Production of Bioactive Substances in Fed-batch and Semi-continuous Culture by *Pleurotus Ostreatus*

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Therapeutic properties of *Pleurotus* genus are associated with the polysaccharides present in the mycelium cell wall and culture broth of submerged cultures. The production of extracellular polysaccharides (EPS) by *Pleurotus ostreatus* using fed-batch and semi-continuous culture with 50 and 75 % medium replacement was investigated. The assays were performed in 4L bioreactor with 40 g L^{-1} initial glucose concentration. Medium replacements were performed when residual glucose concentration reached 20 - 25 g L^{-1}.

The highest fungal biomass productivity was obtained in semi-continuous culture with 75 % medium replacement (61.02 mg L^{-1} h^{-1}) followed by fed-batch (30.00 mg L^{-1} h^{-1}) and 50 % medium replacement (23.4 mg L^{-1} h^{-1}). However, the highest global EPS productivity (6.06 mg L^{-1} h^{-1}) was obtained in semi-continuous culture with 50 % medium replacement. 75 % medium replacement and fed-batch led to EPS productivities of 4.03 and 3.78 mg L^{-1} h^{-1}, respectively.

The highest EPS productivity was promoted by semi-continuous culture with 50 % medium replacement, in the second batch (9 mg L^{-1} h^{-1}). This process can be improved in order to maintain this high productivity value in further batches. Fed-batch culture under tested conditions was not effective for EPS production by *P. ostreatus*.

1. Introduction

Fungi of the *Pleurotus* genus are well known due to their nutritional value. It contains high levels of proteins, fibers, carbohydrates, vitamins and minerals, while presenting low calorie, fat and sodium levels (Reis et al., 2012). In recent years they have also received attention for their therapeutic properties such as antimicrobial (Wolff et al., 2008), antiviral (Zhang et al., 2004), antitumoral (Assis et al., 2013), immunomodulation (Carbonero et al., 2012), anti-inflammatory (Patel et al., 2012), antioxidant (Finimundy et al., 2013) among others, associated to the polysaccharides, specially β-D-glucans, present in the fruiting body cell wall, mycelium and culture broth in submerged cultures. Although many researchers have studied the medicinal properties of these fungi, few studies have been conducted to identify the best cultivation conditions and processes for extracellular polysaccharide (EPS) production (Papaspyridi et al., 2012).

Pleurotus can be cultivated in different agro industrial wastes as they are able to produce and excrete lignocellulosic enzymes such as laccases, cellulases, hemicellulases and xylanases (Kuforiji and Fasidi, 2008). However, they can also be cultivated in submerged culture enabling the production of large amounts of mycelium in a short time, under a most efficient control of the culture conditions such as pH, nutrient concentrations, oxygenation etc (Rosado et al., 2003). The polysaccharides obtained from the mycelium, culture broth and fruiting bodies have similar medicinal properties. However, the steps for extracting the polysaccharides from culture broth (Wolff et al., 2008) and their subsequente purification are relatively simpler than those required for mycelium (Sarangi et al., 2006) and fruiting bodies (Dalonso et al., 2009).

Based on the above, the objective of this work was to investigate the performance of *P. ostreatus* in producing extracellular polysaccharides in fed-batch and semi-continuous culture.

2. Materials and methods

2.1 Microorganisms and maintenance
P. ostreatus was purchased from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" under the code DSM 1833 and kept in Petri dishes containing WDA medium (1 L of wheat extract, 20 g of dextrose and 15 g of agar), at 4 °C (Furlan et al., 1997).

2.2 Culture medium
POL medium (5 g L^{-1} $(NH_4)_2SO_4$; 0.2 g L^{-1} $MgSO_4.7H_2O$; 1.0 g L^{-1} K_2HPO_4; 2.0 g L^{-1} yeast extract; 1.0 g L^{-1} peptone; 1.0 g L^{-1} $CaCO_3$) (Cavazzoni and Adami, 1992) with 40 g L^{-1} initial glucose concentration was used (Furlan et al., 2008).

2.3 Inoculum
Inoculum was prepared in 2 L Duran flasks, containing 400 mL of POL medium with 20 g L^{-1} glucose. The flasks were sterilized, inoculated with all the mycelium obtained from a Petri dish after 7 days growth, and incubated at 30 °C and 120 min^{-1} for 6 days (Furlan et al., 2008).

2.4 Culture conditions
A stirred tank bioreactor Biostat MD B. Braun with 4 L working volume was used. Initial K_La was 15 h^{-1}, while pH and temperature were kept constant at 4.0 and 30 °C, respectively, during the cultivation (Furlan et al., 2008).

2.4.1 Fed-batch culture
The process started as batch cultivation. However, when glucose concentration in the culture broth reached about 20 - 25 g L^{-1}, a concentrated glucose solution was added, restoring the original volume and increasing the concentration to 30 g L^{-1}. This concentration was defined based on the results obtained from *P. ostreatus* cultivation using the same culture conditions described above, and simple batch process (Furlan et al., 2008). The incubation was carried out for 14 days and the concentrated glucose solutions were fed into the bioreactor four times.

2.4.2 Semi-continuous culture
This process also started as batch cultivation. However, when glucose concentration in the culture broth reached about 20 - 25 g L^{-1}, 50 or 75 % of the fermented broth was removed and replaced by fresh medium with sufficient glucose concentration to reach initial concentration (40 g L^{-1}). For both cultures, the medium replacements were performed three times after initial batch culture.

2.5 Analytical methods
Fungal biomass concentration was estimated using the gravimetric method. Glucose concentration was determined through the enzymatic glucose-oxydase-peroxidase method (Free, 1963). Extracellular polysaccharides were extracted by adding 3 volumes of acetona refrigerated to 8 °C (Maziero et al., 1999) to 1 volume of the culture broth. The mixture was maintained at 4 °C for 24 h to precipitate the polysaccharides, that were separated by centrifugation at 4,500 x g for 5 min. The precipitate was washed twice with a 3:1:1 (v/v/v) acetone:ethanol:distilled water solution (Cavazzoni and Adami, 1992) and solubilised in 10 mL of a 75 % H_2SO_4 solution. Polysaccharide concentration was determined by measuring the total reducing sugars according to the phenol-sulphuric method (Dubois et al., 1956).

3. Results and discussion

3.1 Fed-batch cultivation
The concentration profiles of fungal biomass, glucose and EPS in the experiment carried out in fed-batch culture are shown in Figure 1. The maximum fungal biomass concentration (12.8 g L^{-1}) was reached at about 330 h cultivation while the maximal extracellular polysaccharide concentration (0.8 g L^{-1}) and productivity (3.5 mg L^{-1} h^{-1}) were achieved after 230 h, when the extracellular polysaccharides started to be degraded. Furlan et al. (2008), using the same microorganism strain in batch culture reached a maximum extracellular polysaccharide production and productivity of 1.3 g L^{-1} and 7.5 mg L^{-1} h^{-1}, respectively, after 175 h cultivation. The results obtained in this work suggest that fed-batch process under the tested conditions is not effective for the production of extracellular polysaccharides by *P. ostreatus* when compared with the simple batch.
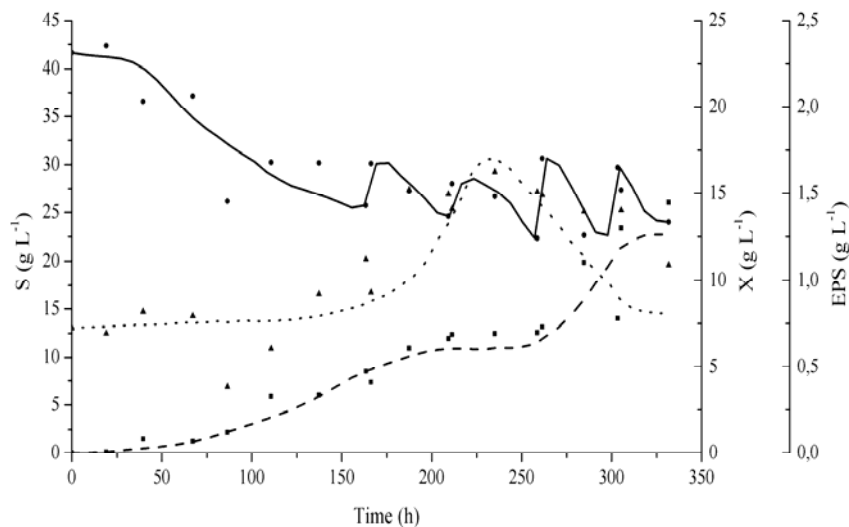
Figure 1: Profiles of fungal biomass - X (dashed line), glucose uptake - S (continuous line) and extracellular polysaccharide concentration - EPS (dotted line) with time in fed-batch culture.Glucose was added at 169, 217, 264 and 304 hours of cultive in order to restore the glucose concentration to 30 g L^{-1}

3.2 Semi-continuous culture

Concentration of biomass (X), glucose uptake (S) and extracellular polysaccharide concentration (EPS) in semi-continuous culture with 50 and 75 % medium replacement are presented in Figures 2A, 2B and 3, respectively.

It can be observed in Figure 2A that the time for maximum biomass concentration, in each batch, was greater for the culture using 50 % medium replacement than for that using 75 %. The volume removed and replaced at the beginning of each batch was greater in the culture using 75 % medium replacement, thus providing the cells with a higher amount of nutrients and lowering the amount of inibitors and/or toxic metabolics. As it can be seen in Table 1, this can result in higher maximum specific growth rates than those obtained in the culture with 50 % medium replacement. Maximum biomass concentration obtained in each batch was also higher in the culture using 75 % medium replacement than in the culture using 50 %. The comparison of Figures 2 A and B suggests that the biomass growth is associated to substrate consumption as the glucose consumption rates for the culture using 75 % medium replacement were higher than those for the culture using 50 % medium replacement (Table 1). This is probably due to the fact that the substrate added in the process using 75 % medium replacement is more easily assimilated as the concentration of cells remaining in the medium after the replacement is lower.

Figures 2A and 2B also show that in both cases (50 and 75 % medium replacement) the first batch was longer, with 216 and 163 hours respectively, due to the period required for microorganism adaptation to culture conditions in bioreactor. As expected, the duration of the second batch was shorther as the fungus had already adapted to the culture medium and conditions. However, in the latter two batches, glucose consumption was slower, which could be attributed to difficulty in glucose transfer to the cells (Burns et al., 1994).

It can be observed in Figure 3 that the extracellular polysaccharide production in the culture using 75 % medium replacement was faster than that using 50 % medium replacement. However, in the culture using 75 % medium replacement the concentration of the polysaccharide produced at each cycle was lower than in the culture using 50 % medium replacement (Table 1). In Figure 3 it is also possible to observe, in almost all batches, a decrease in polysaccharide concentration after attaining maximum value, independent of the batch or replacement condition. This is probably due to the synthesis and excretion of β-glucanase in the culture medium, breaking down the polysaccharides that were adhered to the mycelium biomass, releasing glucose in the culture medium, thus enabling the dissolved glucose to enter the cells (Burns et al., 1994).
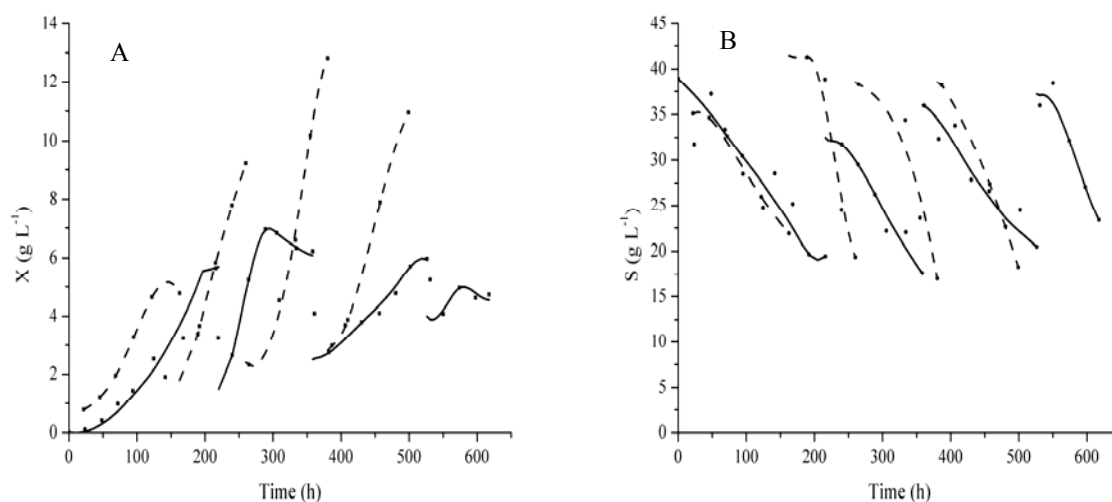
Figure 2: A: Profile of fungal biomass concentration B: Profile of glucose concentration

Figure 2: A reports: Profile of fungal biomass concentration – X with time in 50 % (continuous line) and 75 % (dotted line) medium replacement cultures.
Figure 2: B reports : Profile of glucose concentration - S with time in 50 % (continuous line) and 75 % (dotted line) medium replacement cultures. 50 % or 75 % of the volume of the culture medium were removed when glucose concentration was about 20 – 25 g L-1, and the same volume of POL medium containing glucose in a sufficient amount to restore the glucose concentration to 40 g L-1 was added. A total of 4 residence times (cycles) were obtained.
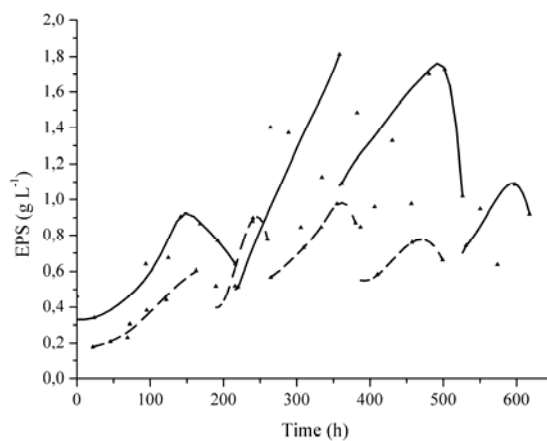

Figure 3: Profile of extracellular polysaccharide concentration

Figure 3 reports the profile of extracellular polysaccharide concentration – EPS with time (h), in 50 % (continuous line) and 75 % (dotted line) medium replacement cultures. 50 % or 75 % of the volume of the culture medium were removed when glucose concentration was about 20 – 25 g L^{-1}, and the same volume of POL medium containing glucose in a sufficient amount to restore the glucose concentration to 40 g L^{-1} was added. A total of 4 residence times (cycles) were obtained.

Table 1: Maximum specific growth rate (μX$_{max}$), maximum fungal biomass concentration (X$_{max}$), glucose uptake rate (V$_S$) and concentration of extracellular polysaccharide produced at each cycle (EPS) obtained for 50 and 75 % medium replacemnet.

	Cycle	Medium Replacement	
		50 %	75 %
μX$_{max}$ (h^{-1})	1	0.015	0.020
	2	0.017	0.021
	3	0.006	0.021
	4	0.007	0.015
X$_{max}$ (g L^{-1})	1	5.7	5.2
	2	7.0	9.2
	3	6.0	12.4
	4	5.0	10.9
V$_S$ (g L^{-1} h^{-1})	1	0.086	0.097
	2	0.121	0.302
	3	0.091	0.179
	4	0.152	0.172
EPS (g L^{-1})	1	0.60	0.42
	2	1.30	0.51
	3	0.67	0.41
	4	0.35	0.22

Table 2 presents the mean values of biomass and extracellular polysaccharide yield factors on substrate as well as biomass and extracellular polysaccharide global productivity. These kinetic parameters were calculated at the respective maximum EPS concentration points. While 75 % medium replacement favored micelial growth, 50 % medium replacement favored extracellular polysaccharide production.

On analyzing the extracellular polysaccharide productivity of each batch (4.09, 9.25, 5.20 and 5.70 mg L^{-1} h^{-1} for first, second, third and fourth batches, respectively, for 50 % medium replacement, and 2.68, 7.08, 4.07 and 2.32 mg L^{-1} h^{-1} for first, second, third and fourth batches, respectively, for 75 % medium replacement), it can be observed that the maximum values were achieved during the second batch, under both conditions. The extracellular polysaccharide productivity of second batch for 75 % medium replacement was about 25 % higher than that obtained in *P. ostreatus* simple batch cultivation (7.5 mg L^{-1} h^{-1}) (Furlan et al., 2008). Therefore, it would be necessary to evaluate the reasons for the drop in productivity after the second batch, in order to improve the process and keep productivity at the highest level.

Table 2: Yield factor of biomass (Y$_{X/S}$) and extracellular polysaccharide (Y$_{P/S}$) on substrate, biomass (P$_X$) and extracellular polysaccharide (P$_P$) productivity

Replacement	Y$_{X/S}$ (g g^{-1})	P$_X$ (mg L^{-1} h^{-1})	Y$_{P/S}$ (g g^{-1})	P$_P$ (mg L^{-1} h^{-1})
50 %	0.43	23.37	0.06	6.06
75 %	0.22	61.02	0.03	4.03

4. Conclusions

The semi-continuous culture with 75 % medium replacement favored micelial growth while 50 % medium replacement favored extracellular polysaccharide production. In the second batch of semi-continuous culture using 50 % medium replacement, EPS productivity reached 9 mg L^{-1} h^{-1}, which is higher than the productivity obtained in simple batch (Furlan et al., 2008) and fed-batch cultivation. This suggests that the

semi-continuous process using 75 % medium replacement can be improved for maintaining this productivity value in subsequent batches.

The fed-batch culture under tested conditions was not effective for extracellular polysaccharide production by *P. ostreatus* when compared to semi-continuous culture.

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