQs content (μmol/g FW) of lighted suspension cultures under shear stress was 1.6 times higher than that of control cultures. However, the maximum AQs accumulation was obtained in the sheared cells kept in the darkness, whose AQs content was 2.2 times higher than that in non-sheared cells

at the same time, with a volumetric production and volumetric productivity of 718.4 μmol/L and 51.31 μmol/L.d, respectively. These production parameters values signify a AQs enhancement of 129% comparing to control conditions (Table I). This maximum value of AQs content 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)*.
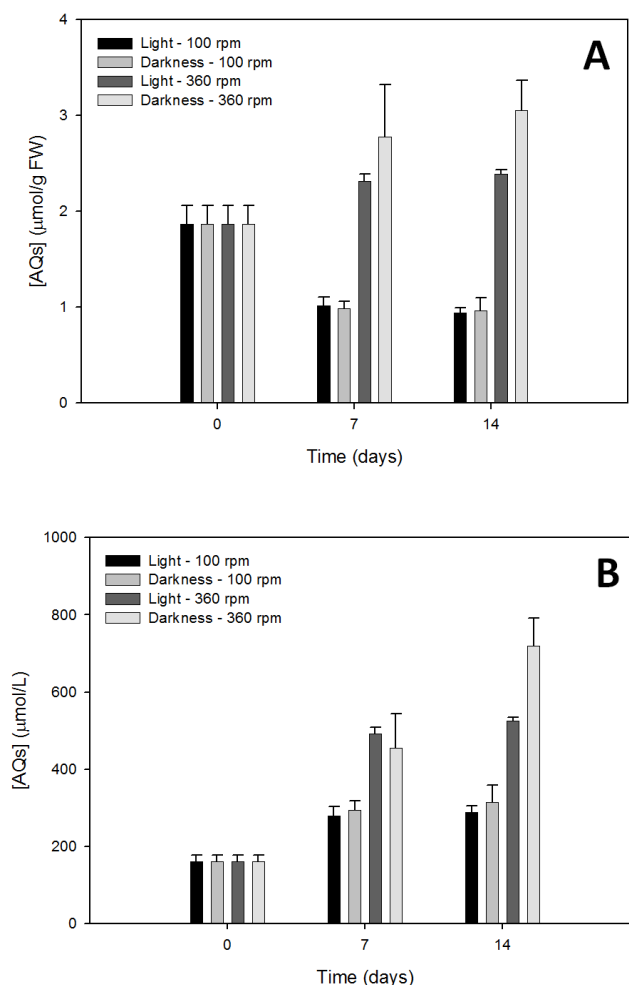

Figure 4. Time course of AQs content of *R. tinctorum* suspension cultures grown in baffled Erlenmeyer flasks agitated at 100 and 360 rpm, and in presence or absence of light. Specific production (μmol/g FW) (A) and volumetric production (μmol/L) (B). All values are the mean ± standard deviation (SD) of three independent experiments.

The AQs production obtained in unbaffled flasks (previously mentioned preliminary experiments) and baffled flasks under light/darkness conditions and in a bioreactor system *(17)* are shown in Figure 5. In this figure the AQs production is presented as the extent of improvement over that corresponding to the control, the flask shaked at 100 rpm. It should be mentioned that the results obtained with the baffled flasks at

100 rpm are very close to those reported previously for unbaffled flasks, which were the control in our previous publication *(17)*. 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 AQs produced in baffled flasks are 2.2 times higher, and almost reach the productivity of the bioreactor at 450 rpm. Unbaffled Erlenmeyer flasks at the same shaking frequency were not able to increase AQs content in the same extent as a stirred tank bioreactor at 450 rpm.
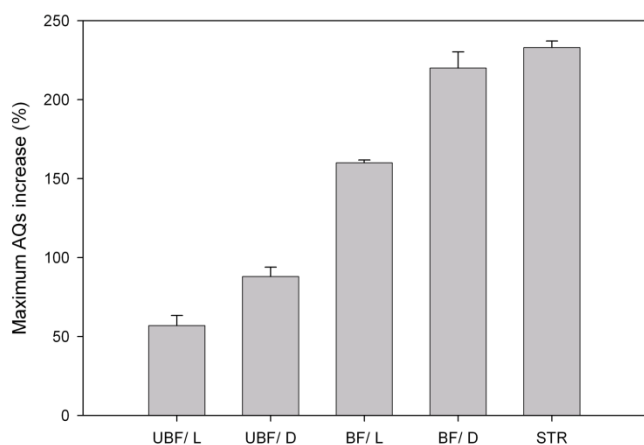

Figure 5. AQs increases obtained in *R. tinctorum* suspension cultures grown in unbaffled (UBF) and baffled Erlenmeyer flasks (BF) at 360 rpm under light (L) /darkness (D) conditions, and in a stirred tank bioreactor system (STR) operating at 450 rpm (Busto et al., 2008). The reference value is the AQs production in the control, flasks shaked 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.

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 *(53, 54)*. Nevertheless, most of reports point towards 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 *(55)*. 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 *(56)*. In our case, the presence of light did not affect AQs production in control conditions, but, a significant difference of approximately 40% was observed between stressed cells with or without light

irradiation at 14 days of culture. 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 could be associated with shear stress. The similar AQs production is a strong indication that baffled Erlenmeyer flasks agitated at 360 rpm in absence of light are a helpful 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. We could even consider that l in our flasks is approximately $l = 0.007$ mm, as indicated by the AQs production. This is not information that can be used directly by 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. Provided that in the future standard flasks with well defined geometry become widely available in all usual sizes, the work by Peter *et al. (40)* could extended and be used for the calculation of the conditions leading to hydrodynamic stress similar to that generated in a standard stirred bioreactor.

CONCLUSIONS

Suspension cultures of *R. tinctorum* were able to grow under shear stress generated using four-baffled shake flasks agitated at 360 rpm. The biomass concentration and AQs production obtained were similar to that 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 after 14 days of culture. Absence of light irradiation played an important role on AQs synthesis, and its effects could be associated with shear stress.

Four-baffled shake flasks agitated at 360 rpm were able to induce a similar intensity of shear stress as that reached using a stirred tank bioreactor operating at 450 rpm. The shear stress generated was able to elicit mechanically AQs synthesis in *R.*

tinctorum suspension cultures. The present results can not be quantitatively used by others because of the lack of repeatability in the geometry of hand-fabricated baffled flasks, but it can be concluded that this down-scaled and simple culture system is a suitable and valid alternative to study intracellular mechanisms 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.

ACKNOWLEDGEMENT

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