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Rheology and mixing analysis of plant cell cultures (*Azadirachta indica*, *Borojoa patinoi* and *Thevetia peruviana*) in shake flasks

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ABSTRACT

Plant cell culture is a useful technology for the production of secondary metabolites with commercial and pharmaceutical value. The rheology, morphology or aggregation of cell suspension have different applications in flow systems, bioreactor design and unit operations. This suggests solutions to mixing, mass transfer, hydrodynamic stress and cell growth problems. In this study, some morphological aspects associated with elliptical form factor and the tendency to form cell aggregates of *Azadirachta indica*, *Borojoa patinoi* and *Thevetia peruviana* cell cultures were evaluated in shake flasks. The rheological behavior through rheograms for cell concentrations of 0, 4, 8 and 12 g DW l^{-1} was also evaluated. From this data, properties and parameters like apparent viscosity, Reynolds number, phase state and volumetric power were calculated for shake flasks of 250 and 500 ml. The results showed a dilatant behavior of the culture medium and pseudo-plastic behavior of cell cultures. This last behavior increases with cell concentration and size of the cell aggregates. Rheograms for all cell concentrations of *A. indica*, were significantly different from the other two species. Culture conditions like shaker orbit diameters and flask volumes were recommended in order to favor an adequate cell growth and mass transfer in plant cell cultures.

1. Introduction

Plant cell culture has emerged as an alternative for the production of secondary metabolites and therapeutic proteins with a commercial interest. It is an important alternative production technology for plants which are difficult to cultivate in farms and have long periods of cultivation or low yields [1]. This technique allows the control of the culture conditions and makes it possible to evaluate different variables (temperature, oxygen, CO_2, concentrations of substrates, etc.) in order to obtain higher yields of a specific product. Its potential application has been focused on obtaining biomass, secondary metabolites such as taxol, shikonin, berberine and ginsenosides and therapeutic proteins [2]. These cultures are important in the pharmaceutical industry and they have served as a base for studying the behavior of plant suspensions in industrial processes. The simplicity of the culture facilitates medium evaluation and strain selection, which need many replicate experiments [3]. Some reports of mixing and transport phenomena like power consumption, flow regimes and energy dissipation have been observed for microorganism cultures in shake flasks [3,4] and few investigations analyze engineering aspects of the plant cell culture in shake flasks [5], despite being critical for the scale up of these bioprocesses [6]. Rheological behavior of the broth is related to the bioreactor operation conditions which should ensure an adequate oxygen transfer, mixing and delivered power intensity to the culture medium. This characterization in shake flasks is useful for subsequent scale up to bigger bioreactors, e.g. stirred tank bioreactors. The transfer of momentum, mass and energy are affected among other factors, by the culture viscosity. The previously mentioned factors depend on the characteristics of the culture, such as cell concentration and morphology [7].

Plant cell cultures are usually grown between 90 and 120 rpm, using different shaker orbit diameters and flask volumes, but without a technical criterion to set specific conditions. Aggregation is common in plant cell suspensions due to failures in cell separation during cell division, the secretion of extracellular polysaccharides in the final stages of batch cultures, the age of culture and maintenance conditions [8]. Cell morphology varies from round to elongated shapes and has been studied using the elliptical shape

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Nomenclature

τ	Shear stress (Pa)
γ	Shear rate (s^{-1})
K	Consistency index (Pa s^n)
n	Flow behaviour index (dimensionless)
τ_o	Yield stress of the fluid (Pa s)
μ_c	Plastic viscosity (Pa s)
Vi	Linear velocity on the wall of the flask (m s^{-1})
n_s	Shaking frequency in the agitator (s^{-1})
d_i	Diameter of the flask (cm)
$V_{spindle}$	Spindle speed (m s^{-1})
$n_{spindle}$	Frequency (s^{-1})
$d_{spindle}$	Diameter of the inner cylinder in rheometer spindle (mm)
Re	Reynolds number
ρ_c	Continuous phase density (kg m^{-3})
Ph	Phase number
d_o	Shaker orbit diameters (m)
Re_f	Reynolds number flask
η	Fluid viscosity (Pa s)
V_L	Effective volume (m^3)
PV^{-1}	Power consumption (W m^{-3})
Ne'	Modified power number

factor (ESF). This factor represents the ratio of the width and length of the cells [9]. In industrial processes, an essential step in the biotechnological production of a compound is the scale up of the culture from flask to bioreactor [10]. However, many cases of scale up are accompanied by a reduction in production. The maximum concentration of product obtainable in the biochemical process is influenced by the degree of mixing and hydrodynamic conditions of the bioreactors [2].

If the applied stress and the strain rate are related by a complex function, the fluid is non-Newtonian and an apparent viscosity is defined [11]. This is not constant but adequately describes the properties of the fluid. If the apparent viscosity decreases with an increase on the stress, the fluid is pseudo-plastic and in the opposite case, the fluid is dilatant. These behaviors can be integrated into the power law model. Fluids are described as Bingham or Casson when it is required to overcome an initial stress (yield stress) before starting the movement [12]. Some rheological studies suggest that the majority of plant cell cultures exhibit non-Newtonian behavior with shear thinning characteristics (pseudo-plasticity). This is a consequence of the biomass concentration, cell aggregation and cell morphology [13]. On the other hand, the rheology of the culture medium free of cells is influenced by some extracellular components such as polysaccharides and proteins, and the conditions used during the culture [7]. The knowledge of plant cell culture rheology helps to create solutions to several problems: mixing, mass transfer, expansion stress, cell growth and production of metabolites. All of them interact with each other in a bioreactor [8]. Finally, this knowledge allows one to set up the different factors of cell cultures like design and operational features of bioreactors, pipe dimensions, aeration and pumping system, type of stirrer, etc. These factors greatly influence the cell growth and formation of certain products of interest [10].

Some characteristic factors for mixing and mass transfer in a bioreactor are Reynolds number, oxygen transfer rate, power consumption, dissipation energy and, specifically in shake flask, the phase state. Depending on the magnitude of the Reynolds number in stirred tank and in unbaffled shaking flasks, the flow is classified as turbulent above 50 000 and 60 000 or laminar below 100 and 5000 Re, respectively, and there is a transitional region between the corresponding two values [3,14,15]. Buchs proposed a model to describe the relation between the movement of the liquid and the orbital shaker table. This phenomenon is analyzed with the dimensionless phase number (Ph) [16].

The model establishes that if the liquid within the flask circulates "in-phase" with the shaking table then the Ph value is greater than 1.26. In contrast, if the liquid remains at the base of the flask and it shows only little relative movement, Ph < 1.26. This last operating condition is termed as "out-of-phase". Thus, if the amount of liquid increases and the viscosity is the main influencing factor, the liquid does not follow the movement of the shaking table and the specific power consumption is reduced [3,17]. This "out of phase" unfavorable hydrodynamics condition usually occurs in small stirring diameters, low volumes of medium culture and high viscosities. This phase also leads to a significant reduction in the mass transfer area in the cell cultures, avoiding good cell viability, causing morphological changes and metabolic alterations and affecting the product. The power consumed in the system is presented in terms of volume (PV^{-1}), relating the dissipated energy and the density of the medium. According to Buchs, in shake flasks the power consumed is related to the power number (Ne), which is a function of Reynolds number. In a two-phase liquid system, the interfacial area available for mass transfer depends on the maximum rate of energy dissipation or energy consumption [6], which is related to the volumetric power. Furthermore, it has been shown that PV^{-1} is one of the best criterions of scale up to maintain constant interfacial area in a process that employs geometrically similar stirred tanks. PV^{-1} is an important factor of study, even though shake flasks generate lower hydrodynamic stress compared to stirred tanks [18].

There are no known reports in which mixing in plant cell cultures is analyzed at shake flask level. This study aims to determine the rheological behavior of some plant cell cultures of interest for the production of insecticides and medicinal compounds (*Azadirachta indica*, *Thevetia peruviana* and *Borojoa patinoi*) in shake flasks. In addition, the relationship between the rheology of with the mixing the cell suspension is studied, which could be determinant for the cell survival. For this, flowcharts were constructed at different plant cell culture concentrations and the power law was determined. The data obtained were related with the morphology of the cultures using the shape factor and the size of cell aggregates. Finally, based on engineering concepts such as apparent viscosity, Reynolds number, in-phase state and the volumetric power previously described for microorganism cultures, some adequate conditions were proposed for a suitable mass transfer and survival of the plant cell cultures. The methodology presented in this work could be extrapolated to other plant systems of interest at different laboratories or industries.

2. Methodology

2.1. Culture and maintenance of plant cell suspension

Cell suspension of *Azadirachta indica* (neem), *Borojoa patinoi* (borojó) and *Thevetia peruviana* (yellow oleander) were established from cultured calli at Laboratorio de Biotecnología Vegetal of the Universidad Nacional de Colombia, Medellín campus. The suspensions of *B. patinoi* and *A. indica* were grown on Murashige and Skoog medium. *T. peruviana* grew in Shenk and Hildebrandt medium. Growth regulators and vitamins were added. 250 ml shake flasks with 20% working volume were used and carried on Centricol shakers (KS501) at 120 rpm for periods of 15 days in darkness before the subculture. *A. indica* cells were screened monthly due to the forma-