AIRLIFT BIOREACTOR FLUID-DYNAMIC CHARACTERIZATION FOR THE CULTIVATION OF SHEAR STRESS SENSITIVE MICROORGANISMS

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ABSTRACT

Airlift bioreactors are considered very efficient for aerobic cultivation of microorganisms. In particular, for the cultivation of filamentous fungi, where low shear rates may be required. In this context, the main aim of this study was to design an airlift bioreactor built with an external loop, adequate for biomass production of microbial organisms, including ectomycorrhizal fungi. A 5-L airlift bioreactor was constructed and experimental studies performed under an air-salt solution system (0.15 mol/L NaCl), in order to characterize the reactor´s fluid-dynamic in relation to the superficial gas velocity (U_g) in the range of 0.001 to 0.020 m/s. In order to evaluate the performance of the reactor, a preliminary assay was conducted with the ectomycorrhizal fungus *Rhizopogon nigrescens*. Better gas separation reflected in better liquid circulation and higher oxygen transfer (0.0197 1/s at 1 vvm) when compared to an operating, 2.3-L prototype airlift. According to this study, volumetric coefficients for oxygen transfer (k_La) up to 0.020 1/s (specific airflow rates of 1.0 vvm) are sufficient to promote the growth of shear stress sensitive microorganisms, such as ectomycorrhizal fungi.

Indexing terms/Keywords

Airlift bioreactors, Fluid-dynamic, Gas hold-up, Mass transfer, Sensitive microorganisms

Academic Discipline and Sub-Disciplines

Chemical Engineering; Bioprocesses

SUBJECT CLASSIFICATION

Airlift bioreactor design; oxygen transfer; kinetics of fungi

TYPE (METHOD/APPROACH)

Experime

INTRODUCTION

The majority of the bioreactors employed under aerobic conditions in the industrial sector are conventional stirred-tanks. The remaining are those without a mechanical agitation, such as the airlift and the bubble column bioreactors. Nevertheless, stirred-tanks are not the most suitable bioreactors for the cultivation of certain microorganisms [1-3]. The degree of stirring required to reach the ideal mass transfer may, in many cases, damage the cells and reduce productivity [2-4]. Moreover, the agitation required by these bioreactors may imply high-energy consumption. Additionally, in the majority of the bioprocesses known, the cultivation must remain free of contaminants for long periods, requiring the installation of complex mechanical seals. Conventional bioreactors are then more expensive and more problem-prone than those without mechanical agitation are.

The disadvantages presented by conventional bioreactors have stimulated the pursuit for alternatives, such as the airlift bioreactor [5-8]. Due to the fluid-dynamic characteristics, these bioreactors are more convenient for the cultivation of several types of microorganisms, such as ectomycorrhizal fungi [6]. In an airlift bioreactor, the volume of the liquid is divided into two different zones. Only one of these zones receives the injection of air. The difference in terms of gas hold-up between the aerated (riser) and the non-aerated zones (downcomer) creates a difference in the liquid´s density and, as a result, promotes the circulation of the fluid [5].

Gas hold-up and the velocity of circulation of the liquid phase, whose source and values depend on the gas flow, are among the most important parameters in fluid-dynamics when studying and designing airlift bioreactors [5,9-12]. Gas hold-up is the gas fraction in the gas-liquid dispersion that, combined with the size of the bubbles, determines the lastingness of the gas in the liquid phase, and will increase the size of the interface area available for oxygen transfer [5]. Gas hold-up is an important parameter during the designing of a bioreactor, since the total volume is proportional to the maximum hold-up intended capacity.

The difference in gas hold-up between the riser and the downcomer affects the velocity of the circulating liquid, which, in turn, will affect hold-up. This is mainly due to the velocity variation of the rising bubbles [1]. Aside from this, liquid

circulation has an effect on the turbulence, in the fluid-reactor wall heat transfer coefficient, in the gas-liquid mass transfer, and in the shearing rate force to which the microorganisms are submitted [5].

The mixing, which is affected by the turbulence, has to promote a rapid dispersion of the acid/base pulses in order to control the pH. It has also to offer homogeneous oxygen transfer conditions in order to avoid the formation of anoxic zones [5]. A good qualitative index of the mixture is represented by the time of mixture, defined as the time necessary for the bioreactor to reach a new stationary state after a change in the nutrient feeding [13].

Studies in different parts of the world have showed a significant increase in plant survival and development when inoculated with ectomycorrhizal fungi [14,15]. In order for this effect to be spread into large areas, the production of high amounts of fungal biomass are necessary to serve as inoculum. Modern techniques of inoculant production employ submerged cultivation processes followed by mycelium immobilization in a gel form of calcium alginate. Although these techniques are considered a good approach to obtain good quality inoculum, they present some drawbacks. Amongst the most important are the serious difficulties imposed by the poor growing conditions presented by most of the ectomycorrhizal fungi cultivated in conventional bioreactors. To reduce such difficulties, airlift bioreactors are considered a promising alternative [6].

In this context, an airlift bioreactor with external circulation was built. Before employing it for the cultivation of microorganisms, the characteristics of the bioreactor were studied on an air-salt solution system. Among the parameters studied are the effects of the gas flow rate on the superficial liquid velocity, on the gas hold-up and on the mass transfer coefficient. Values obtained experimentally were compared with different bioreactor systems available in the literature. To evaluate the performance of the bioreactor, an assay was performed with the ectomycorrhizal fungus *Rhizopogon nigrescens*.

MATERIAL AND METHODS

Equipment

A 5-liter external-loop airlift (ELA) bioreactor was built in stainless steel (Fig.1), based on H/D and A_r/A_d ratio similarities of a 2.3-L, previously built glass prototype airlift (glass ELA) [6]. Main adaptations are related to the top of the bioreactor, where the riser was elongated beyond the entrance of the downcomer, allowing for better gas separation, and the horizontal section that connects the riser to the top of the downcomer inclined to a 45º angle, as showed in Fig. 1. A porous ceramic sparger was placed at the base of the riser.

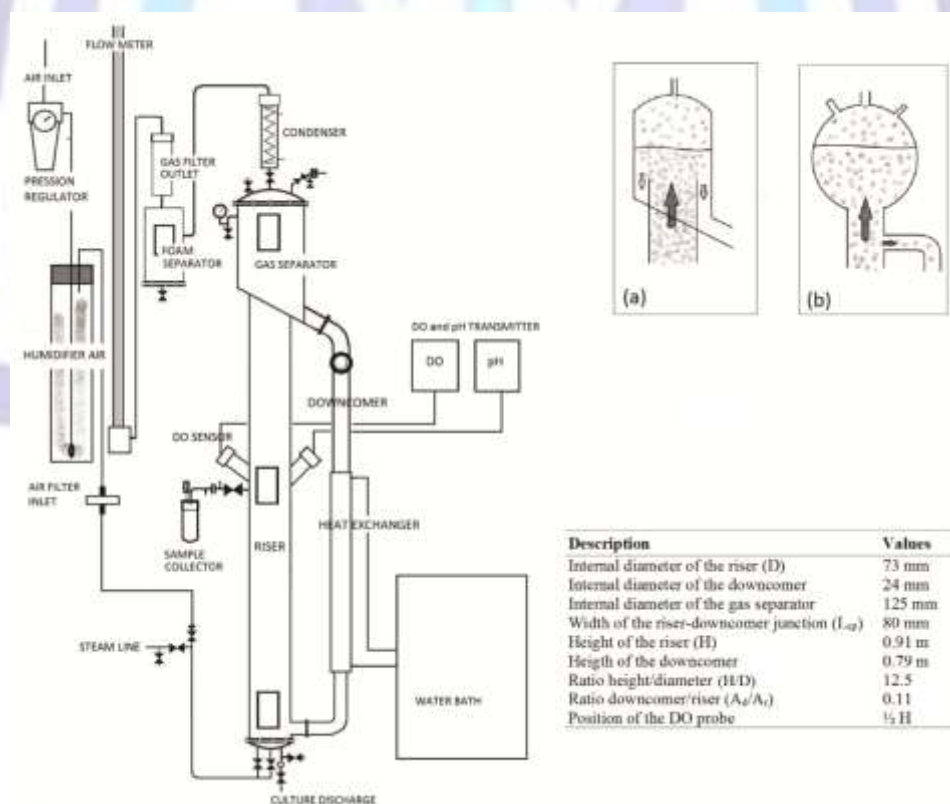| Description	Values
Internal diameter of the riser (D)	73 mm
Internal diameter of the downcomer	24 mm
Internal diameter of the gas separator	125 mm
Width of the riser-downcomer junction (L_{rd})	80 mm
Height of the riser (H)	0.91 m
Heigth of the downcomer	0.79 m
Ratio height/diameter (H/D)	12.5
Ratio downcomer/riser (A_d/A_r)	0.11
Position of the DO probe	½ H

Fig. 1 5-L external-loop airlift (ELA) bioreactor design displaying important specifications. Details of the modification introduced in the gas separator (a), compared to the 2.3-L glass ELA bioreactor with downcomer entrance located before the gas separator (b)

An attached water bath was installed to control the internal temperature through a heat exchanger installed in the downcomer. Three rectangular glass screens (40x80 mm) were mounted in different parts of the bioreactor to observe the air distribution. An extra screen was located just after the 45º curb of the downcomer, right at the liquid entrance. A polarographic probe InPro6000 and a pH probe InPro3250, with 4100e and 2100e transmitters, respectively (Mettler-Toledo International Inc.), were used to measure the dissolved oxygen and the pH. The pH and DO probes were carefully and shallowly mounted into the riser in order to avoid any interference with the circulation of the liquid. All feeding lines, sample collector, material dispenser and air entrance and exit were built with steam seals.

Fluid-dynamic studies were performed utilizing an air-water system simulating a microbial fluid with a Newtonian behavior. For that, a solution of 0.15 mol/L NaCl (viscosity similar to that of water) was used. Simple techniques were employed for the bioreactor hydrodynamic characterization, avoiding the building of several ports and accessories. A Newtonian behaving fluid was chosen for the fluid-dynamic characterization because the reactor was primarily built for the cultivation of ectomycorrhizal fungi. These fungi, to the best of our knowledge, do not significantly alter the viscosity of the medium throughout the cultivation.

For the fungal culture, the bioreactor was sterilized (121 °C) using direct steam created by a 25-L autoclave, connected through a derivation of the air outlet. During the sterilization of ports, like feeding, sampling, and air inlet, the bioreactor was kept at 110 °C by depressurization, created by exiting steam. After this 30-minute phase, the bioreactor was pressurized and kept at 121 °C for an extra 30 minutes.

EXPERIMENTAL PROCEDURES

Airflow and gas hold-up

The airflow was measured with a glass gas-bubble meter ($\varnothing$=1.72 cm and V=220 cm^3), taking into account the time necessary to fill the tube with air coming from the bioreactor. This time was controlled observing the movement of a soap gas bubble along the gas-bubble meter tube. Specific airflow rates from 0.04 to 1.00 vvm (volume of air/volume of the bioreactor, per minute) were applied, which are considered suitable for the majority of microbial cultivation systems, corresponding to a range of superficial gas velocity (U_g) from 0.001 to 0.020 m/s.

The overall gas hold-up (ε) was determined by the volume expansion technique [5], taking into consideration the values of the columns heights of the static liquid and the gas-liquid dispersion under aeration. The measuring was performed by draining the volume of liquid corresponding to the expansion (V_E), according to the following equation:

$$\varepsilon = \frac{V_E}{V_D}$$
(1)

where V_D is the volume of the gas-liquid dispersion.

Superficial liquid velocity

In order to determine the superficial liquid velocity, a polystirene-epoxi sphere, presenting a specific mass of ca. 1 g/cm^3 (corresponding to the density of the liquid in the downcomer) was employed. According to the "flow-follower" technique, this sphere was freely placed in suspension in the liquid and the time it required to move through the length of the downcomer was measured for every value of airflow. The values of superficial liquid velocity in the riser (U_{Lr}) were obtained according to the continuity criterion [16], as shown in equation 2:

$$U_{Lr}A_r = U_{Ld}A_d$$
(2)

where U_{Ld} is the superficial liquid velocity in the downcomer, and A_r and A_d are the areas of the transversal section of the riser and the downcomer, respectively.

Time of mixture

To measure the time of mixture, a heat technique was employed [13]. In this technique, hot water (~700 mL) introduced at the top of the bioreactor was utilized as a marker. The temperatures at the top and at the bottom of the bioreactor were measured with two digital identical thermometers, and the values obtained in both thermometers recorded until they showed equal temperatures. The time elapsed in this procedure was then considered as the time of mixture. The measurements were performed at three different specific airflows: 0.20, 0.36 and 0.52 vvm.

Determination of the pressure at the top of the bioreactor

The pressure (P) at the top of the bioreactor was determined for different values of specific airflows. For that purpose, a "U" manometer, containing water, was installed at the top of the bioreactor and the P values were calculated using the following equation:

$$P = \rho g h + P_{atm}$$
(3)

Determination of the volumetric coefficient of oxygen transfer ($k_L a$)

The dynamic method was employed for the determination of the volumetric coefficient of oxygen transfer [2,17]. This method considers the response signal of a probe plunged into a liquid media under aeration. Firstly, nitrogen was injected into the liquid media to eliminate dissolved O_2. At that moment, aeration of the liquid was initiated (under the airflow, temperature and pressure previously established), and the signal registered by the probe recorded. The $k_L a$ was calculated according to equation 4, which is the result of the mass balance in the system, considering a well-mixed gas-liquid mixture in the riser, where the air is injected, and a plug-flow regime in the downcomer, where the gas phase was neglected.

$$ ln\left(1 - \frac{C}{C_S}\right) \cdot \left(1 - \frac{V_d}{V_r(1-\varepsilon)}\right) = -k_L a.t \tag{4}$$

where C is the oxygen concentration in the liquid media (mg/L), C_S is the concentration of oxygen saturation and V_r and V_d are the volumes (L) of the riser and the downcomer, respectively. Since the gas phase is absent in the downcomer, its volume, which represents circa of 7% of the working volume, did not contribute to the transfer of oxygen. The term $V_d/V_r(1-\varepsilon)$ represents the loss caused by the lower volume available for this transfer to take place. When the values of $ln[(1-C/C_S).(1-V_d/V_r(1-\varepsilon)]$ from the data obtained in the dynamic assay are plotted against time (t), a linear curve is obtained. The slope values in this curve give the value of $k_L a$. The fraction C/C_S is the signal of the probe itself, calibrated in the interval from 1 to 100%, so it is not necessary to determine the oxygen concentration in saturation (C_S).

Biomass production for the ectomycorrhizal fungus *Rhizopogon nigrescens*

A preliminary study employed the ectomycorrhizal fungal isolate *Rhizopogon nigrescens* Coker & Couch (UFSC-Rh90). The isolate was obtained from a publicly accessible culture collection (CBMAI 1472). During the study the isolate was kept at 25±1 °C in Modified Melin-Norkrans (MMN) solid medium [6] with glucose as the sole source of carbon. Mycelium from a previous cultivation, preserved under refrigeration (8±2 °C) in a saline solution (0.85 % NaCl) for three days, was utilized to prepare the inoculum. In order to promote a suitable homogeneous mycelial suspension for the starting up of the bioreactor cultivation process, 50 g of biomass (~1.5 g of dry weight) were fragmented in a blender for 20 s at 3600 rpm, in 300 mL of MMN liquid medium.

For the bioreactor cultivation, we used a variation of the PGK medium [6], containing (g/L): glucose 10.0; soy peptone 1.5; malt extract 1.0; NH_4NO_3 0.85; KH_2PO_4 0.264; K_2HPO_4 0.628; $MgSO_4.7H_2O$ 0.33; $CuSO_4.5H_2O$ 0.0021; $MnCl_2.4H_2O$ 0.0006; $ZnSO_4.7H_2O$ 0.0005; and $FeSO_4.7H_2O$ 0.0004. The initial pH was adjusted to 5.8 with an equimolar solution of 0.15 mol/L citric acid and sodium citrate prior to sterilization. Additionally, we added 0.25 mL/L of polypropylene glycol to prevent foam production.

Under a laminar flow, 350 mL of the mycelial suspension were inoculated into 4.7 L of culture medium previously sterilized at 121 °C for 30 min using a Mariotte flask. Afterwards, 5-L of the inoculated medium were aseptically transferred to the bioreactor using a sterilized connection. The bioreactor was then set for operation at 25±1 °C with the flow rate of 0.36 vvm, equal to an U_g=0.007 m/s. The injected air was purified with the aid of a filtering hydrophobic PTFE (Millipore Corporation; (Billerica, MA, USA) membrane, presenting pores of 0.22 μm diameter. To compensate evaporation, the air was humidified before filtering, by dispersing it through a 700 mL distilled water tube, as can be seen in Fig. 1. Cultivation was carried out until total consumption of glucose.

Eight samples of approximately 40 mL (6.5% of the medium total volume) were collected in different time intervals, more spaced in the beginning of the cultivation when the biomass concentration was lower. They were subsequently filtered in nº 1 filter paper (Whatman, Madison, UK), and the resulting biomass air dried at 75±1 °C until constant weight. The filtered biomass pH was determined conventionally and the residual glucose concentration determined by spectrophotometry set at 505 nm, utilizing the GO-POD method (colorimetric enzymatic kit; Biodiagnostica, Pinhais, BR).

RESULTS

Gas hold-up and superficial liquid velocity

The bioreactor designed during this study (5-L ELA) was built with a see through glass visor, located in the entrance of the downcomer. Using this device, we could verify that, for the airflow rate levels employed, no gas bubbles were drifted to the downcomer, confirming the effectiveness of the separator. Fig. 2a shows the superficial liquid velocity in the riser (U_{Lr}) and the overall gas hold-up (ε) as a function of the superficial gas velocity (U_g) for the 5-L ELA bioreactor. As can be seen, on a typical scale gas hold-up increased with the increase of U_g. The curve inflexion observed for U_{Lr} suggests a transition from a homogeneous to a heterogeneous gas bubble regime. Larger bubbles, generally formed by coalescence, tend to rise in higher velocities when compared to smaller ones (Fig. 2a). With the change in flow pattern, there is a continuous rise of U_{Lr}, driven by the increasing velocities of the rising bubbles. Instead, increases in gas hold-up occur at declining rates, as the superficial gas velocity augments, since there is a reduction in contact time between the bubbles and the liquid. Fig. 2b shows the results for the comparison between the overall gas hold-up obtained for both bioreactor designs. With a complete separation of gas at the top of the 5-L ELA bioreactor, associated to a higher velocity of liquid circulation, there is a reduction of more than 50% in gas hold-up for larger gas flow regimes.

Fig. 3a compares the data for the liquid circulation velocity obtained in the 5-L ELA bioreactor, with the data from the 2.3-L glass ELA. As demonstrated, there is around 43% increase in performance in the 5-L bioreactor design, obtained mainly due to the modifications implemented in the gas separator (Fig. 1a), when compared to the glass ELA prototype (Fig. 1b). Fig. 3b shows significant divergences in the values of gas hold-up obtained experimentally, with those empirically calculated employing two of the main correlations predicted and available in the current literature for an airlift bioreactor fitted with an external circulation loop.
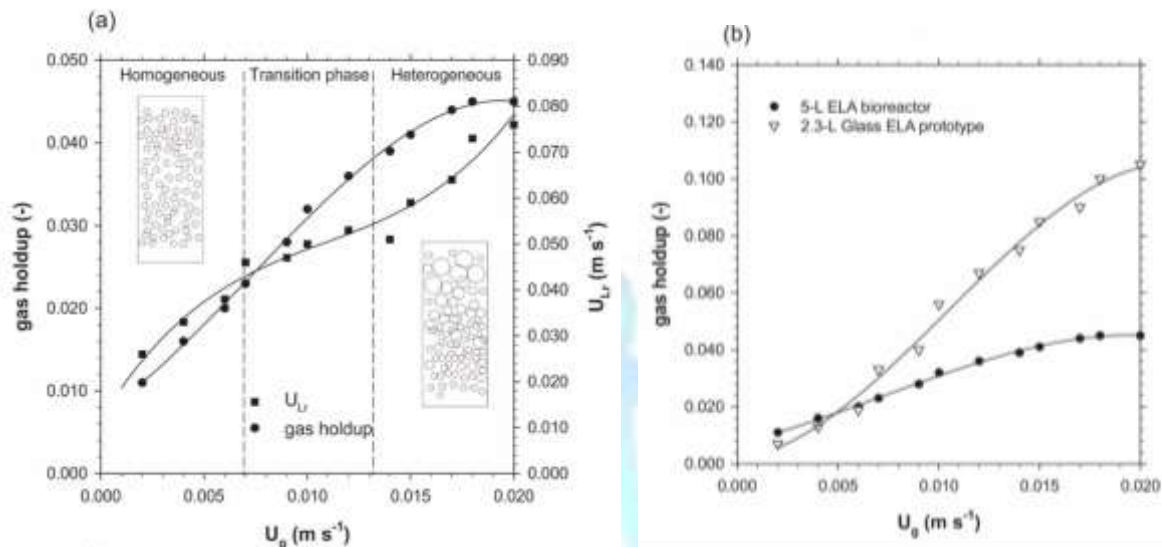

Fig. 2 Comparison between the superficial liquid velocity and the overall gas hold-up as a function of different superficial gas velocities in the 5-L external-loop airlift (ELA) bioreactor (a). Comparison between the values of overall gas hold-up for 5-L and 2.3-L ELA bioreactor designs (b)
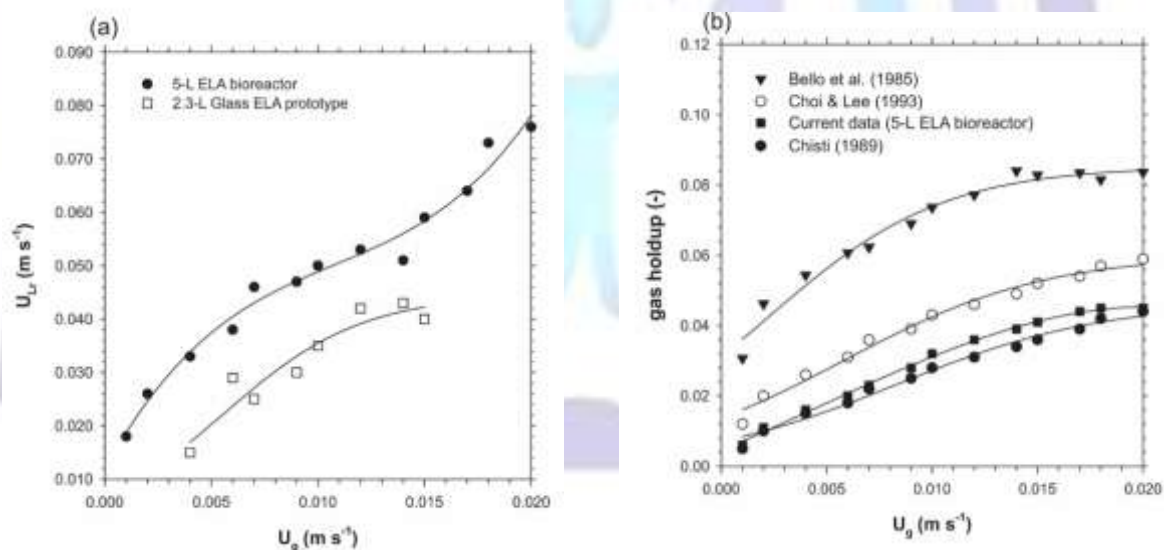

Fig. 3 Relationship between the superficial liquid velocity and the superficial gas velocity in the riser of the 2.3-L and 5-L external-loop airlift (ELA) bioreactors (a). Comparison between the experimental and theoretical values for the overall gas hold-up, as a function of the superficial gas velocity for the 5-L external-loop airlift (ELA) bioreactor (b)

Time of mixture

Fig. 4a presents the values for temperature at the top and at the bottom of the 5-L ELA bioreactor, while performing an assay to determine the time of mixture at an airflow rate of 0.20 vvm. The results show that it takes 65 s for a complete mixing (100%) of the liquid (Fig. 4b). For flow rates of 0.36 and 0.52 vvm, this time decreased to 60 and 55 s, respectively.

The precise determination of the mass transfer coefficient, using the dynamic assay during cultivation, depends upon the time of mixture, since there will potentially be deposition of cells at the bottom, especially cell pellets, with consequent cell

rarefaction in the upper levels of the bioreactor. This is particularly important, since until the complete establishment of the hydrodynamic parameters (for example, 65 s for a flow rate of 0.2 vvm), the readings for the oxygen concentration are dependent on those conditions. The results of this assay are crucial to define the initial experimental points to determine the values of k_La during the dynamic assessment. This time is yet again important for the re-establishment of the pressure within the bioreactor (caused by the pressure losses during filtering), and for the stabilization of the correct reading values performed by the DO probe.
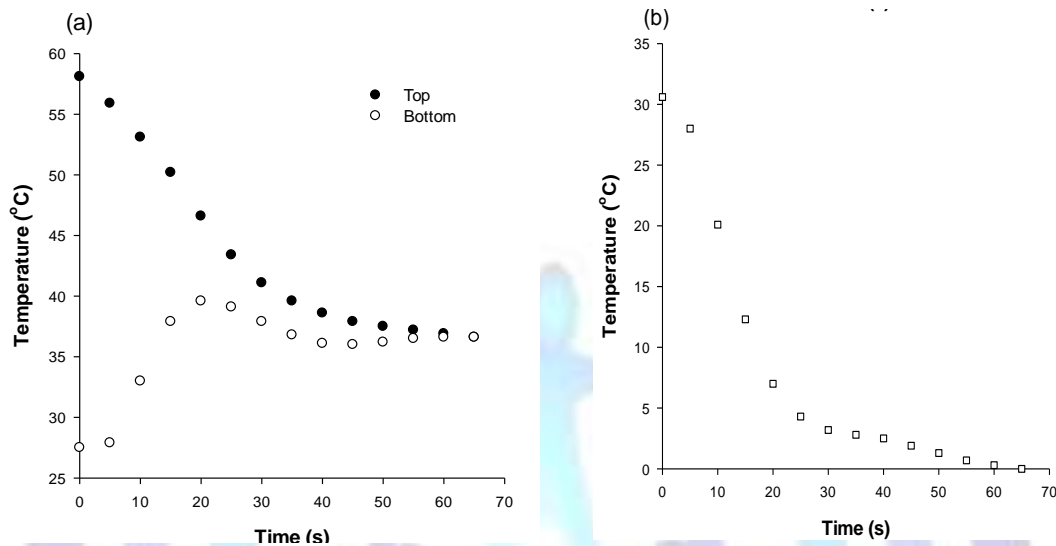

Fig. 4 Determination of the time of mixture in the 5-L external-loop airlift (ELA) bioreactor for an airflow rate of 0.20 vvm. Temperature at the top and at the bottom of the riser, along with the time of mixture (a). Temperature differences between the top and the bottom of the bioreactor (b)

Pressure at the top of the bioreactor

Fig. 5 shows the variation in pressure at the top of the bioreactor, determined for different superficial gas velocities. The resistance imposed to the gas as it passes through the exiting filter is the main factor responsible for this variation. It also demonstrates the superficial liquid velocity, which presents the same pattern observed for the pressure. This indicates a probable change in the gas bubble behavior, from a homogeneous to a heterogeneous condition starting at U_g of ~0.007 m/s. Information about the operating pressure is also important to calibrate de DO probe, allowing for readings that are more accurate. This fine-tuning may be necessary to detect the small variations that can take place, while determining the specific oxygen uptake rate presented by slow growing microorganisms.
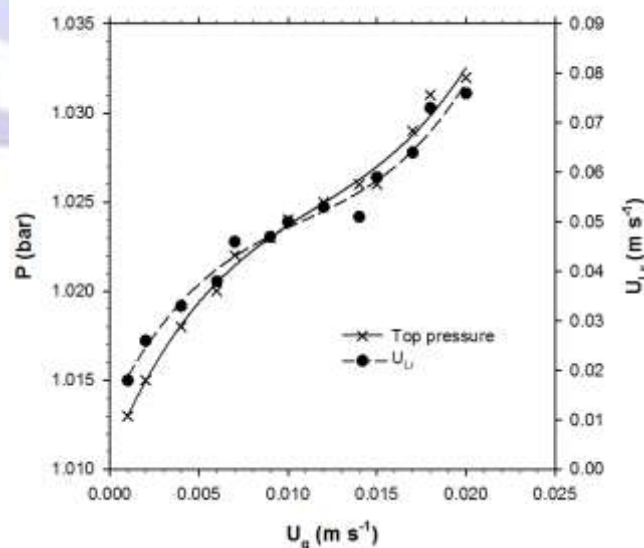

Fig. 5 Pressure at the top of the 5-L external-loop airlift (ELA) bioreactor, and superficial liquid velocity, in relation to the superficial gas velocity, determined at 25±0.2 ºC

Volumetric oxygen transfer coefficient

Experimental values for the volumetric oxygen transfer coefficient (k_La) were in agreement with those obtained for the gas hold-up assessment (k_La=0.455ε-0.0013; R^2=0.991), as specified in Fig. 6a. These values differed from those obtained by way of empirical equations (Fig. 6b), utilized to estimate the values of k_La as a function of the superficial gas velocity for an airlift operating under external loop circulation.

Correlations that can adequately predict the k_La values in a particular process are fundamental for bioreactor scaling up [2,5,18,19]. A correlation for the k_La can be obtained as a function of the superficial gas velocity {k_La=α[1+A_d/A_r]$^{-2}$(U_g)^{ß}} [20], such as the one presented in Fig. 6b, where the parameters α and β were adjusted using the experimental data, producing the following equation:

$$k_La = 0.844 \left[1 + \frac{A_d}{A_r}\right]^{-2} U_G^{0.88} \qquad (R^2 = 0.992) \qquad (5)$$

(k_La in 1/s and U_g in m/s)

This equation was used to define the specific airflow rates for the operation of a pilot, 110-L ELA bioreactor (data not shown), using the constancy of k_La as criterion for scaling up.
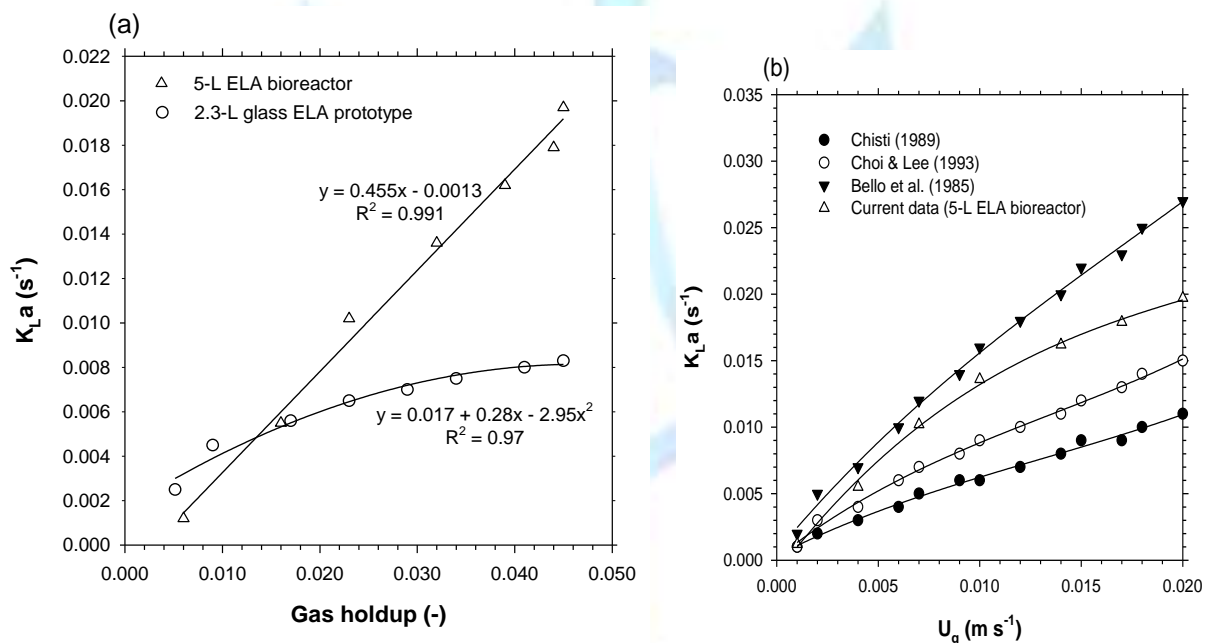

Fig. 6 Variation in the volumetric oxygen transfer coefficient (k_La) for different values of overall gas hold-up in the 2.3-L and 5-L external-loop airlift (ELA) bioreactors (a). Comparison between experimental and theoretical values of k_La for the 5-L ELA bioreactor (b)

Biomass production by *Rhizopogon nigrescens*

The growth kinetics for the ectomycorrhizal fungus *R. nigrescens* in the 5-L ELA bioreactor under an airflow rate of 0.36 vvm is shown in Fig. 7. Even thought one may find important to verify the performance of a specific microorganism under different fluid dynamics, this cultivation was conducted at the above specified conditions, under a homogeneous gas bubble regime (U_g=0.007 m/s). Although filamentous fungi do not show a regularly exponential growth phase, a good approximation was observed (R^2=0.98), showing a specific maximum growth rate of 0.48 1/d, which resulted in a duplication time of about 35 h. The index for substrate conversion in biomass ($Y_{X/S}$) was 0.42 g_X/g_S. The biomass developed in pellets, which reached diameters close to 3 mm, with total glucose consumption happening in a week. The dissolved oxygen concentration was kept over 6.15 mg/L, equivalent to 80% saturation, considering that oxygen solubility determined (by Käppeli and Fiechter method [21]) for the medium was around 7.5 mg/L.
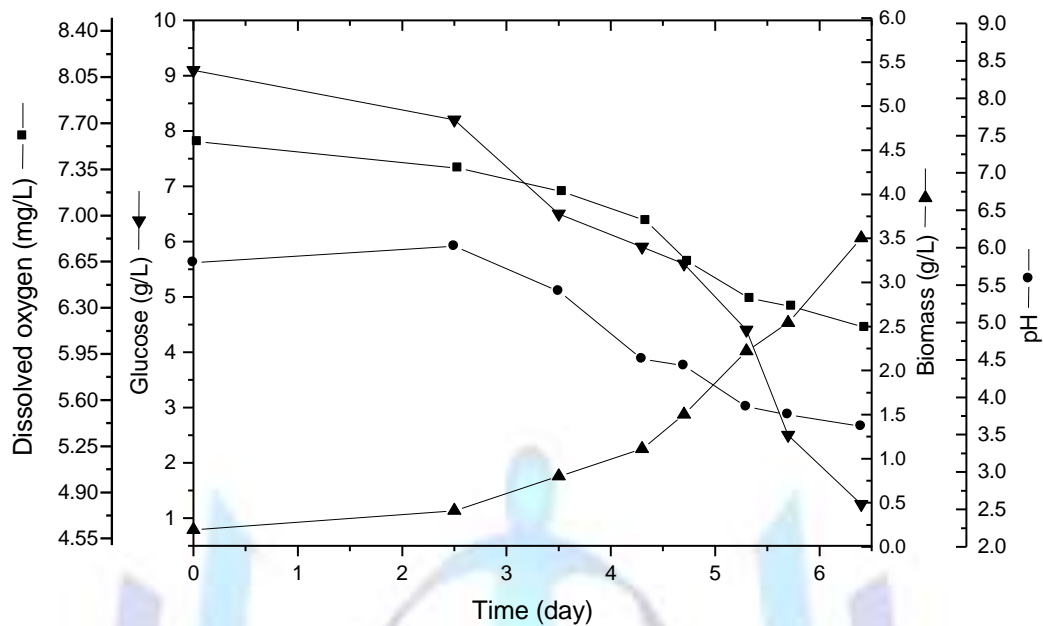
Fig. 7 Glucose consumption, biomass production, pH and dissolved oxygen variation during the cultivation of the ectomycorrhizal fungus *Rhizopogon nigrescens* at 25±1 °C in the 5-L external-loop airlift (ELA) bioreactor, with airflow rate of 0.36 vvm

DISCUSSION

In airlift bioreactors, increments in gas hold-up, associated to increasing turbulences, favors the coalescence of air bubbles. This, in turn, increases the ascending velocity of gas bubbles, and the circulation of the liquid, which will affect gas hold-up. Similar patterns are commonly observed, even for different bioreactor geometrical designs [22-27]. For the glass ELA prototype, the gas that enters the downcomer, due to its lower density, put forth a counter flux effect, which is detrimental to the circulation of the liquid, as observed in Fig. 3a. This is due to the small diameter of the downcomer that creates an effect of prevailed coalescence of bubbles (a large bubble the size of the downcomer may be formed), restricting the passage of liquid and creating fluctuations in the circulation flow pattern.

Gas hold-up values were higher for the 2.3-L ELA bioreactor (Fig. 2b). For this design, there is gas that accumulates in the downcomer and the velocities of the circulating liquid are smaller than those observed for the 5-L ELA. Higher hold-up values for the 2.3-L ELA reflect in smaller mass transfer capacities (Fig 6a), since they correspond to gas that is stagnated in the downcomer, not contributing effectively to k_La.

Even though there are correlations that can be used to estimate the gas hold-up as a function of the superficial gas velocity, this estimation is dependent on a few parameters, such as the type of fluid, H, L_{cp}, A_d/A_r and U_g. Consequently, the differences observed between the experimental values from this study and the values obtained from different theoretical correlations proposed by others [5,20,28] (Fig. 3b), are due to the fact that not all the parameters included in the current bioreactor design have their values within the range of the parameters for those proposed correlations (U_g, for example). Another reason that contributes to these differences maybe the complete separation of gas at the top of the riser.

The transition from a homogeneous to a heterogeneous flow regime is a gradual process that depends on the size of the air bubbles. This happens mainly because of the coalescence of the smaller bubbles. Pneumatic bioreactors function differently while working under a homogeneous or a heterogeneous flow regime, since the mass transfer velocities are dependent on the system hydrodynamics [2,5]. Therefore, for the designing and building of a bioreactor, it is important to know the conditions to which a specific regime prevails [29]. A classic method to determine the limits between the flow regimes using the gas hold-up and U_{Lr} measurements can be obtained via drift-flux analysis, as shown in Fig. 8. The graphic location of U_g/ε against U_g+U_{Lr} [30], confirms that from 0.05 m/s (U_g=0.007 m/s) the heterogeneous regime prevails in the bioreactor, indicated by the change in the curve's slope.
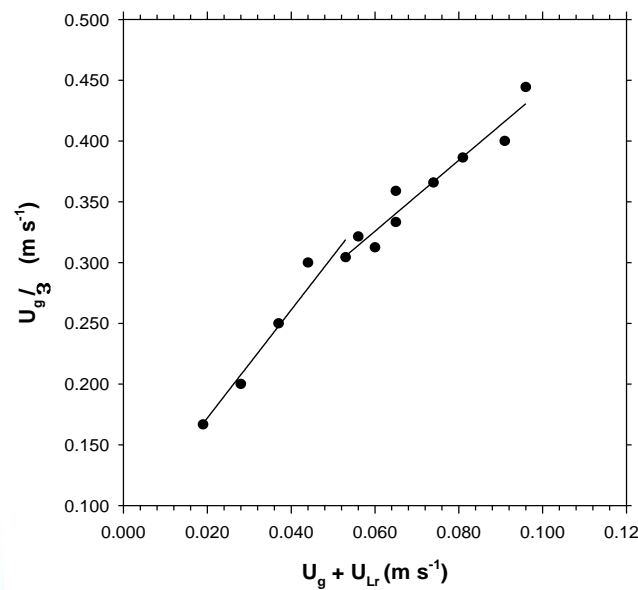
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Fig. 8 Identification of the flow regime pattern in the 5-L external-loop airlift (ELA) bioreactor thorough drift-flux analysis

The interesting results observed in Fig. 4 reveals that the circulation of the liquid puts into effect an important delay in the time necessary for the mixing to take place. A practical implication of this phenomenon is seen in the determination of the k_La, in which there is inconsistencies in the initial measurements of the dissolved oxygen during the dynamic assay. Another yet important implication can be seen during a particular cultivation batch, in which there is the need to control specific variables, such as pH, for example. In this case, it is necessary to establish the correct time of action for the control system, so it will be cyclically turned on (duty cycle) only after the necessary time for a complete mixing has been reached (cycle time). If this is not observed, the system may not perform as predicted.

The qualitative aspects of the mixing are crucial for the bioreactor effectiveness. A good mixing must supply enough oxygen and nutrients, besides maintaining a stable pH balance, avoiding, by default, parallel reaction to the process, which could culminate with cell death. Even though anoxic conditions in the downcomer (only place where oxygen consumption takes place) of airlift bioreactors are remote during the cultivation of ectomycorrhizal fungi, it is important to consider the improvements in the liquid circulation velocity achieved by the current design. During controlled conditions, ectomycorrhizal fungi present metabolic growing curves several times smaller than other microorganisms, such as bacteria like *Escherichia coli*. Therefore, when employed for the growth of more oxygen-demanding microorganisms, or when applied for larger batches, it is important to take into account the possibility of having a lower oxygen concentration in the downcomer, even below the critical levels. This may happen mainly because of its extra length.

The well-mixed assumption for the oxygen balance resulting in equation 4 is justified by the fact that the measurements in different sampling points did not affect more than ca. 10% of the reproducibility of typical k_La values [5], and it is, therefore, indicative of a fully mixed behavior. The experimental values of k_La diverged from those produced by the theoretical correlations (Fig. 6b) for the same reasons already pointed out for the gas hold-up. The higher k_La (0.0197 1/s) value obtained for an airflow of 1.0 vvm, was around five times smaller than the probe delay constant (0.1081 1/s, determined experimentally). Therefore, as suggested by Schmidell [17], there is no need for k_La value adjustments as a function of the probe response time [31].

Table 1 presents the values of k_La and other hydrodynamic parameters, obtained in the literature for different bioreactor designs. It is important to compare the results obtained in this study with those of Gouveia et al. [27], who worked with an internal-loop 6-L airlift bioreactor. The differences in gas hold-up values between the bioreactor designed by Gouveia et al. [27] and the bioreactor from this study, are due primarily to the fact that for the latter there is total gas separation, and the downcomer (absence of gas bubbles) occupies around 7% of the bioreactor entire volume. Despite these differences, the k_La values were similar for both bioreactor designs, proving the efficiency of the current design. Furthermore, Gouveia et al. [27] used the sulfite method instead of the nitrogen method, employed in the present study, for the determination of k_La. Thus, it is likely that the real k_La value obtained by those authors is lower than the reported value. It is known that high sulfite concentrations, commonly utilized to remove oxygen, may limit the liquid coalescence, a phenomenon that does not happen in culture media, which, in turn, may induce an over estimation of k_La [2,17].

Table 1 Comparison between the hydrodynamic properties of different bioreactors systems, for a superficial gas velocity (U_g) of 0.02 m/s

ε ( )	U_{Lr} (m/s)	t_m (s)	k_La (1/s)	Bioreactor	Liquid phase	V (L)	References
0.02	0.32	25	-	Internal-loop airlift	Tap water	55	[22]
0.015	0.60	-	-	External–loop airlift	Tap water	725	[23]
0.10	0.15	25	-	External-loop airlift	Water and calcium alginate beads	60	[32]
-	-	-	0.0694	Stirred tank with 700 rpm and 0.3 vvm	Culture medium	9	[33]
0.03	-	-	0.0025	Hybrid internal-loop airlift (no mechanical agitation)	2% cellulose fiber solution	1200	[25]
0.03	-	-	0.0042	Hybrid internal–loop airlift (170 rpm)	2% cellulose fiber solution	1200	[25]
0.03	-	-	0.0119	Hybrid internal-loop airlift (260 rpm)	2% cellulose fiber solution	1200	[25]
0.025	0.20	-	-	External-loop airlift with a Nylon packed bed	Tap water	12	[26]
0.075	0.063	24	0.0200	Internal-loop airlift	Sulfite solution	6	[27]
0.045	0.076	25	0.0197	External-loop airlift	0.15 mol/L NaCl	5	Current data

ε=total gas hold-up; U_{Lr}=superficial liquid velocity; t_m=time of mixture; k_La=volumetric coefficient for oxygen transfer; V=volume; CMC=carboxymethyl-cellulose

Even though the mass transfer coefficients for stirred-tank bioreactors are higher than for pneumatic bioreactors, the latter still have some advantages over the former. Among them, the possibility to cultivate sensitive and slow growing cells, like those of ectomycorrhizal fungi. The technology for large-scale production of this inoculum is still limited, mainly due to the unavailability of fungal biomass. One likely solution to this problem is the development of proper equipment that allows for the cultivation of these fungi, as the one discussed here.

The performed preliminary application reveals the potential of this technology to supply the market with enough biomass material to attend the ever-growing demands for fungal inoculum. Although the assay took around a week to reach production values near 3.5 g/L, due to the employment of a diluted medium and lower volumes of initial inoculum (~0.2 g/L), an assay with a more concentrated medium and larger inoculum volumes could still be performed within a week, reaching higher biomass contents. At first, periods of up to one week for the cultivation of microbial cells may seem long, but it is not if one considers the nature of the microorganism involved.

It is noteworthy to mention that pellets may form a compact mass in longer cultivation batches [6], decreasing growth rates due to intrinsic difficulties in mass transfer into the hyphae inside the pellet. In this study, due to the low biomass concentration, the airflow rate of 0.36 vvm was sufficient to provide high oxygen levels, surely higher than the critical values. The critical oxygen concentration value is very difficult to be determined due to a progressive increase in diameter of the pellets, which requests consequent increasing needs for dissolved oxygen, in order to reach the internal hyphae demands. In this study, around 50% of the oxygen transfer capability (k_La~0.010 1/s) was used, which indicates a capacity to attend cultivations that have concentrated biomass. Around 4% variation in k_La values were noticed during cultivation batches. This difference very likely comes from the accumulation of biomass in the form of pellets, which reduces available space and increases air bubble coalescence, rather than an effect from the variation in viscosity of the cultivation medium. In addition, the airflow control implementation will allow operations with variable airflow rates as to attend an adequate oxygen demand, saving energy and, especially, avoiding biomass compression. This is a function of the fluid turbulence and rotation, which increases proportionally to increases in airflow. Furthermore, optimal pH control will bring better results for more pH-sensitive microorganisms.

CONCLUSIONS

A total separation of the gas in the headspace of the bioreactor improved significantly the fluid dynamics of the system, promoting a better liquid circulation and, consequently, an efficient oxygen transfer. The different airflow values promoted an increase in the pressure at the top of the bioreactor. These values of pressure may be utilized to set the transmitter parameters, while calibrating the dissolved oxygen probe to different operational flows. Since external-loop airlifts have higher U_{Lr}, they generally present lower ε and k_La values than internal-loop bioreactors. However, they have shorter mixing times, which is quite important for the implementation of a control system (pH, for instance). An appropriate

conceptual design can compensate these differences, as demonstrated in the current study. Additionally, the absence of bubbles in the downcomer allow the installation of an online optic sensor to measure and intervene with the microbial growth. The design adopted for this study was able to attend the needs of stress sensitive microorganisms, such as ectomycorrhizal fungi. Furthermore, knowing the fluid-dynamic characteristics is fundamental for the development of systems that can control the entire process.

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Nomenclature

A_r, A_d	transversal sectional areas of the riser and downcomer, respectively (cm^2)
C, C_e, C_S, C_{crit}	oxygen concentration in the liquid phase (e=downcomer, S=saturation, crit=critical) (mmol O_2/L)
D	internal diameter of the riser (m)
F_L	liquid volumetric flow (L/h)
G	gravity acceleration (m/s^2)
H	height of the riser (m)
H	height of the liquid column of the manometer (m)
k_La	volumetric coefficient for oxygen transfer (1/s)
L_{cp}	width of the riser-downcomer junction (cm)
P	pressure (atm)
P_{atm}	atmospheric pressure (atm)
S	glucose concentration during cultivation (g/L)
T	time (min; day)
t_m	time of mixture (s)
U_g	superficial gas velocity (m/s)
U_{Lr}	superficial liquid velocity in the riser (m/s)
U_{Ld}	superficial liquid velocity in the downcomer (m/s)
V_d, V_r	volumetric dimensions of the downcomer and riser, respectively (L)
V_E	volume of the liquid corresponding to the expansion (L)
V_D	volume of the gas-liquid dispersion (L)
X	biomas concentration (g/L)
$Y_{X/S}$	index for substrate (glucose) conversion into biomass (g_x/g_s)
μ_X	cell growth specific velocity (1/day)
P	specific mass (g/cm^3)
E	overall gas hold-up (dimensionless)

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