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Hydrodynamic stress and limonoid production in *Azadirachta indica* cell culture

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ABSTRACT

Azadirachta indica cell culture has been used for the production of antifeedant limonoids in shake flasks and stirred-tank bioreactors (STBs). However, the hydrodynamic environment may lead to low product yield, affecting growth and cell viability. The aim of this paper was to determine the effect of three different hydrodynamic conditions, corresponding to shake flasks (120 rpm) and STB (400 and 800 rpm) on *A. indica* cell culture. Reynolds stress, turbulent eddy length, cell growth and viability, limonoid production, reactive oxygen species (ROS), and guaiacol peroxidase (GPX) response were analyzed. The most suitable treatment for biomass generation corresponded to shake flask (maximum of 15.69 ± 0.47 g DW/L), whereas limonoid production was favored at 400 rpm (8.37 ± 3.81 mg/g DW). Reynolds stress reached 62.21 ± 8.65 Pa at 800 rpm, and turbulent eddies were similar to the aggregate size. This affected cell growth and viability, while ROS concentration increased significantly (156.1 ± 104.2 FU/min) and GPX activity remained low, 0.04 ± 0.00 μmol TG/(mg protein min), suggesting hydrodynamic stress conditions. As far as we know, this study is the first that relates hydrodynamic stress conditions to limonoid production, cell growth and viability, ROS, and GPX in an *A. indica* cell culture.

1. Introduction

The extensive use of chemical pesticides represents an environmental hazard, since it pollutes soils, water bodies, vegetation, and food [1] and has adverse neurological, respiratory, and hormonal effects on the human body [2]. As an ecologically friendly option, the formulation of biopesticides derived from the neem tree (*Azadirachta indica*) has been gaining interest [3]. The main secondary metabolites responsible for the pesticide or antifeedant effect in *A. indica* are limonoids, or tetranortriterpenoids [4], azadirachtin being the most active compound [5]. *A. indica* cell culture is seen as an interesting alternative for the production of these secondary metabolites. In particular, stirred-tank bioreactors (STBs) have been used for this purpose [6], although other reactor systems have been employed [7–9]. Additionally, shake flasks play an important role in the stages previous to the scale-up and as a method for inoculum preparation. However, the hydrodynamic environment resulting from the agitation speed and the bioreactor configuration affects the plant cell growth and the metabolite yield in STBs [10,11]. Therefore, it is important to establish the relationship between the operating conditions of the bioreactor and the culture response under hydrodynamic stress.

From an engineering point of view, the energy dissipation rate, the turbulent eddy length, and the Reynolds stress are parameters that are useful for analyzing the hydrodynamic stress under certain culture conditions [10,12]. For agitated systems, Kolmogorov's theory of isotropic turbulence has been used to identify the smallest turbulent eddy involved in the hydrodynamic stress, in terms of the dissipation energy rate and the viscosity of the culture [11,13]. Reynolds stress, on the other hand, accounts for turbulent changes of fluid momentum in an agitated vessel. It describes the interactions between particles (aggregates and cells) and randomly interacting turbulent eddies in the culture broth [12].

Quantification of cell damage due to hydrodynamic stress can be done in several ways: measurement of cell viability, release of extracellular compounds, increase of cell respiration rate, change

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Nomenclature

Re	Reynolds number
n	Agitation speed (s^{-1})
d	Diameter of the flask (m)
D	Impeller diameter (m)
T	Bioreactor vessel diameter (m)
l	Turbulent eddy length (m)
x	Impeller disk thickness (m)
ρ	Culture density (kg/m^3)
Ne'	Modified power number for shaking flasks
μ	Culture viscosity (Pa s)
ε	Maximum energy dissipation rate (W/kg)
ε_{avg}	Average energy dissipation rate (W/kg)
τ_R	Reynolds stress (Pa)
$\tilde{\upsilon}(d_C)$	Velocity between two points separated at a distance equal to the mean aggregate diameter (m/s)
d_C	Mean aggregate diameter (m)
V_L	Working volume (m^3)
P	Power input (W)
P_g	Gassed power input (W)
P_o	Power number

in cell wall structure and composition, changes in cell morphology and cell aggregate size, and aggregation pattern [14]. There are two possible outcomes due to hydrodynamic stress: a lethal effect, leading to cell apoptosis or necrosis, and sublethal effects, noticeable as positive or negative metabolic alterations [11]. At the cellular level, there exists a relationship between hydrodynamic and oxidative stress responses, where reactive oxygen species (ROS) and antioxidative mechanisms (enzymatic and non-enzymatic) are involved [15]. ROS are small molecules, mainly oxygen ions or free radicals, which unavoidably result from aerobic plant metabolism, and they are generated from both organic and inorganic sources. ROS serve as signal molecules for triggering protective responses, but they may lead to oxidative damage. Among the principal ROS active in signaling in plants are superoxide ion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), singlet oxygen ($^1O_2$), alcoxy radicals (RO), and peroxy radicals (ROO) [16]. The principal non-enzymatic actors in plants are ascorbic acid, glutathione, proline, α–tocopherol, carotenoids, and flavonoids. On the other hand, the antioxidative enzymatic mechanisms in plants mainly consist of superoxide dismutase (SOD), peroxidases such as ascorbate and guaiacol peroxidases, catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [15]. Guaiacol peroxidase (GPX) is a hemo-protein that catalyzes the hydrogen peroxide reduction using aromatic compounds as electron donors. It participates in the lignin synthesis pathway, which is also a stress indicator [17], and takes part in auxin metabolism and cell growth and development [18].

Some authors have studied the effect of hydrodynamic stress on plant cell culture and its relationship with secondary metabolite production in STBs [19–21]. However, reports on antioxidative mechanisms and ROS generation in plant cell culture under hydrodynamic stress in bioreactors are scarce. In particular, there are no reports of the effect of hydrodynamic stress or dissipation energy on *A. indica* cell culture. Consequently, the aim of this investigation was to determine the *A. indica* cell culture response to hydrodynamic stress under different agitation speeds, and therefore Reynolds stress levels in shake flasks and STBs. These responses were evaluated in terms of cell growth and viability, limonoids, ROS synthesis, and GPX enzymatic activity. This study will help to broaden the understanding of the relationship between mechanical stimulation and secondary metabolite production. Additionally, hydrodynamic stress could be used for increasing limonoid yield or as a scale-up strategy for *A. indica* cell culture.

2. Materials and methods

2.1. Plant material

An *Azadirachta indica* cell culture was established using calli obtained from seed kernels. The seeds were collected in the Cotové agricultural center of Universidad Nacional de Colombia (Santa Fé de Antioquia, Colombia, geographical coordinates: 6°33′23″N, 75°49′39″W).

2.2. Cell suspension establishment, cell growth and viability

Calli were cultured using an MS medium with 30 g/l of sucrose, 1 mg/L of benzil amino purine (BAP), 2 mg/L of indolebutyric acid (IBA), and 2 g/L of Phytagel at pH 5.8, 25 °C in total darkness. Friable calli were used to obtain suspensions, which were grown using 500 ml shake flasks, 100 ml of MS liquid medium, and an orbital shaker at 120 rpm with a cotton plug. Operative conditions were the same as the calli culture. The subculture to fresh medium was carried out every 10 days. Cell viability was estimated using Evans blue dye. Cell count was done using a Leica CME optical microscope, and cell growth was determined via the dry cell weight (DW) [22]. Homogeneous samples of 10 ml were filtrated in a vacuum, dried for 24 h at 60 °C, and then weighed. Samples were harvested for analysis every two days and stored in liquid nitrogen.

2.3. Hydrodynamic stress assays

A. indica cell culture was carried out using a shake flask and a stirred-tank bioreactor (STB). For the shake flask, the same conditions as previously mentioned were used. In the stirred-tank bioreactor, the culture was carried out using a 3 l bioreactor (Applikon, eZ Control) with 2 l of working volume, a six pitched blade impeller with upward flux, 3 baffles, and a porous stainless steel diffuser. Dissolved oxygen (DO) was maintained at 30% during the whole fermentation, and the pH was maintained at 5.8, using NaOH 0.3 M. Two agitation speeds were evaluated, 400 and 800 rpm 50 ml samples were taken for analysis. ROS and GPX were evaluated every two hours for eight hours on the first day of cultivation, and then every two days. Cell growth, cell viability, and limonoids were analyzed every two days. The inoculum had a volume of 300 ml of sedimented cells. Fig. 1 depicts the geometrical configurations of the bioreactor and the impeller used in the present study.

2.4. Turbulent eddy length, cell aggregate, and cell size estimation in shake flask

In order to evaluate the turbulent eddy length, it is necessary to analyze the energy dissipation rate in the flasks under different operating conditions. The estimation of the energy dissipation rate in the shake flasks was made using empirical correlations [23–25]. First, the laminar flow regime in the shake flasks was verified using the Reynolds number (Re) (Eq. (1)).

$$Re = \frac{\left(\rho n d^2\right)}{\mu} \tag{1}$$

The power input (P) was calculated from Eq. (2). Then Eq. (3) was used to calculate the maximum energy dissipation rate (ε), as