OXYGEN LIMITATIONS TO GROW *Azadirachta indica* CELL CULTURE IN SHAKE FLASKS

LIMITACIONES POR OXÍGENO PARA CRECER UN CULTIVO DE CÉLULAS DE *Azadirachta indica* EN MATRACES

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Abstract

It was evaluated the growth of *Azadirachta indica* cell suspension in different conditions of oxygen delivery in Erlenmeyer shake flask. Oxygen transfer rate (OTR, kg O_2 m^{-3} day^{-1}) for the closures utilized were: silicone foam (1.04), cotton (0.58), and aluminum foil (0.07). *A. indica* cells growing during 6 weeks of subculture showed that lower OTR reduced cell viability, the pH of broth medium, and *Azadirachtins* production. While, higher OTR induced the formation of aggregates. Using a stirred tank bioreactor, it was determined that *A. indica* cells had an oxygen consumption of 0.100 kg O_2 kg DW^{-1} day^{-1}, a higher value than other plant cell cultures. These results show that OTR generated in Erlenmeyer shake flasks is lower to oxygen uptake rate of *A. indica* cells and it is a limiting factor to grow this plant.

Keywords: *Azadirachta indica* cell culture, shake flask, oxygen transfer rate, oxygen transfer resistance, closures.

Resumen

Se evaluó el crecimiento de suspensiones celulares de *Azadirachta indica* en diferentes condiciones de suministro de oxígeno en matraces. Las velocidades de transferencia de oxígeno (OTR, kg O_2 m^{-3} $día^{-1}$) obtenidas con los tapones utilizados para tapar matraces Erlenmeyer fueron: espuma de silicona (1.04), algodón (0.58) y papel aluminio (0.07). Las células de *A. indica* crecidas en las condiciones anteriores durante 6 semanas de subcultivo, mostraron que la OTR menor reduce la viabilidad celular, el pH del medio y la producción de azadiractinas. Por su lado, las OTR mayores favorecen la formación de agregados celulares. En un biorreactor de tanque agitado, se determinó que las células de *A. indica* tienen un consumo de oxígeno de 0.100 kg O_2 kg CS^{-1} $día^{-1}$, valor superior al que tienen otros cultivos de células vegetales. Estos resultados muestran que la OTR generada en matraces Erlenmeyer es menor a la demanda de oxígeno de las células de *A. indica* y es un factor que limita su crecimiento.

Palabras clave: cultivo de células de *Azadirachta indica*, matraz agitado, velocidad de transferencia de oxígeno, resistencia a la transferencia de oxígeno, tapones.

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1 Introduction

Microorganisms and plant cells are cultured in shake flasks with several biotechnological purposes such as culture medium optimization, elicitation or simply for the development of inocula for larger bioreactors. These aerobic systems demand oxygen to grow. However, low biomass yields and variation in metabolite production may occur as a result of an insufficient oxygen supply (Mantzouridou *et al*., 2005; Suresh *et al*., 2009). Several reports about oxygen transfer rate (OTR) and oxygen limitation in bacteria, yeast and fungus cultures have been published. These reports indicate that, in particular, variables such as the size and flask volume, agitation speed, and porous closures, affect OTR values (Veglio *et al*., 1998; Büchs, 2001; Nikakhtari and Hill, 2006; Suresh *et al*., 2009). Particularly, OTR determinations for plant cell culture in shake flask and the effects over cell growth and metabolite production have received little attention (Kieran *et al*., 1997).

Previous studies of Lee and Shuler (1991), reported that the type of closure affects plant cell metabolism and the headspace gas composition. *Catharanthus roseus* cells cultured in shake flasks plugged with Parafilm (American National Can, Greenwhich, Ct, USA) covered foam caps and Reynolds aluminum foil (Reynolds Metals Company, Richmond, Va, USA) accumulated ethylene and CO_2, whereas oxygen limitations were detected. In this case, ajmalicine production was favored only by the use of aluminum foil. Despite that, there is no criterion for choosing the type of closure. Information about a possible effect of aluminum foil or other type of closure upon oxygen transfer limitations for other plant cell cultures is not available. Additionally, the relationship between OTR and oxygen uptake rate (OUR) of plant cells is not well known. In consequence, it is necessary continue studies that consider the effect of OTR in the establishment of cell cultures, their effects on growth and over the production of secondary metabolites.

It is reported that plant cell cultures such as *Beta vulgaris* (Rodríguez-Monroy and Galindo, 1999) and *Solanum chrysotrichum* (Rodríguez-Monroy *et al*., 2004) can be cultivated for a long time in shake flasks with aluminum foil cap. In contrast, an *Azadirachta indica* cell line incubated in shake flasks with aluminum foil cap, showed limitations to grow during its culture, and it was necessary periodically begin new suspension cultures from callus. This specie had interest because cell cultures producers of insecticide compounds classified as limonoids and *Azadirachtin*s (Orozco-Sánchez and Rodríguez Monroy, 2007). In order to determine if the problems to grow *A. indica* in shake flask with aluminum foil cap was due to oxygen limitation, several OTR conditions were evaluated. These conditions resulted by using different types of closures (aluminum foil, cotton and silicone foam). This study reports the behavior of *A. indica* culture (growth index, pH medium, cell viability, aggregate morphology and secondary metabolites) during serial cultivation in shake flasks under different OTR conditions (0.07, 0.58 and 1.07 kg O_2 m^{-3} day^{-1}).

2 Materials and methods

2.1 *Azadirachta indica* culture

Cell suspension cultures of *A. indica* were obtained according to the methodology reported by Capataz-Tafur *et al*. (2007) using seeds for callus formation. Cells were grown in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with sucrose (30 g/L), indole butyric acid (4 mg L^{-1}) and benzylaminopurine (1 mg L^{-1}). The medium pH was adjusted to 5.7 prior to sterilization. Cultures were grown in Pyrex 500 mL Erlenmeyer flasks (ref. 4980-500; Corning, New York, USA) with 100 mL of culture medium and subcultured every 7 days. Shake flasks were incubated in darkness, in an orbital shaker at 120 rpm with orbital diameter of 24 mm (model 6040, Vichi, México) at 25 °C .

B. vulgaris was maintained according to the methodology reported previously by Rodríguez-Monroy and Galindo (1999).

2.2 *Oxygen transfer in shake flasks*

In order to estimate OTR and the relative relationship between the resistances to oxygen transfer through the plug and the gas-liquid phase, we used the following analysis:

Oxygen diffuses across the closure flask and from the gas phase to the liquid medium (Fig. 1). The oxygen transfer rate through the closure, $\Phi_{closure}$ (kg O_2 h^{-1}), can be expressed by the following equation (Boon and Heijnen, 1998):

$$\Phi_{closure} = k_1 \left(po_{2,air} - po_{2,in} \right) \qquad (1)$$

where $pO_{2,air}$ (kg m^{-1} h^{-2}) is the partial pressure of oxygen of the air, $pO_{2,in}$ (kg m^{-1} h^{-2}) is the

partial pressure of oxygen of the gaseous phase in the headspace of the flask and k_1 is the oxygen transfer coefficient through the closure (m h). The oxygen transfer rate in the gas-liquid interface, $OTR_{gas-liq}$, is given by:

$$OTR_{gas-liq} = k_L a \left(Co_{2,in} - Co_2 \right) \qquad (2)$$

$Co_{2,in}$ (kg m^{-3}) is the oxygen concentration of the liquid phase at equilibrium with the oxygen in the headspace of the flask at the temperature system ($po_{2,in}$), Co_2 is the oxygen concentration of the liquid phase (kg m^{-3}) and $k_L a$ is volumetric oxygen transfer coefficient in the gas-liquid interface (h^{-1}). $po_{2,in}$ and $Co_{2,in}$ are related according to Henry's Law (Doran, 1995) as:

$$po_{2,in} = H \times Co_{2,in} \qquad (3)$$

where H is Henry's constant (3.18×10^{13} m^3 h^{-2}). Additionally, $pO_{2,air}$ can be expressed by:

$$po_{2,air} = H \times Co_{2,air}^* \qquad (4)$$

where $Co_{2,air}^*$ is the oxygen concentration of the liquid phase at equilibrium with air at pressure and the temperature of the system, 6.829×10^{-3} kg O$_2$ m^{-3} (Doran, 1995; Büchs, 2001). If the same quantity of oxygen enters to gas phase inside the shake flask equals to oxygen enters to liquid phase, it means, the "buffer capacity" of the gas amount in the head space of the flask is neglected:

$$\frac{\Phi_{plug}}{V_L} = OTR_{gas-liq} \qquad (5)$$

where V_L is the liquid volume inside the flask (m^3). The before condition has been called "steady state" for the headspace in the flask (Boon and Heijnen, 1998; Veglio *et al.*, 1998; Amoabediny and Büchs, 2007). Thus, using the previous equations, the following equation is obtained:

$$OTR = \frac{k_1 \times k_L a \times H}{k_1 \times H + V_L k_L a} \left(Co_{2,air}^* - Co_2 \right) \qquad (6)$$

Defining K_o as follows

$$K_o = \frac{k_1 \times k_L a \times H}{k_1 \times H + V_L k_L a} \qquad (7)$$

The following equation describes the OTR of a shake flask plugged:

$$OTR = K_o \left(Co_{2,air}^* - Co_2 \right) \qquad (8)$$

where K_o is the overall oxygen transfer coefficient (h^{-1}). Similar criteria were previously reported by other authors, to estimate OTR in shake flasks (Boon and Heijnen, 1998; Veglio *et al.*, 1998; Gupta and Rao, 2003). The maximum OTR (OTR_{max}) is given by equation (8) when CO_2 is equal to zero. Therefore, Ko can be evaluated using the "dynamic gassing out" method and measuring CO_2 in the liquid medium (Fig. 1) against time, according to equation (9) (Doran, 1995):

$$\ln \left(\frac{Co_{2,ee} - Co_{2,i}}{Co_{2,ee} - Co_2} \right) = K_o(t - t_i) \qquad (9)$$

where $Co_{2,ee}$ is the steady-state oxygen concentration in the liquid medium, $Co_{2,i}$ is the oxygen concentration at the initial time (t_i) and Co_2 is the oxygen concentration at time (t). Shake flasks of 500 mL with 100 mL of culture medium were used for such measurements. MS basal medium supplemented with 30 g sucrose L^{-1} was used. Dissolved oxygen (DO) was determined using a polarographic electrode (Applisens dissolved oxygen sensor Z010032520, Applikon, Schiedam, Netherlands) connected to a recorder device (biocontroller ADI 1030, Applikon, Schiedam, Netherlands). K_o and OTR_{max} were determined using the closures previously mentioned: aluminum foil, cotton and silicone foam. Value of $k_L a$ was also determined without the closure under the agitation conditions before described.

The overall resistance to oxygen transfer, R_t (h), is the inverse of K_o and is equal to the resistance to oxygen transfer through the closure (R_1), plus the resistance through the gas liquid interface (R_2). They can be expressed using the following equations:

$$Rt = \frac{1}{K_o} = \frac{V_L k_L a + k_1 H}{k_L a \times k_1 H} = R_1 + R_2 \qquad (10)$$
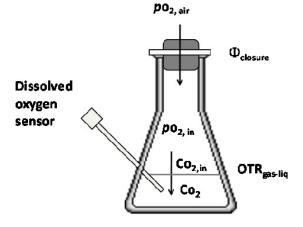

Fig. 1. Oxygen transference diagram in shake flask.

$$R_1 = \frac{V_L}{k_1 \times H} \qquad (11)$$

$$R_2 = \frac{1}{k_L a} \qquad (12)$$

2.3 Effect of closure type on the growth and Azadirachtin production of A. indica cell culture

A. indica cell culture was grown in 125 mL shake flasks and three different closures: Reynolds aluminum foil (Costco Wholesale Corporation, Issaquah, WA, USA), cotton plugs made using 6.50 ± 0.03 g, and silicone foam (ref. C1046; Sigma, St. Louis, MO, USA). Previous experiments showed that that the cells conserved in shake flasks with cotton plugs and subcultured every 7 days, can conserve the growth and viability. So, for the three different closures, the measurements of cell growth, cell viability, pH medium, and *Azadirachtin* content were made every subculture of 7 days, during a 6-week period using three samples.

Biomass was determined by filtration of 10-mL samples through filter paper (grade 1; Whatman, Maidstone, UK). Cells were dried to constant weight at 70 °C, 1 day. Growth index (GI) was calculated with equation (15):

$$GI = \frac{X_t - X_0}{X_0} \qquad (13)$$

where X_t is biomass in t time (dry weight, DW, or kg DW m^{-3}) and X_0 is the initial biomass.

Cell viability was determined considering the membrane integrity by Evan's blue dye exclusion test (0.25% w/v) (Withers, 1985). Additionally, cell cultures was observed under a stereoscopic zoom microscope (SMZ1500; Nikon, New York, USA).

Azadirachtin evaluation was realized after of six subcultures. Procedure of extraction was done according to previously reported methodologies (Schaaf *et al.*, 2000; Balaji *et al.*, 2003; Capataz-Tafur *et al.*, 2007) and the content in biomass and cell-free medium was determined. Biomass was separated by filtration through a filter paper (Whatman 1) and lyophilized. The lyophilized powder (0.5 g), was extracted with methanol (3 × 10 mL). Extracts were concentrated under low pressure distillation at 45 °C. The extract was fractionated by using 1.0 mL of sodium chloride (0.5% w/v), water (10 mL) and dichloromethane (3 × 10 mL). The organic phase was evaporated to dryness, and the dry powder dissolved in 2 mL of methanol for HPLC analysis.

A similar procedure was carried out for cell-free medium. *Azadirachtin*s were determined by HPLC analysis, using a 2695 Waters Separation Module (Milford, MA, USA), 2996 Waters Photodiode Array Detector and Empower Pro Software. Separation was performed with a LiChroCART 125-4 LiChrospher 100 RP-18 column (125 mm × 4.6 mm, pore diameter 5 μm) (Merck, Whitehouse Station, NJ, USA). Mobile phase flow was 1.0 mL min^{-1} and a gradient was used beginning with 35:65 v/v acetonitrile/water (ACN/H$_2$O), increased to 45:55 ACN/H$_2$O (10 min), 70:30 ACN/H$_2$O (11 min) and returned to 35:65 ACN/H$_2$O (14 min). Fifty-microliter samples were injected to the HPLC and UV signal was recorded during a 15-min period (λ = 213 nm). *Azadirachtin* (A7430; Sigma) was used as standard.

2.4 OUR measurement of different plant cell cultures

In order to determine the oxygen demand by each plant cell species, a 7-L stirred tank bioreactor (Applikon) operated with a 45° inclined six-blade impeller, Di/Dt = 0.4 (400 rpm) and 0.08 vvm of aeration was used. Oxygen was measured using a polarographic electrode (Applisens dissolved oxygen sensor Z010032520, Applikon, Schiedam, Netherlands). Cell suspension cultures produced in shake flasks (500 mL) were used to inoculate the bioreactor with a working volume of 4 L, obtaining a suspension with 7 g DW/L. OUR was determined by the dynamic method (Doran, 1995). Determination of oxygen consumption was done during the first hour after cells were inoculated. Cultures were subjected to conditions without oxygen limitation (DO > 30%). OUR (kg O$_2$ m^{-3} day^{-1}) was calculated considering DO variation with time, dCo$_2$/dt (without aeration) and the biomass concentration present in the bioreactor, according to the following equation:

$$\frac{dCo_2}{dt} = OUR = QO_2 \times X \qquad (14)$$

where QO$_2$ represents the specific oxygen consumption (kg O$_2$ kg DW^{-1} day^{-1}) and X is the biomass concentration (kg DW m^{-3}). DW corresponds to dry weight of cells. Using a graph of CO$_2$ vs. time, OUR (QO$_2$ × X) is obtained calculating the slope of the strength line (Doran, 1995).

2.5 Maximum QO₂ achievable in shake flask

The maximum oxygen consumption that *A. indica* cells can have in shake flasks with a particular OTR_{max} was calculated using the equation:

$$QO_2, max = OTR_{max}/X \qquad (15)$$

The maximum QO₂ (achievable by oxygen transfer) vs. biomass was plotted for the three cases of shake flasks closers (plugged with silicone foam, cotton and aluminum foil). These values were compared with the QO₂ measured in the bioreactor (without oxygen limitation).

2.6 Statistical analysis

The experiments were carried out in triplicate and each sample was analyzed twice. Mean values ± standard error are reported. Statistically significant differences between groups were evaluated with one-way analysis of variance and Fisher's least significant difference (LSD) procedure at the 95 % confidence level using Statgraphics for Windows 4.1.

3 Results and discussion

3.1 Oxygen transfer in shake flasks

OTR_{max} obtained with shake flasks covered with cotton and silicone foam was higher than that obtained with aluminum foil, 8 and 15 times, respectively (Table 1). So, resistance to oxygen transfer using silicone foam was lower than using aluminum foil and cotton plug (Fig. 2). The resistance of the closure represents 95.4 %, 60.7 % and 31.2 % of the total resistance with aluminum foil, cotton and silicone foam, respectively. In particular, with aluminum and cotton closures the main resistance to oxygen transfer is in the closure and not in the liquid gas interface. In any case, resistance in the closure cannot be neglected. The mass transfer occurring in shake flask, neglecting the closure resistance has been modeled (Maier *et al*., 2004); however, the data obtained in the present research indicate that this consideration may lead to significant errors in the calculations of oxygen transfer.

In this research, the dynamic gassing out method for estimate the oxygen transfer rate was used. There are other methods to determine the oxygen transfer in shake flasks which use non-invasive or invasive
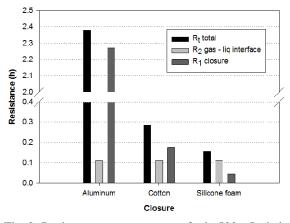

Fig. 2. Resistances to oxygen transfer in 500 mL shake flask. Total resistance (R_t), resistance in the closure (R_1) and resistance in the gas liquid interface (R_2).

Table 1. Overall oxygen transfer coefficient (Ko) and OTR_{max} in 500-mL shake flask using different closures.

Closure	Ko (h^{-1})	OTR_{max} (kg O_2 m^{-3} day^{-1})
Aluminum	0.42 ± 0.03^a	0.07 ± 0.00^a
Cotton	3.51 ± 0.0^b	0.58 ± 0.01^b
Silicon foam	6.37 ± 0.1^c	1.04 ± 0.03^c

Mean values followed by different letters in the same column are different according to Fishers LSD ($p \leq 0.05$).

electrodes, approximation of steady state or non-steady state in the oxygen balance for the headspace in the flask, sulfite oxidation method, on-line monitoring system with oxygen sensor, etc. (Boon and Heijnen, 1998; Veglio *et al*., 1998; Anderlei *et al*., 2004; Anderlei *et al*., 2007; Amoabediny and Büchs, 2007; Deshpande and Heinzle, 2009). The method used to determine oxygen transfer rate can affect strongly the obtained value and each method has its advantages and disadvantages. So, the use of invasive electrodes can introduce a baffle effect but measure directly into de medium the oxygen concentration. Hansen *et al*. (2011) found a reduction of 13.7 % in OTR in a 250 mL shake flask (150 rpm, cotton as closure, 40 mL of sulphite sodium solution) using a non-invasive electrode. Amoabediny and Büchs (2007) found between 1.1 and 25 % longer OTR considering unsteady-state conditions in shake flasks with sulphite sodium solution as oxidation system. Although the results of these researchers contribute to improve the correct OTR determination in shake flasks, they used the sulphite oxidation as system, which is complex

and strongly dependent of sulphite sodium and cobalt (used as catalyst) concentrations (Puskeiler *et al.*, 2005; Poughon *et al.*, 2003). Maier *et al.* (2004) found that OTR of a *Pichia pastoris* culture was 2.8 times OTR$_{max}$ of the sulphite sodium system in shake flasks. Taking into account that the biomass presence can increase or decrease the oxygen transfer coefficient and this variation depend of cell concentration and extracellular compounds production (Garcia-Ochoa and Gomez, 2009) we decided to do the measurements in this study with a culture medium free of cells. Considering besides the main purpose of estimate the oxygen transfer coefficients and the relationships between the different resistances to oxygen transfer, we do not numerically compare our results with other researchers.

3.2 Effect of the type of closure on A. indica cell culture performance

A. indica cell culture has positive growth index (GI) after of six subcultures using cotton and silicone foam, with pH variations between 4.9 and 5.3, and cell viability higher than 50% (Fig. 3). However, using aluminum foil the GI was zero after of the subculture 4 and was negative at subculture 6. In this case, the pH of the medium decreased from 5.1 to 4.0, and cell viability fall until 16 %. Cell suspension cultures with pH < 4.0 and cell viability lower than 25 % did not show cell growth further. Additionally, it was observed differences in aggregate after of 6 subcultures (Fig. 4). Cells cultured in flasks covered with aluminum foil produce aggregates with a diameter lower than 1 mm, whereas aggregates growing in flasks covered with cotton had a diameter between 3 and 5 mm, and aggregates obtained with silicone foam cap were the largest (reaching 10 mm).

On the other hand, the content of *Azadirachtin*s evaluated after 6 subcultures is shown in Fig. 5. The lowest production of *Azadirachtins* was observed using aluminum foil (0.52 ± 0.19 mg L $^{-1}$) and the highest content was observed with silicone foam closure (1.51 ± 0.38 mg L $^{-1}$). The result was similar considering the *Azadirachtins* production by gram of biomass (0.06, 0.07 and 0.12 mg/g using aluminum, cotton plug and silicone foam closure, respectively). The level of *Azadirachtins* produced in the cultures *in vitro* is lower than that reported by the fruits of *A. indica* 5.13 mg g^{-1} DW (Prakash *et al.*, 2005).

A. indica culture developed in Erlenmeyer flask covered with aluminum foil had the lower OTR value and cell culture present a reduction of their pH (Fig.

3). This phenomena may be a consequence of lactic acid accumulation produced by the cells as a result of hypoxic or anoxic conditions by the low oxygen availability (Geigenberger, 2003; Tadege *et al.*, 1999). It is possible that *A. indica* cells under anoxia cannot withstand this pH reduction in the long term, and they lose viability and die. Then again, cells with low oxygen availability reduce their aggregates size. This result suggests that oxygen limitation stress produce a reduction of the aggregate size and could be similar to that observed in plant tissues (Potters *et al.*, 2007). It was reported that plants exposed to abiotic stresses such as flooding, dryness and UV radiation stress induced variations in the cells (size and forms) in plant tissues.
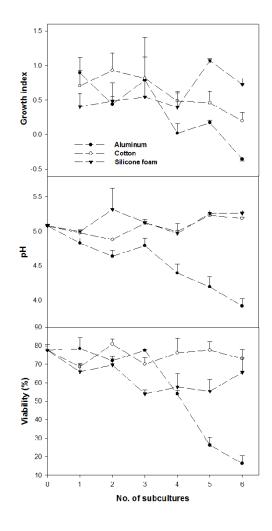

Fig. 3. Growth, pH and cell viability evaluated during continuous subcultures of *A. indica* cells in shake flask using different types of closures.
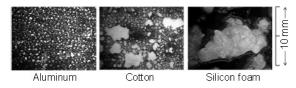
Fig. 4. *A. indica* aggregates size obtained in shake flask using different types of closures after 6 subcultures.
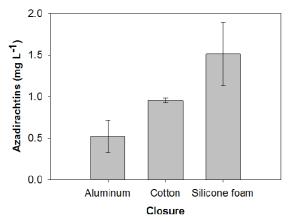

Fig. 5. *Azadirachtins* content of *A. indica* cells cultured in shake flask using different types of closures after 6 subcultures.
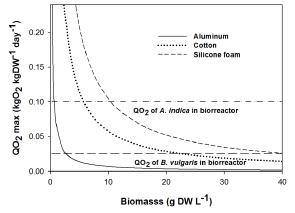

Fig. 6. $QO_{2,max}$ as a function of biomass concentration of *A. indica* and *B. vulgaris* cultured in shake flasks using different closures (aluminum, cotton, silicone foam) and comparison with values measured in bioreactor.

The reduction of *A. indica* aggregates may be associated with a culture response to oxygen limitations. Kebler *et al.*, (1999) proposed a relationship to know the maximum diameter for unlimited respiration throughout the aggregate. This report indicates that the diameter of the aggregate is proportional to the square root of the oxygen

concentration at the surface, and different ajmalicine concentrations was found among the different aggregate sizes. The relationship between aggregate size and oxygen concentration is in agreement with our results. Higher OTR_{max} are related with a higher oxygen concentration in the liquid medium and allow larger aggregates to developed, as occurred with cultures growing in shake flasks using silicone foam closures. The higher content of *Azadirachtin*s measured under this condition with a better oxygen supply or with cell differentiation, could lead to enhanced production of secondary metabolites (Zhao *et al.*, 2003).

3.3 OUR of A. indica cultured in a bioreactor and comparison with $QO_{2,max}$ obtained in shake flask

In order to show that the poor growth and azadiracthins production in shake flasks covered with aluminum foil (the lower OTR) could be related with a high oxygen consumption by *A. indica* cells, the OUR of cells was measured in a bioreactor without oxygen limitations. So then, *A. indica* cell culture had a consumption of 0.100 ± 0.016 kg O_2 kg DW^{-1} day^{-1}. The OUR for *B. vulgaris* cell culture was 0.027 ± 0.010 kg DW^{-1} day^{-1}. These measurements were done using the same conditions and the results indicated that *A. indica* cells consume four times more oxygen than *B. vulgaris* ($p \leq 0.05$).

OTR$_{max}$ values for the different closures (Table 1) were used to calculate the $QO_{2,max}$ in shake flasks using different closures (aluminum, cotton, silicone foam). $QO_{2,max}$ was plotted as a function of the biomass concentrations and this is showed in the Fig. 6. In the same figure, it is showed the QO_2 measured in a culture developed in a bioreactor (horizontal lines), as a comparative condition without oxygen limitations. In this condition, for *A. indica* cultures QO_2 was of 0.100 kg O_2 kg DW^{-1} day^{-1}, and for *B. vulgaris* was of 0.026 kg O_2 kg DW^{-1} day^{-1}. In shake flasks, QO_2 decrease as the biomass increase and this behaviour is independent of the type of closure used. The QO_2 profile of *A. indica* cultures developed in shake flaks with the different closures indicated that there are oxygen limitations when biomass concentrations increase. These oxygen limitations using aluminium foil closure was at 0.7 g DW L $^{-1}$, for cotton was at 5.7 g DW L $^{-1}$, and for silicone foam was with 10.4 g DW L $^{-1}$. Doing a similar analysis with *B. vulgaris*, which can be cultivated for long terms in shake flasks, this specie presented oxygen limitations

by biomass concentrations of 2.6 g DW L $^{-1}$ with aluminium foil, 21.3 g DW L $^{-1}$ with cotton, and 40.0 g DW L $^{-1}$ with silicone foam. This consideration allows understand how *B. vulgaris* cell cultures could be developed in shake flasks with different covers, reaching high biomass concentrations, while *A. indica* cell had limitations to grow by oxygen limitations.

B. vulgaris does not lose viability when using aluminum foil as a close (Rodríguez-Monroy y Galindo, 1999) and consume only 25 % of oxygen that *A. indica*. These results agree with those reported by Geigenberger (2003) who showed that plant tissues can reduce oxygen consumption when external oxygen concentration decrease considerably or when oxygen concentration within the tissue falls even in well-oxygenated atmospheres. Plants can reduce the respiration rate, change their metabolism and protein degradation or modify their morphology to avoid internal anoxia. Only when oxygen concentration is close to zero, the cells turn on the mechanism to produce ATP in anaerobic conditions, using the lactate dehydrogenase and alcohol dehydrogenase enzymes (Geigenberger, 2003). Therefore, the lowest aggregate size and content of *Azadirachtin*s observed in shake flasks covered with aluminum foil may be related to insufficient oxygen supply and a reduction in their metabolism.

It is necessary to analyze oxygen consumption of different plant species especially for the in vitro establishment of new plant cell cultures. If the plant cell culture has high oxygen consumption, the effect of low OTR in primary and secondary metabolism and its morphology should be studied because growth and secondary metabolism may be limited. For increasing the OTR in shake flasks, it may be necessary to use a closure with high oxygen transfer or modified other variables that affect the OTR (e.g., volume of culture medium in the flask or agitation speed).

Conclusions

OTRs of 0.07, 0.58 and 1.07 kg O$_2$ m $^{-3}$ day $^{-1}$ were obtained using several closures of shake flask: aluminum, cotton and silicone foam, respectively. OTR lower affected *A. indica* cell culture in shake flasks (cell viability, pH medium, aggregate morphology and *Azadirachtins*) decreasing the cell growth and the *Azadirachtins* production. These results show the oxygen demand of this plant cell culture may be a limiting factor to grow in Erlenmeyer shake flasks.

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