



A new bioreactor design for culturing basidiomycetes: Mycelial biomass production in submerged cultures of *Ceriporiopsis subvermispora*



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HIGHLIGHTS

- A single device was developed for agitation and air injection into a bioreactor for aerobic fermentations.
- Rounded surfaces of agitation device avoid adherence during submerged filamentous fungi cultivation and increases homogeneity.
- High homogeneity of culture broth promotes decrease in chlamydospores formation during submerged cultivation of a basidiomycete.
- A specially designed bioreactor provides higher fungal biomass concentration compared to STR bioreactor.

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ABSTRACT

Oxygen transfer in submerged cultures of basidiomycetes is a key factor for efficient fungal growth. However, conventional stirred tank and airlift reactors are not utterly suitable for basidiomycetes culturing because they promote high shear stress to the mycelial hyphae and favor rapid agglomeration of mycelial pellets, compromising the diffusion of oxygen to the inner side of mycelium. We describe an original reactor design that overcome some of the limitations of conventional bioreactors used in submerged cultures of basidiomycetes. The strategy was to use a mechanism that permits simultaneous axis rotation and air injection, being the agitation promoted by an L-shaped tube. Adherence of mycelium and shear stress is avoided once only rounded surfaces exist inside the bioreactor. *Ceriporiopsis subvermispora* was selected as a model basidiomycete. The most productive system employed sucrose/corn steep liquor as the culture medium and pulsed addition of sucrose during the culturing. This approach provided efficient mycelial growth (maximum of $14.1 \text{ g} \cdot \text{L}^{-1}$), and avoided pH increase and pellet agglomeration throughout the fungal cultivation for 7 days, resulting in a biomass productivity of $1.72 \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$. Microscopic evaluation of chlamydospores accumulation in the grown mycelium confirmed that minimal fungal stress occurred in the cultures performed in the new designed bioreactor, contrasting with cultures carried out in conventional stirred tank bioreactors.

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1. Introduction

Submerged cultures of basidiomycetes have been used to produce mycelial biomass for inoculation of solid-state cultures used in large-scale processes such as biopulping and fungal pretreatment of lignocellulosic materials (Ferraz et al., 2008; Singh and Singh, 2014; Rouches et al., 2016). Submerged cultures of basidiomycetes also find application in production of extracellular enzymes, exopolysaccharides and bioactive metabolites (Fang and Zhong, 2002; Tinoco-Valencia et al., 2014; Braga et al., 2015). *Ceriporiopsis subvermispora*, used as a model basidiomycete in the current work, is a white-rot fungus recognized by its great

selectivity in lignin degradation, which has been broadly explored in biopulping and lignocellulose pretreatment processes (Akhtar et al., 1998; Ferraz et al., 2008; Machado and Ferraz, 2017). This species produces several extracellular enzymes such as manganese peroxidases, laccases, xylanases, mananases, endoglucanases, β -glucosidases and cellobiose dehydrogenases (Ruttimann-Johnson et al., 1993; Ferraz et al., 2008; Harreither et al., 2009; Chmelova and Ondrejovic, 2016). *Ceriporiopsis* Dom. is a poroid genus belonging to the Polyporaceae family (Burdall, 1998). *C. subvermispora* is a rare species, found in temperate regions and distributed in southern Canada, northern North America and Central Europe. The development of appropriated technologies for producing large amounts of inoculum from this and other basidiomycete species may be a key step in biopulping and lignocellulose pretreatment processes.

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Oxygen transfer in submerged cultures is a key factor for efficient fungal growth (García-Ochoa and Gomez, 2009). However, some of the efficient reactors currently used for submerged cultures, such as the stirred tank reactor (STR) and the airlift reactor, are not utterly suitable for basidiomycetes culturing. These fungal class usually produces extracellular exopolysaccharides (Tang and Zhong, 2002; Kim et al., 2005) that performs as a glue, promoting adhesion of the growing mycelium to the prominent parts of the reactor and sensors, resulting in mycelial agglomeration, which limits oxygen transfer to the core of the large formed pellets (Krull et al., 2013; Silvério et al., 2013).

Submerged culturing of basidiomycetes could be advantageous to overcome some of the problems related to the control of large-scale solid-state fermentation such as low homogeneity and formation of temperature, nutrient and product gradients associated with the low water availability in the cultures (Krishna, 2005). However, few efforts have been performed to develop new bioreactors design that could avoid high shear stress (García-Ochoa and Gomez, 2009; Fazenda et al., 2010; Silvério et al. 2013; Tinoco-Valencia et al., 2014) and agglomeration of mycelial pellets (Krull et al., 2013; Silvério et al., 2013) inside the cultures. Some improvements in the STR and airlift reactors have been described in patents claiming to diminish shear stress to the microorganisms or cell cultures, maintaining high efficiency in oxygen transfer to the culture broth (Prave and Sittig, 1985; Familletti, 1987; Schilling et al., 1998). However, the problems related to the high adhesion capacity of the basidiomycete's mycelia in bioreactor prominent parts and sensors have not been addressed up to date.

In the present work, we described the growing performance of a model basidiomycete, *Ceriporiopsis subvermispora*, in submerged cultures. Media improvement and the design of a new bioreactor are presented. An original reactor design was developed to overcome limitations imposed by STR and airlift bioreactors to basidiomycetes growing in submerged cultures.

2. Material and methods

2.1. Fungus, culture media and analytical procedures

C. subvermispora (Pilát) Gilbn. & Ryv. (L14807 SS-3 strain) was maintained at 4 °C in 2% (w/v) agar slants containing 2.4% (w/v) potato-dextrose broth (DIFCO, Maryland), 0.7% (w/v) yeast extract (Vetec, Brazil) (PD/YE medium). A culture medium composed of 2% (w/v) sucrose and 3.2% (w/v) corn steep liquor was used for fungal growth in submerged cultures. Corn steep liquor is a commercial product containing 47% (w/v) solids composed mainly of corn protein hydrolysate, minor amounts of lactic acid and reducing sugars and micronutrients as reported elsewhere (Akhtar et al., 1998; Agarwal et al., 2006; Masarin and Ferraz, 2008).

Analytical procedures used for monitoring fungal growth in the cultures included pH and mycelial dry weight determinations. Residual sugars in the cultures was determined by the dinitrosalicylic acid procedure (Dubois et al., 1956). When sucrose was used as the carbon source, the liquid medium was acidified with 1 mol·L⁻¹ HCl to a final concentration of 0.5 mol·L⁻¹ followed by heating at 100 °C for 10 min. After heating, the mixture was neutralized with 1 mol·L⁻¹ NaOH and analyzed by the dinitrosalicylic acid method (Dubois et al., 1956). Light microscopic evaluation of the grown mycelium was used for estimating chlamydospores accumulation in the fungal hyphae. Freshly collected mycelium was dispersed in a 5% (w/v) sodium hydroxide solution and stained with 1% phloxine B (Alic et al., 1987). Chlamydospores counting was semi-quantitatively ranked from – (none) to ++++ (copious). Volumetric oxygen transfer coefficient (k_La) was determined using the procedure described by Schmidell (2001): after

filling the bioreactor with the sucrose/corn steep liquor medium nitrogen was sparged abundantly, under stirring, until the dissolved oxygen reached zero, verified by an O₂ polarographic electrode previously calibrated to oxygen saturation. After that, the desired agitation and aeration were started. The variation of dissolved oxygen concentration with time was registered and k_La was calculated according to the equation $\ln(1 - C/C_S) = -k_La \cdot t$, where C and C_S are the oxygen concentration in the liquid and oxygen saturation concentration, respectively. By plotting $\ln(1 - C/C_S)$ against time (t) k_La was obtained by linear regression.

The influence of the culture pH on the *C. subvermispora* growth rates was estimated in Petri dishes with PD/YE, 2% (w/v) agar medium at pH 3.5, 4.0, 4.5, and 5.5. Culture broths were adjusted to the defined pH by adding 1 mol·L⁻¹ HCl or 1 mol·L⁻¹ NaOH before autoclaving at 121 °C for 30 min. The colony diameter was measured every day up to reach the Petri dishes border. Triplicate cultures for each pH were performed. Growth rates were determined as the slope in the curves relating the colony diameter versus growing period. Average values followed by standard deviations are reported in the text.

2.2. Submerged-unshaken cultures

Unshaken submerged cultures of *C. subvermispora* were conducted in 250 mL Erlenmeyer flasks maintained at 27 °C using PD/YE or the sucrose/corn-steep liquor media. Each culture flask contained 20 mL of medium that was inoculated with 2 discs (8 mm in diameter) of *C. subvermispora* precultured in PD/YE solid medium. For each culturing period, fungal biomass, residual sugars and pH were determined from three independent culture flasks.

2.3. Cultures in STR bioreactor

An 1.25-L (working volume) STR bioreactor (Bioflo III, New Brunswick) was inoculated with *C. subvermispora* mycelium previously grown in sucrose/corn-steep liquor for 15 days. For inoculum preparation, the mycelium mass from several cultures grown on 250 mL Erlenmeyer flasks were blended in autoclaved water by 10 cycles lasting 15 s each cycle. A 45 s resting time between each blending cycle was used to avoid excessive heating of the mycelium suspension. The bioreactor contained 1.25 L of sucrose/corn-steep liquor medium and was inoculated with blended mycelium at the ratio of 250 mg·L⁻¹ (mass of mycelium at the dry weight basis per L of medium). Cultures were agitated at 166 rpm and aerated at 1.16 vvm, based on the work from Lee et al. (2004), who used submerged culture to produce mycelial biomass and exopolysaccharides by basidiomycetes. Adecanol was used as an anti-foaming agent at 0.77 mL·L⁻¹. Periodic sampling of the culture was performed using the bioreactor facilities. Samples were assayed for mycelial and residual sugar contents and for pH.

2.4. New bioreactor design and culture conditions

The bioreactor consisted of a simple 14-L Marriote's bottle with a single-bottom outlet for sampling. The main top entrance of the Marriote's bottle was fitted with an innovative cap as briefly described in previous patent (Prata et al., 2011), performing several features: (a) to close the main bottle entrance providing one gas-exhaust tube and one liquid inlet tube; (b) to hold one air inlet tube connected to the central air inlet chamber; (c) to hold an L-shaped stainless steel tube, fixed with two ball bearings, that cross the air inlet chamber (Fig. 1). The L-shaped tube had the top aperture closed and was fixed into an external electric motor providing its rotation. Inside the air inlet chamber, the L-shaped tube has a ring of small holes permitting air entrance to the inside of the

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