Research & Reviews: Journal of Microbiology and Biotechnology

e-ISSN: 2320-3528
p-ISSN: 2347-2286

Biomass and Intra-polysaccharide Production from Starchy Waste Using *Agaricus brasiliensis* in a Bench-scale Bioreactor

Sandra Montoya*, LM Vargas-del-Rio and Oscar J Sanchez

Bioprocess and Agro-industry Plant, Institute of Agricultural Biotechnology, Caldas University, Manizales, Colombia

Research Article

Received date: 31/12/2018

Accepted date: 18/01/2019

Published date: 25/01/2019

***For Correspondence**

Sandra Montoya, Bioprocess and Agro-industry Plant, Institute of Agricultural Biotechnology, Caldas University, Manizales, Colombia, Tel: +57-6-8781500x13436.

E-mail: sandra.montoya@ucaldas.edu.co

Keywords: *Agaricus brasiliensis*, Bench-scale, Gelatinized starch, Intra-polysaccharides, Mycelial biomass, Submerged fermentation

Abbreviations: SmF: Submerged fermentation; EPS: Exopolysaccharide; IPS: Intra-polysaccharide; GS: Gelatinized starch; SSF: Solid-state fermentation; G: Industrial glucose

ABSTRACT

Agaricus brasiliensis is an edible and medicinal mushroom used worldwide by its pharmacological potential. The objective of this work was to study the submerged fermentation (SmF) of *A. brasiliensis* for production of mycelial biomass and intracellular polysaccharides (IPS) from laboratory scale up to bench scale in a 7 L stirred-tank bioreactor considering the addition of olive oil and two carbon sources (industrial glucose and gelatinized starch from waste potato) in combination with different initial medium pH (4.5, 5.5 and 6.5) and fermentation times. Three experimental designs were performed to study the effect of the strain, olive oil, carbon source, initial pH of the medium, and time on production of biomass and IPS. SmF with GS-based (Gelatinized starch) medium was studied in the bioreactor with and without pH control compared to the medium with glucose. Response variables were as follows: biomass, reducing sugars, polysaccharides, IPS, viscosity, and broth pH. The time profiles of the cultivations were obtained. Best fermentation conditions at bench-scale were as follows: 10 g/L olive oil in a gelatinized-starch-based medium with initial pH 4.5 without pH control at 25°C, 250 rpm and 1 vvm aeration rate during 6 days of cultivation. These conditions enabled an average 33.2% increase in biomass production and 40.4% increase in IPS related to the control fermentation using glucose. The outcomes found in this work demonstrated that gelatinized starch extracted from waste potato is a suitable low-cost carbon source for this process and could be the base to design a SmF technology for *A. brasiliensis* cultivation at industrial scale.

INTRODUCTION

The submerged fermentation (SmF) of edible and medicinal mushrooms has become a promising technology to produce substances with nutraceutical potential as the mycelial biomass and a variety of fungal metabolites like glucan-type polysaccharides, polyketides, terpenoids, and fatty acids [1-5]. Among this group of bio-compounds, fungal polysaccharides represent a great interest in the pharmaceutical industry. These primary metabolites from fungi can be intracellular polysaccharides (intra-polysaccharides, IPS) or extracellular polysaccharides (exopolysaccharides, EPS) and exhibit differences in their structure and molecular weight depending on the sub-cellular structure where they are found or the liquid or solid medium to where they are excreted. In the case of SmF, several of these substances are expelled

e-ISSN: 2320-3528
p-ISSN: 2347-2286

into the medium and solubilized in it, but a large amount of polysaccharides is found as IPS making part of the structure of the mycelium [5]. In addition, the Macromycetes cell wall is a dynamic structure that may change according to the availability and type of nutrients and environmental conditions [1]. Nevertheless, it is considered that the main components of this cell wall are chitin, glucans, mannoproteins, and glycoproteins [6,7]. The fungal polysaccharides have important medicinal properties that could be considered to depend mainly on their chemical structure, monosaccharidic composition, type of glycosidic bonds (α or β), and molecular weights [4,8-12]. Several reports [11,13-16] indicate that EPS production from different Macromycetes species changes when pH is between 4.0 and 7.0 and that the obtained EPS have different composition and molecular weights. Furthermore, the studies in SmF of Macromycetes have analyzed different variables like pH, temperature, agitation speed, aeration, carbon sources, type and composition of nutrients in the culture medium, and process time in dependence on the cultivated species and interest products [2,4,5].

The Macromycetes studied in this work can be found under three names in the literature: *Agaricus subrufescens Peck*, *Agaricus blazei Murrill ss. Heinem*, and *Agaricus brasiliensis* [17]. Common names used for this species are "*Himematsutake*" or "*Agarikusutake*" in Japan, "*cogumelo de Deus*" (mushroom of God) or "*Seta del sol*" (sun mushroom) in Brazil, royal sun Agaricus, Murrill's Agaricus, ABM or almond Portobello in the United States, and "*Jisongrong*" in China [16,18]. *Agaricus brasiliensis* (*A. blazei Murrill ss. Heinem.*, *Agaricaceae*, *Agaricomycetes*) [15,19-21] is a medicinal and edible mushroom representing interest for pharmaceutical and food industries because of its content of potential biologically active compounds and its biological activities. According to Gern [16], this Macromycetes is used as a food, but it is mostly consumed as a tea. Therefore, it is sold fresh, but mostly dehydrated or pulverized, in capsules, tablets and infusions, and is also used as an ingredient in cosmetic products [18]. The production of this mushroom in Japan is about 100-300 tonnes dry basidiome per year and is used by 300,000-500,000 people to prevent cancer and as a co-adjuvant of other drugs during chemotherapy after removing a malignant tumor. In China, 500 tonnes of this dry mushroom are produced every year [16]. The cultivation of *A. brasiliensis* at industrial scale is performed mostly in Brazil, Japan, China, and South Korea, but its production is currently expanding due to its high price in the international market considering its pharmaceutical properties and pleasant almond flavor [18,22].

The production of *A. brasiliensis* basidiomes is carried out by solid-state fermentation (SSF) at industrial scale. The processes and techniques previously established for *Agaricus bisporus*, the most commercialized mushroom in the world, have been adapted for cultivation of *A. brasiliensis*. However, the different cultivation parameters for *A. brasiliensis* during all production stages (temperature, relative humidity, carbon dioxide concentration, and lighting) as well as feed stocks, compost preparation, and particularities regarding the covering layers and fructification should be studied to increase the yields adapting the production processes to the specific condition of each country [18]. Regarding the production of bioactive polysaccharides from *A. brasiliensis*, SSF cultivation offers some advantages compared to SmF such as low water requirements and a solid support for the development and growth of the mushroom similar to the natural medium. Nevertheless, the quantification and separation of fungal biomass and IPS are more difficult and the EPS remain attached to the solid substrate. In addition, polysaccharide synthesis strongly depends on the substrate composition and quality of fungal inoculum and the carpophore production requires long periods of time increasing the costs at industrial scale [1]. Moreover, SSF of *A. brasiliensis* is still under development and is performed at open fields with high temperatures (21°C-30°C during the different cultivation stages) and relative humidity (60-100%). These climatological conditions can be achieved in Brazil and other countries limiting the cultivation to the summer season.

Submerged fermentation has emerged as a promising alternative for production of biomass and EPS from *A. brasiliensis*. In this context, the utilization of agro industrial waste for the SmF of this fungus has in the last years. The usage of waste insoluble in water from breweries along with wheat bran and lignocellulosic hydrolyzates has been analyzed [1]. The hydrolyzates can release not only fermentable sugars like xylose, arabinose and glucose, but also toxic compounds like hydroxymethyl furfural, furfural, acetic acid and phenolic compounds resulting from the degradation of hexoses, pentoses, hemicellulose and lignin. Therefore, a detoxification treatment prior to fermentation might be required; among these treatments, overliming, adsorption with activated carbon and ionic exchange have been proposed. Other materials used for submerged fermentation of Macromycetes that do not imply any detoxification procedure are molasses, whey, casein hydrolyzates and soy cake, among others [4]. Thus, the research on culture media based on alternative low-cost waste feed stocks, which do not require costly detoxifying treatments for producing mycelial biomass, fungal IPS, EPS and other biologically active compounds with potential pharmacological use from *A. brasiliensis* is needed [4,22-27]. Several works performed at laboratory scale have studied the effect of medium composition, usage of vegetable oils (mostly oils rich in oleic and palmitic acids), carbon source, and environmental conditions (pH, temperature, agitation speed and aeration of culture broth) on the production of mycelial biomass and other metabolites by SmF [1,10,12,16,28-35]. On the other hand, it has been assessed that the utilization of soluble starch as a carbon source alternative to glucose and other sugars for production of biomass and EPS from *A. brasiliensis* obtaining good results [12].

The potato is the fourth food crop in the world after wheat, rice and corn. Its production represents the half of worldwide production of tubers and roots. World potato production reached about 392 million tonnes in 2017 being

e-ISSN: 2320-3528
p-ISSN: 2347-2286

China the first producer. In Colombia, a middle-income country, 70% potato is consumed directly as a food and private companies demand about 6% of potato production for producing food products, 7.9% for seed, 1.9% for feed (without processing), and 1.6 for export [36]. A significant amount of potato commercialized in traditional market places is discarded and wasted or employed for animal feed due to its low quality and size. However, the starch contained in such residual potatoes could be effectively utilized as an inexpensive carbon source for fermentation purposes, which does not require detoxification procedures. In this way, valued-added products like mycelial biomass, EPS or IPS could be produced. The objective of this work was to study the SmF of *A. brasiliensis* for production of fungal biomass and IPS from two carbon sources (glucose and gelatinized starch extracted from waste potato) at laboratory and bench-scale considering the addition of olive oil as well as the influence of the culture medium pH during the fermentation in order to generate knowledge useful for subsequent scale-up research intended for the future design of an industrial process.

MATERIALS AND METHODS

Organisms

Two *A. brasiliensis* strains were employed in this work: PSWC838 provided by Pennsylvania State University (USA) coded as WC838, and a strain from Fujian Agriculture and Forestry University (China) coded as ABC. The strains are deposited at Culture Collection of Macrofungi at Universidad de Caldas (Manizales, Colombia) and were maintained on potato dextrose agar (PDA; Scharlau, Spain) with 0.2% activated carbon (AppliChem Panreac, Germany) with periodic transfer on PDA according to the procedure established in a previous work [21].

Strain Selection and Use of Olive Oil in the Culture Medium

In order to choose the *A. brasiliensis* strain and define whether olive oil was needed as a component of the culture medium, a three-factor experimental design was performed. The factors (and their levels) were as follows: *A. brasiliensis* strain (ABC and WC838), olive oil (0% w/v and 1% w/v), and fermentation time (2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days) as shown in **Table 1**. For each treatment, the following variables were measured: biomass concentration (in mg/mL), total carbohydrates (in mg/mL), reducing sugars (in mg/mL), viscosity (in cP) and pH of the culture broth. The composition of the liquid medium was as follows (in g/L): industrial glucose, 30; KH_2PO_4, 1.0; $MgSO_47H_2O$, 0.5; $MnSO_4H_2O$, 0.3; $CaCl_2$, 0.1; yeast extract, 4.0 [3,16]. In dependence of the treatments, commercial extra virgin olive oil was employed. Initial pH of the media prepared was in the range 5.65 ± 0.07. Several 125 mL flasks with 30 mL of the culture media were employed which were sterilized in autoclave at 121°C during 15 min and inoculated with a 5 mm square piece of mycelium from plate with PDA in laminar chamber. Then, the flasks were stirred in an orbital shaker model MD-4000-CT (Actum, Colombia) at 100 rpm and 25°C for 20 days. Samples were taken every two days; the first sample (day 0) was taken in the day of inoculation.

Table 1: Effect of the initial pH of the culture medium and presence of olive oil on cell biomass and total carbohydrates in the culture broth during 20 day old cultivation in 125 mL Erlenmeyer flasks using a synthetic medium at 25°C, 100 rpm agitation for two *A. brasiliensis* strains. Data reported corresponded to the mean value of two replications and standard deviation.

Strain	ABC		WC838	
Time [days]	Olive oil [% w/v]			
	1	0	1	0
Biomass [mg/mL]				
0	2.1	2.1	1.4	1.4
2	1.8	1.7	2	1.2
4	1.3	0.6	0.9	0.6
6	1.8	0.8	0.7	0.7
8	2.3	1.2	0.5	0.7
10	2.6	1.3	0.6	0.8
12	4.7	1.7	1.3	0.5
14	6.2	0.4	0.8	0.4

e-ISSN: 2320-3528
p-ISSN: 2347-2286

16	4.1	1.4	1.6	0.7
18	6.4	1.3	1.9	1
20	2.9	0.9	2.6	0.5
Total carbohydrates [mg/mL]				
0	31.10 ± 2.99	30.84 ± 1.27	31.10 ± 3.00	30.84 ± 1.27
2	26.0 ± 0.08	22.80 ± 0.02	26.93 ± 0.00	32.80 ± 0.06
4	31.89 ± 0.08	22.63 ± 1.94	23.34 ± 0.26	30.31 ± 2.32
6	29.60 ± 1.57	23.83 ± 0.92	32.47 ± 1.08	27.43 ± 3.21
8	30.52 ± 3.63	31.19 ± 0.95	35.26 ± 2.18	35.75 ± 0.56
10	34.40 ± 0.03	34.59 ± 0.33	33.12 ± 2.46	33.79 ± 0.07
12	32.36 ± 0.80	29.97 ± 5.34	33.08 ± 1.48	34.38 ± 0.76
14	34.86 ± 0.30	35.54 ± 0.37	35.31 ± 2.02	30.45 ± 0.61
16	32.32 ± 1.31	33.40 ± 1.01	33.02 ± 0.56	33.98 ± 0.02
18	32.32 ± 2.37	33.40 ± 0.33	33.02 ± 0.69	33.98 ± 0.95
20	33.49 ± 0.69	35.20 ± 0.31	34.38 ± 1.08	33.04 ± 1.11

Extraction of Starch from Residual Potato

Starch extraction was performed from 300g waste (discarded) potato (*Solanum tuberosum*). Potatoes of a Colombian variety (brown Pasto potato) obtained from local market place were used. This variety is commonly employed as animal feed since it does not meet quality standards for human consumption, so it is considered as waste potato. The extraction procedure was based on several reports [37-39] as follows: a) Conditioning of waste potato using 1% sodium hypochlorite solution with successive washing with drinking water; b) A solid-liquid mix with 3:4 (w/v) ratio using distilled water was prepared; c) Homogenized potato mix was passed through a filtering cloth to start solid separation with subsequent addition of water to wash the residual starch; the solids were filtered on metal sieves of 150 μm and 106 μm, allowing the passage of the starch granules; d) The starch suspension was decanted for 20 min, then the precipitate was separated with three successive washes until the water became translucent; e) Starch was dried in a vertical oven with forced air model TH 240 (Disenos Electroynicos Especiales, Colombia) at 40°C until constant weight; f) Starch was stored in a hermetic vessel at 4°C until its use. The shape and size of starch granules obtained was studied by using a scanning electron microscope model QUANTA 250 (FEI, Spain) with voltage acceleration of 11 kilo electron volts (KV), in low vacuum mode, using pressure of 70-90 Pa and magnification between 500x-2000x.

Cultivation Conditions for Adaptation Stage

Agaricus brasiliensis strain is maintained on PDA solid medium before its use in fermentation process. For this reason, an intermediate stage should be carried out for the strain to adapt to the liquid medium components and conditions in Erlenmeyer flasks. Firstly, a synthetic liquid medium was inoculated with cell biomass plugged from the plates with PDA. Cultures were performed in 250 mL flasks containing 50 mL liquid medium at 100 rpm and 25°C during 14th day incubation [3]. In this way, the fungal biomass was adapted to the conditions of the liquid cultivation.

To determine the effect of the carbon source and initial pH of the liquid medium on production of mycelial biomass of *A. brasiliensis* strain selected, a completely randomized multifactorial experimental design with three factors were performed. The factors (and their levels) were as follows: carbon source (gelatinized starch from waste potato coded as GS (Gelatinized starch) and industrial glucoses coded as G), initial pH of culture medium (4.5, 5.5 and 6.5), and fermentation time (0, 10, 14, and 21 days) resulting in 24 treatments with three replications (**Table 2**). The fermentations at laboratory scale were performed in 1000-mL flasks with 300 mL culture medium at 100 rpm and 25°C during 21 day incubation. The culture media were inoculated with a 50 mL suspension of previously adapted biomass of the strain selected. The variables measured were as follows: cell biomass (in mg/mL), reducing sugars (RS, in mg/mL), polysaccharides in the culture medium (PS, in mg/mL), IPS from biomass (in mg/mg dry mycelium), viscosity (in cP) and pH of the culture broth.

e-ISSN: 2320-3528
p-ISSN: 2347-2286

The composition of the culture medium used for the adaptation stage of the strain selected was as follows (in g/L): industrial glucose, 30; KH_2PO_4, 1.0; $MgSO_47H_2O$, 0.5; $MnSO_4H_2O$, 0.3; $CaCl_2$, 0.1; yeast extract, 2 and extra virgin olive oil, 10. For cultivation at laboratory scale in 1000 mL flasks, the culture medium had the same composition as that of adaptation stage but varying the carbon source according to the experimental design (GS or G) with the same concentration (30 g/L). GS was prepared from 30 g waste potato starch in 1 L distilled water at 600 rpm and heating up to 80°C, then the gelatinized starch solution was homogenized for 30 sec [3,16].

Table 2: Effect of the initial pH of the culture medium and main carbon source during the adaptation stage of ABC strain of *A. brasiliensis* cultivated in 1000 mL Erlenmeyer flasks at 100 rpm and 25°C on concentration of cell biomass, reducing sugars (RS) and polysaccharides (PS). Culture media contained 1% (w/v) olive oil. G: industrial glucose, GS: gelatinized starch. Data reported corresponded to the mean value of two replications and standard deviation.

Main carbon	Initial pH	Time [Days]	Biomass [mg/mL]	RS [mg/mL]	PS [mg/mL]
Industrial glucose	4.5	0	0.24 ± 0.02	32.93 ± 1.98	0.09 ± 0.04
		10	5.79 ± 2.66	29.84 ± 2.13	0.14 ± 0.08
		14	3.96 ± 1.14	26.24 ± 3.10	0.16 ± 0.10
		21	4.60 ± 1.24	27.17 ± 5.13	0.24 ± 0.07
	5.5	0	0.48 ± 0.14	33.98 ± 2.64	0.11 ± 0.04
		10	4.16 ± 2.40	32.76 ± 0.78	0.12 ± 0.02
		14	4.14 ± 0.91	32.50 ± 2.30	0.12 ± 0.04
		21	4.02 ± 0.56	30.61 ± 4.73	0.20 ± 0.06
	6.5	0	0.34 ± 0.11	35.90 ± 1.63	0.05 ± 0.01
		10	2.33 ± 1.43	30.62 ± 5.17	0.08 ± 0.01
		14	4.67 ± 0.54	32.60 ± 1.22	0.13 ± 0.04
		21	4.79 ± 2.94	29.36 ± 3.13	0.13 ± 0.04
Gelatinized starch	4.5	0	0.87 ± 0.35	2.39 ± 0.27	23.43 ± 1.72
		10	8.85 ± 1.28	14.99 ± 2.33	11.88 ± 2.23
		14	8.27 ± 1.37	21.00 ± 2.89	8.21 ± 4.86
		21	7.96 ± 1.19	15.81 ± 5.27	6.94 ± 1.49
	5.5	0	1.40 ± 1.16	3.42 ± 0.27	24.24 ± 0.62
		10	8.37 ± 1.94	20.59 ± 1.75	8.19 ± 1.80
		14	8.16 ± 1.38	22.83 ± 7.27	2.70 ± 2.00
		21	7.13 ± 1.43	21.20 ± 6.35	2.82 ± 2.00
	6.5	0	0.25 ± 0.02	4.07 ± 0.72	23.81 ± 1.13
		10	6.04 ± 3.97	24.35 ± 0.19	5.36 ± 1.07
		14	8.86 ± 1.54	23.83 ± 6.54	4.11 ± 5.26
		21	7.80 ± 5.46	27.08 ± 5.84	0.37 ± 0.09

Cultivation Conditions at Bench-scale

The equipment used for bench-scale SmF was a 7 L stirred-tank bioreactor model ez (Applikon, Netherlands) with automatic control of pH (0,02 units of tolerance) through a system of peristaltic pumps for adding sterile solutions of 0.5 N NaOH or 0.2 N HCl as needed. Four preliminary assays were performed to determine the behavior of the selected strain in such bioreactor: initial pH of 4.5 using GS or G as carbon source and initial pH of 5.5 using GS or G as carbon source;

e-ISSN: 2320-3528
p-ISSN: 2347-2286

these tests were carried out without pH control during cultivation and with the same composition of the culture medium described in previous section. From these assays, an initial pH of 4.5 and GS as carbon source were selected for the experimental design using the bioreactor. For this, a completely randomized one-way experimental design was performed with the pH of the medium at two levels: controlled pH of 4.5 throughout cultivation and initial pH of 4.5 without pH control during cultivation using 4 L culture medium. The operating condition in the bioreactor were as follows: 250 rpm agitation speed, 1 vvm (volume of air under per volume of liquid per minute), 2 bar pressure of the incoming air from an oil-free compressor model Eco Drain 30 (Kaeser, Germany), 25°C, and 7 day cultivation. A total amount of six runs was performed: two replicates for each treatment plus two control runs using G as carbon source and an initial pH of 4.5 without control. 30 mL samples were taken every day (the sample taken at the day of inoculation corresponded to the zero time). The following variables were measured: cell biomass (in mg/mL), RS (in mg/mL), PS (in mg/mL), IPS from biomass (in mg/mg dry mycelium), and pH. In all fermentation runs, 350 mL inoculum adapted in the corresponding carbon source (GS or G) for 14 days was used.

Analytic and Instrumental Methods

The quantification of *A. brasiliensis* biomass was carried out by dry weight at 60°C until constant weight with previous washing. The mycelium was filtered using qualitative grade filters with 8-12 µm pores (Munktell Inc., Sweden). The dry biomass samples were stored at 4°C until their analysis.

RS determination was performed through modifications of the colorimetric technique [40-44]. On the other hand, the measurement of total carbohydrates was done by modifying the phenol-sulfuric acid method [45]; for this, the volume of the control sample with distilled water and samples to be analyzed were reduced down to 0.5 mL, with 50% reduction of the volume of the reagents with addition of 0.5 mL 5% phenol (Bioquigen, Colombia) and 2.50 mL 95%-97% sulfuric acid (Merck, Germany), and increasing the reaction time up to 1 h. For both measurements, anhydrous D-(+) glucose (Scharlau, Spain) was used for calibration curves, which working range of concentration between 0.01-0.30 mg/mL for RS and 0.02-0.20 mg/mL for total carbohydrates. Absorbance was read at 540 nm for RS and 490 nm for total carbohydrates in a spectrophotometer Genesys 10S (Thermo Scientifica, USA).

Viscosity measurement was performed in duplicate using 16 mL of culture broth agitated at 100 rpm with a low viscosity LCP screw in an Alpha viscometer (Fungilab, USA), with a percentage value of the spring deviation related to the scale greater than 15% and less than 100%. The pH of the media was determined in duplicate in a Lab 850 potentiometer (Schott, Hungary).

Polysaccharide and Intra-polysaccharide Measurement

PS measurement in culture media was modified from the method described in a previous work [3] based on the precipitation of PS insoluble in ethanol from liquid media. The modified procedure to quantify the PS consisted in taking a 0.5 mL aliquot of the culture media, which was mixed with 4 volumes absolute (99.9%) ethanol (Scharlau, Spain), then the mix was stored at -10°C and after 45 min the precipitate was separated by centrifugation at 6,000 rpm for 20 min. The insoluble components were resuspended in 0.5 mL 1 M NaOH at 60°C in a water bath during 1 h. After PS solubilization, 10-100 µL were taken and diluted in distilled water and the measurement of total carbohydrates was performed.

The determination of IPS from fungal biomass was also modified from the method described in a previous work [3] based on the solid-liquid extraction of the polysaccharides contained in the mycelium powder or fruiting bodies that are insoluble in ethanol with the help of ultrasonic energy at low temperature [3,46]. The modified procedure consisted in mashing the biomass previously dried (moisture content less than 5%), weighting 0.01 g-0.2 g, and mixing with absolute ethanol at 1:50 ratio; the samples underwent extraction in an ultrasound bath (Elma-Hans Schmidbauer GmbH & Co, Germany) during 30 min at 10°C and then the mix was left in contact for 20 h at 4°C ± 1°C. The extract obtained underwent extraction again with ultrasonic treatment during 30 min at 10°C, the samples were agitated, and the spent mycelium was allowed to precipitate during a few seconds, then the supernatant containing PS was removed by using a micropipette (Brand, Germany). Finally, 1 mL absolute ethanol was added to wash the spent solid and extract the remaining polysaccharides under the same conditions described above. The two supernatants were deposited in a centrifuge tube and the PS extracted were separated by centrifugation at 6,000 rpm for 20 min in a centrifuge model 1040-1 (Eba 21, Germany). The precipitate obtained was suspended in a 1M NaOH solution at 60°C during 1 h and dilutions were prepared as needed for determination of total carbohydrates.

Statistical Analysis

The analysis of data obtained from the experimental designs was performed by applying an analysis of variance (ANOVA) at a 6% confidence level in order to determine the statistically significant differences for each group of results of

e-ISSN: 2320-3528
p-ISSN: 2347-2286

the following variables: biomass, RS, total carbohydrates, PS, IPS from biomass, viscosity, and pH. For these analyses, the statistical package XLSTAT v. 2014 (Addinsoft, France) was employed.

RESULTS

Strain Selection and Use of Olive Oil

The production of biomass from WC838 and ABC strains of *A. brasiliensis* by SmF was studied in presence or absence of 1% (w/v) olive oil during 20 day incubation. The concentration of mycelial biomass was higher for ABC strain (6.4 mg/mL) when olive oil was added to the culture medium than for WC838 strain (2.6 mg/ml) for 18 and 20 days of incubation in 125 mL flask cultures, respectively (Table 1). The ANOVA of the experimental design showed that there exist statistically significant differences among treatments ($p<0.05$) considering the strain and presence/absence of olive oil in the medium. The RS concentrations also exhibited statistically significant differences with the time factor ($p<0.05$) for the four treatments during 20 days of incubation. For ABC strain, RS varied their concentration between 31.98 ± 1.26 mg/mL (day 2) and 39.40 ± 0.31 mg/mL (day 14) with olive oil in the medium, and between 24.02 ± 0.24 mg/mL (day 2) and 39.76 ± 0.55 mg/mL (day 14) without olive oil. In the case of the WC838 strain, RS varied between 26.99 ± 1.23 mg/mL (day 4) and 40.51 ± 0.10 mg/mL (day 14) with olive oil and between 33.59 ± 0.55 mg/mL (day 14) and 39.71 ± 1.70 mg/mL (day 8) without olive oil. For total carbohydrates, considered as the summation of RS and EPS potentially accumulated in the culture medium, statistically significant differences were obtained ($p<0.05$) with the time during 20 days of cultivation.

The initial values of viscosity of the culture medium with and without olive oil were 2.00 ± 0.03 cP and 1.94 ± 0.01 cP, respectively. During 20 days of cultivation, minimum changes in viscosity for all the treatments were obtained (between 2.02 ± 0.00 and 1.80 ± 0.01) with a mean value of 1.88 ± 0.07 at the end of fermentations. The ANOVA performed for the pH showed that there were statistically significant differences among the treatments for fermentation time and interaction between strain and time ($p<0.05$); these same results were also obtained for the viscosity. The pH reached maximum values of 6.18 ± 0.70 at 18th day for ABC strain with olive oil and 6.17 ± 0.00 for WC838 strain in presence of olive oil. In these assays, no pH decreases were observed for any treatment throughout the cultivation.

Taking into account the outcomes obtained, ABC strain of *A. brasiliensis* was selected and the addition of 1% (w/v) olive oil to the culture media was chosen for subsequent fermentation runs.

Extraction of Potato Starch

For extracting starch from the waste potatoes, an experimental procedure was applied with six replications. The mean mass of starch extracted from 300 g waste potato was 20.54 ± 0.96 g with 9.90 ± 0.77% moisture. The shape and size of the starch granules extracted are shown in the two micrographs obtained with 500x (Figure 1a) and 2000x magnifications. The size of the granules was in the range between 5.74 µm and 81.56 µm.

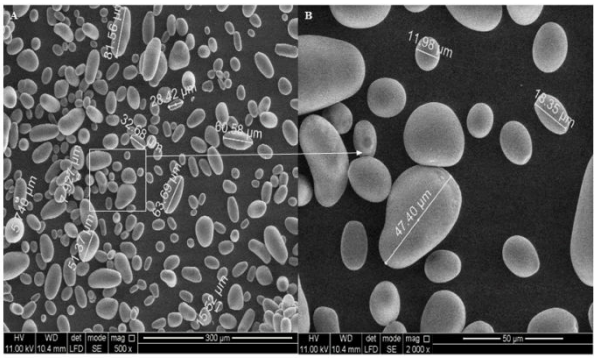

Figure 1: Scanning electron microscopy micrographs of the starch granules obtained from waste potato of Colombian brown Pasto variety by using the extraction procedure developed in this work. Bars: 300 µm A and 50 µm B.

Adaptation Stage of *A. brasiliensis* Mushroom

The initial adaptation of an organism in a synthetic medium containing glucose and the subsequent adaptation to the culture medium until its exponential phase at laboratory scale reduce the lag-phase length during the fermentation process in bench-scale bioreactors [47]. Therefore, in this work, an adaptation stage was proposed and performed for the ABC strain of *A. brasiliensis* during the SmF in flasks in order to define the best conditions for inoculum adaptation and bench-sale fermentation stages. Through the experimental design performed, the influence of the carbon source (G or GS) and initial pH of the medium culture on several variables was established. The results obtained from such experimental design showed that higher biomass concentrations may be reached at 10th day of fermentation for most

e-ISSN: 2320-3528
p-ISSN: 2347-2286

treatments except for those ones with initial pH of 6.5 (**Table 2**). The utilization of GS as main carbon source led to higher values of biomass concentration than the use of glucose did. Under the conditions studied, the culture medium based on SG with initial pH of 4.5 achieved the highest biomass concentration at 10th day of fermentation (**Figure 2a**) along with the medium with initial pH of 6.5 at 14th day; nevertheless, the PS concentration for the latter case was significantly lower. The data of RS concentration in the culture medium presented fluctuations during the 21 days of cultivation for all treatments. The ANOVA performed indicated that there were statistically significant differences ($p>0.05$) in biomass and RS among the treatments for the main carbon source, fermentation time, and interaction between these two factors.

For fermentations using GS, the determination of PS included not only the concentration of potato starch, but also EPS produced by the mushroom and the oligosaccharides formed in the medium from the hydrolysis of polysaccharides through the enzymes released by the fungal cells. In the case of fermentations using glucose, the PS concentration probably included the EPS synthesized by the mushroom and its enzymatic hydrolysis products. PS concentration for treatments with GS showed a higher decrease at 10th day of the process (**Table 2**). In contrast, for glucose-based media, an increase in PS concentration was observed until the end of cultivation except for the medium with initial pH of 6.5 at 14th day. The ANOVA for PS showed that there were statistically significant differences for factors carbon source and fermentation time as well as for their interaction ($p>0.05$).

IPS from biomass achieved a mean value of 0.05 ± 0.01 mg/mg dry mycelium for all the media prepared with glucose and for the medium containing GS with initial pH of 5.5. The GS-based media with initial pH of 5.4 and 6.5 had a mean value of 0.09 ± 0.02 and 0.06 ± 0.03 respectively. The treatment with the highest IPS concentration corresponded to GS as the main carbon source and an initial pH of 4.5. The ANOVA indicated that there were statistically significant differences among the treatment for IPS concentration for the three factors studied with p-value less than 0.05.

The pH of the culture broth decreased from day 0 to day 10 of culture, independently of the initial pH of the medium, and most of the cultures reduced their pH until 14th day (**Figures 2b** and **2c**). The glucose-based media showed a convergence at an average pH value of 4.88 ± 0.05 at the 14th day, while the GS-based media converged to an average pH value of 4.40 ± 0.08 for the same day of cultivation. The ANOVA confirmed that there are statistically significant differences in the pH of the medium when the main carbon source was changed ($p>0.05$). Finally, the viscosity of the glucose-based media exhibited minimum changes with an average value of 1.53 ± 0.04 cP. However, for GS-based media at the beginning of fermentations, viscosity reached significantly higher values of 13.63 ± 2.75, 19.83 ± 4.52 and 23.13 ± 3.43 corresponding to the initial pH of the culture media of 4.5, 5.5 and 6.5 respectively. From the 10th day until the end of cultivations using GS, the average viscosity was 1.97 ± 0.21 cP. The corresponding ANOVA for viscosity showed that there are statistically significant differences considering the carbon source and the fermentation time ($p>0.05$).

e-ISSN: 2320-3528
p-ISSN: 2347-2286

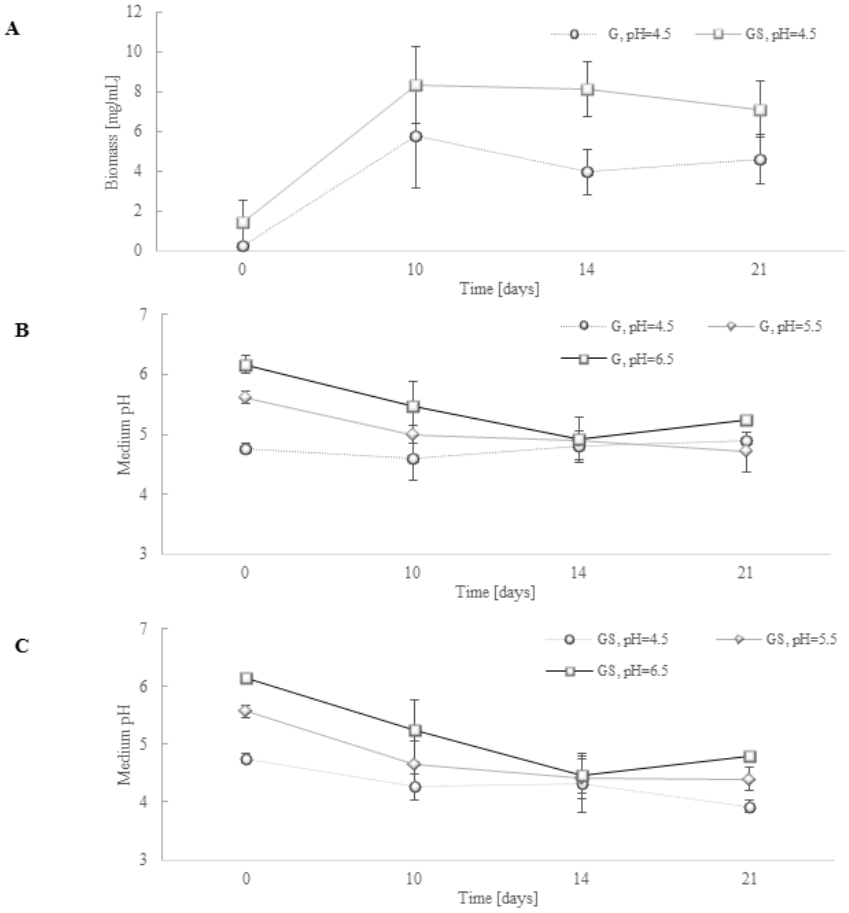

Figure 2: Time profile and effect of initial pH of the culture medium containing industrial glucose (G) or gelatinized starch from waste potato (GS) on biomass concentration of ABC strain of *Agaricus brasiliensis* (**A**)and pH of culture broth during cultivation (**B and C**). The pH indicated in the legend of each graph corresponded to the initial pH value of the culture medium when it was adjusted prior to its sterilization and inoculum addition. The fermentations were carried out in 1000 mL Erlenmeyer flasks at 100 rpm and 25°C using an orbital shaker for 21 days. The data shown are mean from three replications.

Cultivation of *A. brasiliensis* at Bench-scale

Four fermentation assays were carried out for ABC strain in the 7 L stirred-tank bioreactor using as inoculum a previously adapted culture of this strain. As result of these assays, the kinetic profile of cultivations using two main carbon sources (G or GS) and two initial pH of the culture media (4.5 and 5.5) were obtained (**Figure 3**). As can be observed, the length of the lag phase was of 1 day for all the assays, but the kinetic curves had different behavior after the 3rd day of cultivation (**Figure 3a**). The highest biomass concentration (9.05 mg/mL) was reached in the case of the medium based on GS with an initial pH of 4.5; for this case, the stationary phase was clearly observed from 3rd day until the end of fermentation. The curves for glucose-based media reached lower biomass concentrations and did not exhibit a clear stationary phase for both values of initial pH. The concentration of IPS from biomass varied in dependence of the type of carbon source and initial pH. Thus, IPS concentrations obtained were as follows (in mg/mg dry mycelium): G (initial pH 5.5) 0.02 ± 0.02, GS (initial pH 5.5) 0.06 ± 0.03, G (initial pH 4.5) 0.17 ± 0.04, and GS (initial pH 4.5) 0.21 ± 0.06; these values were averaged from the data taken daily throughout the cultivation process. The highest IPS concentration was obtained from the biomass cultivated in the medium based on GS with initial pH of 4.5 as well.

The behavior of the pH of the culture broth for the fermentation runs is shown in **Figure 3b**. pH data were averaged for each process hour from 6 readings taken every 10 min. For glucose-based media, the pH data in the broth converged at a mean value of 4.80 ± 0.14 at the end of fermentation. From **Figure 3b**, it is evident that the pH of the broth for the medium with initial pH of 4.5 fluctuated around 4.8, while the medium pH reduced to 4.8 in the case of the medium with initial pH of 5.5. In contrast, GS-based medium with initial pH of 4.5 showed small variation in the pH during cultivation (final pH value of 4.3). Although the medium containing GS with initial pH of 5.5 had small variations as well, the pH value attained at the end of the process was the same than at the start. Most variations in medium pH corresponded to the phase of vigorous biomass growth (between 24 and 100 h). The data of RS shown in **Table 3** presented oscillations during the 7 days of fermentation employing the glucose-based media. In addition, PS concentrations showed an

e-ISSN: 2320-3528
p-ISSN: 2347-2286

increasing trend for the media with initial pH of 5.5 and 4.5. On the contrary, for GS-based media, a decrease in PS concentration was observed.

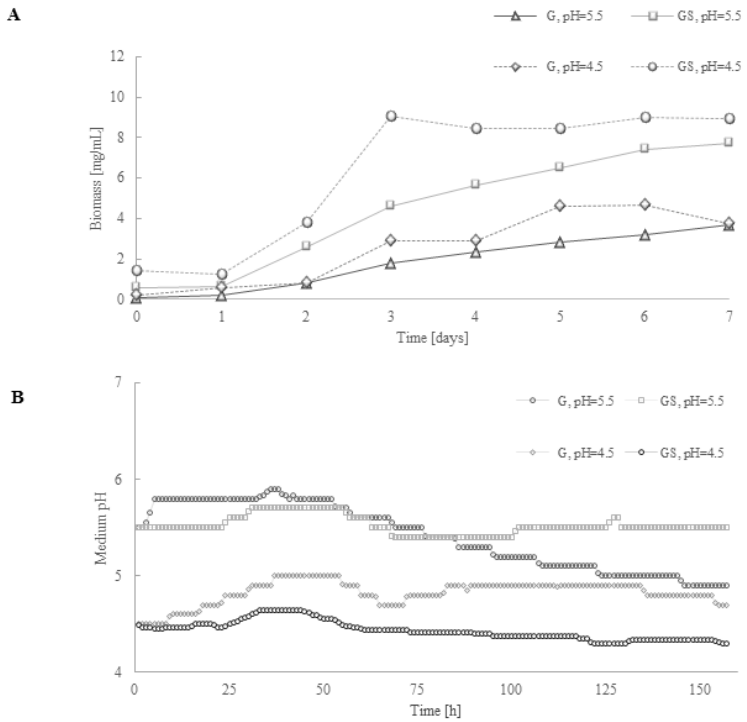

Figure 3: Experimental kinetics of cell biomass A and time profile of pH in the culture broth B during aerobic submerged cultivation of ABC strain of *Agaricus brasiliensis* in a 7 L stirred-tank bioreactor using a culture medium based on industrial glucose (G) or gelatinized starch from waste potato (GS) with two values of initial pH. The fermentation was performed at 25°C, 250 rpm agitation rate, 1 vvm aeration during 7 days.

Table 3: Concentrations of reducing sugars (RS) and polysaccharides (PS) during aerobic submerged cultivation of ABC strain of *Agaricus brasiliensis* in a 7 L stirred-tank bioreactor using a culture medium based on industrial glucose (G) or gelatinized starch from waste potato (GS) with two values of initial pH. The fermentation was performed at 25°C, 250 rpm agitation rate, 1 vvm aeration during 7 days. Data reported corresponded to the mean value of two replications and standard deviation.

Initial pH	Main carbon source	Time [days]							
		0	1	2	3	4	5	6	7
RS [mg/mL]									
4.5	G	35.07 ± 0.05	31.09 ± 1.88	30.76 ± 1.16	32.34 ± 1.70	33.09 ± 0.69	33.10 ± 0.15	33.16 ± 1.41	30.11 ± 1.41
	AG	1.16 ± 0.02	1.36 ± 0.04	0.79 ± 0.03	2.72 ± 0.04	2.73 ± 0.04	3.34 ± 0.05	5.17 ± 0.21	9.34 ± 0.08
5.5	G	32.99 ± 2.52	32.13 ± 4.92	32.82 ± 0.62	34.46 ± 1.66	36.60 ± 1.50	36.65 ± 1.41	37.00 ± 1.41	37.62 ± 0.96
	AG	7.94 ± 0.06	5.16 ± 0.51	7.65 ± 0.74	17.54 ± 0.96	24.53 ± 1.42	19.53 ± 2.95	20.93 ± 3.08	23.11 ± 0.10
PS [mg/mL]									
4.5	G	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.10 ± 0.01	0.14 ± 0.02	0.16 ± 0.01	0.20 ± 0.02	0.28 ± 0.03
	AG	36.00 ± 8.74	27.02 ± 4.85	28.30 ± 1.08	26.10 ± 1.67	25.75 ± 2.12	21.83 ± 0.08	20.94 ± 0.52	17.39 ± 1.13

e-ISSN: 2320-3528
p-ISSN: 2347-2286

5.5	G	0.16 ± 0.11	0.12 ± 0.03	0.06 ± 0.01	0.10 ± 0.00	0.10 ± 0.01	0.11 ± 0.01	0.15 ± 0.01	0.27 ± 0.16
	AG	31.50 ± 0.33	31.01 ± 0.37	27.46 ± 0.55	19.18 ± 0.09	17.45 ± 7.63	16.75 ± 7.28	16.21 ± 6.76	15.28 ± 5.57

The kinetics of production of mycelial biomass of *A. brasiliensis* at bench-scale is depicted in **Figure 4**. One-way experimental design was performed to assess whether stabilizing pH of the medium during fermentation may have an effect on the production of *A. brasiliensis* biomass and polysaccharides using starchy residues as the main carbon source. The results from this design are schematized in **Figure 4**, which includes reference fermentation in glucose-based medium without pH control. The lag phase took place during the first day of cultivation in all the cases. The decay phase, in turn, can be observed during the 7th day of cultivation. From **Figure 4b**, it is evident that controlling pH during fermentation led to lower biomass and polysaccharide concentrations than for the case when no pH control is performed. In fact, cultivation with GS-based medium without pH control reached the highest biomass concentration at bench-scale (7.26 ± 2.43 mg/mL), even above the conventional fermentation using glucose (4.85 ± 0.28 mg/mL). The average value of the concentrations of IPS from biomass for GS-based medium with pH control was 0.18 ± 0.06 mg/mg dry mycelium, very close to the case of glucose-based medium (0.17 ± 0.03 mg/mg dry mycelium), while the value obtained for the medium containing GS without pH control was higher (0.23 ± 0.03 mg/mg dry mycelium). Thus, enhanced production of *A. brasiliensis* biomass and IPS was obtained from a culture medium based on gelatinized starch from waste potato without pH control in a bench-scale stirred-tank bioreactor.

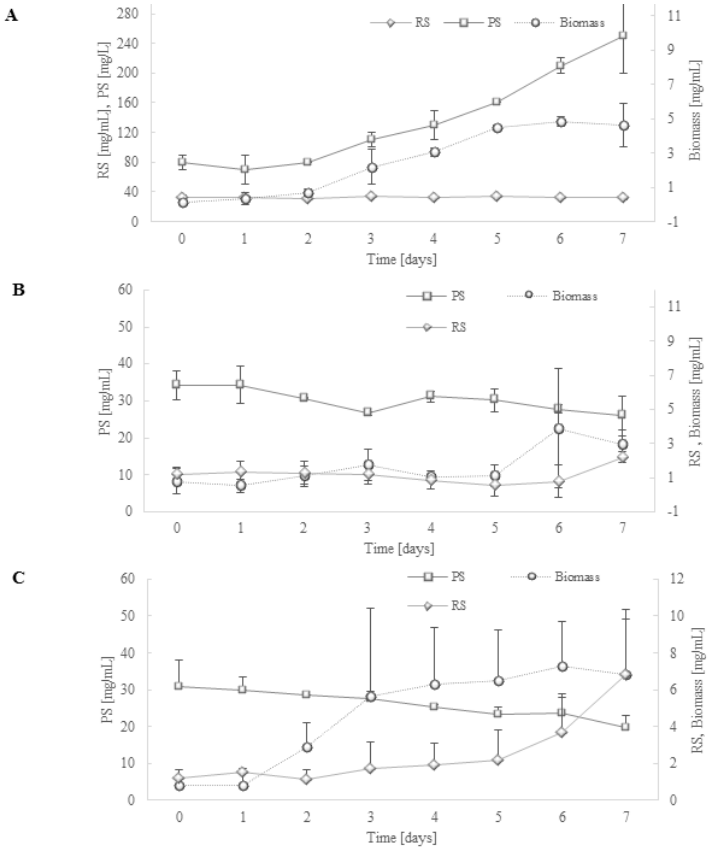

Figure 4: Experimental fermentation kinetics for growth of *Agaricus brasiliensis* by aerobic submerged fermentation in a 7L stirred-tank bioreactor using a culture medium with initial pH of 4.5. A Medium based on industrial glucose, cultivation without pH control. B Medium based on gelatinized starch from waste potato, cultivation with pH control. C Medium based on gelatinized starch from waste potato, cultivation without pH control. The fermentation was performed at 25°C, 250 rpm agitation rate, 1 vvm aeration during 7 days. The data shown are mean from three replications. PS: polysaccharides, RS: reducing sugars.

The changes of pH throughout the cultivations without pH control performed in the bench-scale bioreactor had time-profiles very similar to those described in **Figure 3b** and are not shown because of space constraints. The final pH of the culture broth at the end of fermentation was 4.25 for the process with glucose and 4.62 for the process with residual starch, both without pH control. On the other hand, the behavior of the RS indicates that the concentration of these sugars for the media based on GS remains low during the fermentation though a slight increase at the end of the process can be noticed. The data of PS concentration in the broth showed an increasing trend for the medium based on glucose

e-ISSN: 2320-3528
p-ISSN: 2347-2286

until the end of fermentation. On the contrary, for GS-based media, PS concentration decreased until the end of cultivation reaching 26.01 ± 5.44 for the case with pH control and 19.73 ± 3.32 when pH was not controlled.

The ANOVA of the data obtained from the one-way experimental design at a 95% confidence level showed that there were statistically significant differences ($p>0.05$) in all the variables assessed (biomass, RS, PS, and IPS from biomass) considering the factor evaluated at its two levels (with or without pH control). In addition, if considering the fermentation time, statistically significant differences were found for PS; such differences were also found for concentration of biomass and RS when the factor evaluated was considered along with the cultivation time.

DISCUSSION

The results obtained showed that the best treatment for biomass production of *A. brasiliensis* in Erlenmeyer flasks containing 30 mL medium corresponded to the ABC strain with addition of olive oil. Under these conditions, concentration of *A. brasiliensis* biomass reached 6.2 mg/mL. These results agreed with the data of Gern JC [16] who studied the effect of adding vegetable oils (canola, sunflower, corn, soy, olive and rice) to the culture broth for biomass production of *A. brasiliensis* LPB 03 in 125 mL flasks using a basal medium containing 20 g/L glucose. This author concluded that biomass growth improves when 2% rice oil is added obtaining 8.56 g/L cell biomass compared to the case without addition of any oil (4.68 g/L). The addition of olive or cotton oil at 1% concentration showed similar biomass values. The usage of vegetable oils with different fatty acid composition increases the production of cell biomass by SmF acting, in addition, as antifoaming agents in these processes [16]. It has been suggested that the stimulation mechanism of the biomass by the vegetable oils is due mainly to the fact that the lipids may be partially incorporated in the cell membrane and facilitate the immediate absorption of nutrients from the liquid medium. Moreover, the lipids may have a direct effect on the level of synthesis of the enzymes involved in the polysaccharide production, which are important components of the cell wall [6,16]. For this reason, in this work, the composition of the liquid medium proposed in a previous work [3] was modified in order to study the influence of olive oil on the production of *A. brasiliensis* biomass. On the other hand, the content of minerals in the liquid medium (sulfur, phosphorus, potassium, calcium, magnesium, and manganese) is crucial because of their participation in the metabolic processes leading to the biomass growth and polysaccharide production, especially as enzymatic cofactors and for the biosynthesis of amino acids, nucleic acids, ATP, and secondary metabolites [5]. These mineral components were complemented with the carbon (G or GS) and nitrogen (yeast extract) sources.

In this research, potato starch obtained from starchy waste was employed as a carbon source alternative to the industrial glucose, the most used carbon source in fermentation processes. The procedure applied to extract the starch from waste potato of the Colombian brown Pasto variety was suitable to obtain the amount of starch needed for this research. Nevertheless, although the maximum starch amount for this variety is 16.41% [36], the actual amount of dry starch extracted was 18.49 g and the losses attained 26.77 g (calculated in wet basis) from 300 g waste potato. These losses were mostly due to the starch lost during washing and drying, to the starch retained in the residual mass and, possibly, to the decrease of starch content during post-harvesting as pointed out in [36]. The size of the starch granules determined from scanning electronic microscope micrographs (**Figure 1**) oscillated between 5.74 µm and 81.56 µm, which coincided with the size determined in other works [48, 49] (between 1 and 100 µm). The smallest granules are round and the greatest have elliptic shape [48].

Several assays were performed to determine the suitability of using raw or gelatinized starch in cultivations of the ABC *A. brasiliensis* strain. As expected, it was found that the strain did not grow in the medium with raw starch but exhibited a good growth in the medium with gelatinized starch. During the gelatinization process, the swollen starch granules are partially broken by the heating making the amylose and amylopectin to be dispersed in the medium. The amylolytic enzymes released into the medium by the fungus may take longer to access the amylose and amylopectin polymers contained in the granules of the insoluble raw starch causing the death of the organism in a short time. On the contrary, the enzymes from the mushroom could have greater efficiency to hydrolyze the solubilized amylose and amylopectin of the gelatinized starch as in the case of the enzymatic hydrolysis of starch with exogenous bacterial amylases [50]. In addition, the first adaptation stage of the ABC strain was performed in order to transfer the mycelium into a liquid medium based on glucose similar to that of the PDA agar where the mushroom was extended and maintained previously. During the second adaptation stage, the ABC culture was scaled-up to a 300 mL volume enabling that the mycelium be adapted to the new carbon source, the gelatinized starch. This allowed the reduction of the lag phase down to 1 day in the bioreactor as observed in **Figures 3** and **4**.

The adaptation stage has a paramount importance at the moment of designing the fermentation process at bench and pilot scales especially when alternative carbon sources are employed. The results obtained during the second adaptation stage showed that the best treatment corresponded to medium based on gelatinized starch with initial pH of 4.5 at 10th day of cultivation. However, the length of adaptation to the liquid medium for the inoculum was 14 days to increase the possibility that the pH reach values close to 4.5 in a natural way during the fermentations in the bioreactor. During the

e-ISSN: 2320-3528
p-ISSN: 2347-2286

strain adaptation at 14th day, an average pH value of 4.4 ± 0.08 for GS-based media was reached compared to glucose-based media for which a value of 4.88 ± 0.05 was obtained (**Figures 2b** and **2c**). Under the adaptation conditions studied in flasks, ABC strain produced 30.8% more biomass with GS than with glucose for the same initial pH of 4.5. Furthermore, 80% increase in IPS from biomass was attained for these same conditions related to the average value obtained with glucose.

The first fermentation runs performed in the bench-scale stirred-tank bioreactor showed that the initial pH of the process has influence on the biomass production for both carbon sources used as indicated in other works [1,4,10,12]; this is visualized considering the shape of the curve for biomass concentration with the time (**Figure 3a**). In addition, the results of the one-way experimental design for bench-scale fermentation with an initial pH of 4.5 indicated that the pH control negatively affects biomass production when GS was used in the liquid medium; thus, 46.1% reduction compared to the case when pH was not controlled throughout fermentation can be observed. In this latter case (**Figure 4c**), a maximum biomass concentration can be reached at 6 days of cultivation. Controlling pH makes the cell biomass concentrations in the culture broth be 19.4% lower than for the control treatment (glucose-based medium without pH control, **Figure 4a**). This can be explained by the fact that the mushroom releases a group of enzymes allowing the substrate degradation to obtain its carbon source and each one of these enzymes requires a pH specific to reach their maximum efficiency in transforming the substrate. This could be limited by controlling the pH at a constant value of 4.5 throughout the fermentation and leaving the pH to smoothly oscillate appears to be the best option to harvest the maximum possible amount of cell biomass as depicted in **Figure 3b**.

The outcomes obtained in this work suggest that the concentration of IPS from biomass changes in dependence on the carbon source, initial pH of the medium and pH control during cultivation. In addition, the IPS increases as the cell biomass grows with fermentation time. These changes in IPS could be explained considering that the fungal cell wall is metabolically active and that the intracellular polysaccharides can be considered primary metabolites directly associated to the cell growth. In fact, the cell wall is a multilayered complex composed of polysaccharides, glycoproteins, and proteins with the chitin as the major component; the polysaccharidic fraction of the cell wall includes glucans and mannans in addition to some complex polysaccharides as the glucogalactomannans [6]. More research is needed to assess not only the effect of the medium components and the cultivation conditions (mostly pH) on the IPS concentration, but also on the changes in IPS structures induced by these factors.

The changes in the concentration of RS and total carbohydrates in the culture broth for the medium containing glucose could be clarified considering that the mushroom initially consumes this monosaccharide and, at the same time, releases EPS to the culture medium as a mechanism of adhesion between cells among other functions [47,51]. In addition, the EPS immobilize the enzymes required to degrade different polysaccharides contained in the medium including the EPS themselves as needed. This EPS release occurs even in media based on monosaccharides like the glucose as indicated in different works [3,12,15,35]. The segregation of EPS into the medium is continuously carried out for mycelial growth and could explain the PS increase in the broth when a glucose-based medium is employed (see **Figure 4a**). Thus, when the glucose is depleted in the culture media, the Macromycetes release glucanases, which could hydrolyze the EPS in a non-selective way to obtain their carbon source and provoking the subsequent decrease in the concentration of PS [13]. This phenomenon occurs in the GS-based media in the same way. In this case, the mushroom could initially obtain its carbon (and energy) source from the residual glucose and PS contained in the adapted inoculum added to the medium when fermentation began but, at the same time, the mushroom should hydrolyze the gelatinized starch of the liquid medium used. This polysaccharide is broken down to maltodextrins, other oligosaccharides, disaccharides, and glucose by the amylases released from white-rot fungi as part of their enzymatic pool [5]. These amylases increase the concentration of fermentable sugars, which are immediately consumed as carbon sources for the synthesis of other cell metabolites [8]. This leads to a balance between the rates of formation and consumption of sugars that is evidenced by the behavior of RS curve. The starch hydrolysis by ABC and WC838 *A. brasiliensis* strains was proven in a previous work using solid media containing agar-agar and starch through their hydrolysis halos. In addition, the fermentation data obtained in this work showed that RS increased as PS concentration decreased in GS-based media (**Figure 4** and **Tables 2** and **3**).

In this work, several cultivations were performed using glucose-based media. If considering the EPS as the main fermentation product, the cultivation in Erlenmeyer flasks with 300 mL medium using 14%-inoculum with initial pH of 4.5 reached 240 mg/L EPS in 21 days while fermentation in the bench-scale bioreactor with 4 L medium using 8%-inoculum with the same initial pH reached 250 mg/mL without pH control. These results are similar to those obtained by Gern [16] who added 2% olive oil to the culture medium for *A. brasiliensis* LPB 03 during 5 days incubation. Thus, the highest EPS concentration attained in this research corresponded to 61.1% of EPS produced in that research. Gern also demonstrated that oleic acid may increase cell biomass of this fungus and palmitic acid could increase EPS production. However, linoleic acid suppresses the mycelial growth and EPS production. Olive oil contains a high percentage of palmitic acid (12.99%) and oleic acid is its main component (70%) and has a lower content of linoleic acid (11.14%), which can explain its stimulating effect on EPS production.

e-ISSN: 2320-3528
p-ISSN: 2347-2286

The study on the initial pH of the medium was carried out in range between 4.5 and 6.5 based on the results obtained in other works [10,16]. Most fungi grow better in a slightly acid medium (from 4.0 to 8.0) although large differences may be found among different species and strains [10,16,52]. pH exerts high influence on the enzymatic activity, dissociation of molecules into ions and permeability of membranes. The latter factor is very important for the medium components required by the fungal metabolism to pass through these membranes. The pH values defined for the culture media used in this work could have been the most appropriate for the fungal enzymes released to degrade the substrates and obtain the energy source needed for mushroom growth and development. It should be highlighted that the culture media exhibited a pH of 5.54 for glucose and 5.62 for gelatinized starch before their adjustment for the different treatments of the experimental designs performed. According to the ANOVA performed, the adjustment of the initial pH of the media presented interaction with the fermentation time for biomass production under the conditions studied. For instance, large pH changes during the fermentation at laboratory scale led to higher biomass concentrations.

Results obtained at bench-scale fermentations in the bioreactor without pH control (**Figure 4**) showed that higher pH values were attained at the beginning of the exponential growth phase between 31 h and 44 h of fermentation regardless the initial pH of the medium. Afterwards, the pH decreases until the end of cultivation. These pH changes could be associated to the production of different substances during the degradation of the carbon source and to diverse fungal metabolites. The slight pH increase could be due to the ammonia production related to the catabolic pathways of proteins, amino acids and other nitrogenous compounds from the yeast extract contained in the medium. This generates a pH augment when the ammonia accepts a proton to form ammonium in aqueous solutions with a concentration non-toxic for the strain. Moreover, cell lysis processes could release metabolites increasing pH [53-55]. Subsequent pH decrease in the broth could correspond to the production of organic acids like the oxalic acid characteristic of *Agaricus* spp. and acetic acid released during glucose consumption. On the other hand, pH control in the medium based on GS presented lower biomass production compared to the case without controlling pH. This outcome is similar to those reported by [10], which obtained a lag in the stationary phase using glucose as the carbon source with a controlled pH of 4.0; in that work, a pH controlled between 4.0 and 7.0 enabled an EPS increase with best results at a controlled pH of 7.0.

The viscosity in the culture broth presented small changes during the cultivation process at laboratory scale. For this reason, viscosity was not considered as a response variable during the fermentations in the bioreactor. The relatively low molecular weight (MW) of the EPS from *A. brasiliensis* compared to the EPS produced by other fungi may explain this fact. The exopolysaccharides obtained from this mushroom as low-MW EPS (<100 kDa), medium-MW EPS (100-1000 kDa) and high MW EPS (>1000 kDa) [10]. A 750-kDa EPS was found as well [35]. Therefore, the viscosity caused by these EPS did not provoked a non-newtonian behavior of the culture broth as does the media where other Macromycetes like *Pleurotus eryngii* or *Schizophyllum commune* are cultivated [56, 57].

Regarding the environmental conditions for SmF of Macromycetes, temperatures in the range between 26°C and 36°C are the most suitable considering that an increase in fermentation temperature could enhance the fungal metabolism but decrease the oxygen solubility in the medium leading to a deceleration in the cell growth [4,5,11]. Aeration also has an important influence on the dissolved oxygen in the broth and should be controlled through a constant rate of 1 vvm inside the bioreactor. In the same way, agitation speed plays a crucial role as well taking into account not only the mycelium integrity, but also the concentration of dissolved oxygen. If the agitation speed is too high, the mycelium may be damaged and, therefore, pellet formation is decreased and production of cell biomass and target metabolites like EPS may be negatively affected. In fact, the pellets can be broken up by the interaction between these cell agglomerates and the swirls, impact between them and the internal devices like impellers and baffles, and collision between them [4,5,58]. For these reasons, agitation speeds of 100 rpm at laboratory scale and 250 rpm in the bioreactor were chosen to ensure the full movement of the pellets present in the medium throughout the fermentation and considering that the viscosity did not exhibit a non-newtonian behavior. Under these conditions, the mycelial integrity was guaranteed as well as the dissolved oxygen required for the mushroom survival.

Further research on the combination of factors like the agitation speed and aeration rate in dependence of the medium pH is needed to increase EPS production from *A. brasiliensis*. As demonstrated in this work, pH is a critical factor for biomass accumulation and synthesis of metabolites like EPS and IPS. As an example of such type of research, a higher EPS production and enhanced specific formation rate of intracellular polysaccharides were obtained by gradually decreasing the initial pH of the medium from 6.5 down to 3.5 during SmF of *Ganoderma lucidum* [16]. Nevertheless, operation conditions and formulation of the culture medium should be specifically defined according to the target product (EPS, mycelial biomass, IPS, terpenoids, glycoproteins, etc.) at different scales in order to implement commercial processes.

The results obtained in this work indicate that the mycelial biomass and EPS produced during the submerged fermentation using *A. brasiliensis* are affected by factors like pH, carbon/energy source of the culture medium, and addition of olive oil along with appropriate values of the environmental conditions (temperature, agitation speed and aeration rate). Best fermentation conditions at bench-scale (4 L working volume) stirred-tank bioreactor were as follows:

e-ISSN: 2320-3528
p-ISSN: 2347-2286

10 g/L olive oil in a medium using gelatinized starch from waste potato as the carbon source with initial pH of 4.5 without pH control at 25°C, 250 rpm and 1 vvm of aeration rate during 6 days of cultivation. These conditions enabled an average 33.2% increase in biomass production and 40.4% increase in IPS from biomass related to the control fermentation using industrial glucose. The outcomes found in this work demonstrated that gelatinized starch extracted from waste (discarded) potato is a suitable low-cost carbon source for this kind of processes and could be the base to design a submerged fermentation technology for cultivation of *A. brasiliensis* at industrial scale.

ACKNOWLEDGEMENT

The authors thank the Bioprocess and Agroindustry Plant at Universidad de Caldas (Manizales, Colombia) and Fund of Science, Technology and Innovation of the Royalties General System of Colombia for financial and physical support to carry out this work through the research project implementation of a comprehensive strategy through biotechnological innovation for waste utilization in Caldas province (grants 00HD221 and 0700413). The authors also acknowledge the Fujian Agriculture and Forestry University (Fuzhou, China) for providing the ABC strain of *A. brasiliensis* used in this study.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

REFERENCES

1. Camelini CM. Agaricus subrufescens biomass production by solid and submerged fermentation processes to obtain bioactive polysaccharides. Federal University of Santa Catarina. 2010.
2. Petre M, et al. Biotechnology of mushroom pellets producing by controlled submerged fermentation. Rom Biotechnol Lett 2010;15:50-55.
3. Montoya S, et al. Polysaccharide production by submerged and solid-state cultures from several medicinal higher Basidiomycetes. Int J Med Mushrooms. 2013;15:71-79.
4. Arango CS and Nieto IJ. Biotechnological cultivation of edible macrofungi: an alternative in obtaining nutraceuticals. Rev Iberoam Micol. 2013;30:1-8.
5. Sanchez OJ et al. Polysaccharide Production by Submerged Fermentation. In Ramawat K, Мйrillon JM (eds.), Polysaccharides, Ed. Springer, Cham. 2014;451-473.
6. Moore D. Fungal Morphogenesis, Ed. Cambridge University Press, United States of America. 1998;469.
7. Carlile MJ, et al. The Fungi. 2nd Ed. Academic Press, London. 2001;588.
8. Hyun-Han K, et al. Optimization of submerged culture conditions for mycelial growth and exopolysaccharides production by Agaricus blazei. J Microbiol Biotechnol. 2004;14:944-951.
9. Gao H and Gu WY. Optimization of polysaccharide and ergosterol production from Agaricus brasiliensis by fermentation process. Biochem Eng J. 2007;33:202-210.
10. Shu CH, et al. Effects of culture pH on the production of bioactive polysaccharides by Agaricus blazei in batch cultures. J Chem Technol Biotechnol. 2004;79:998-1002.
11. Shu CH, et al. Effects of culture temperature on the production of bioactive polysaccharides by Agaricus blazei in batch cultures. J Chem Technol Biotechnol. 2007;82:831-836.
12. Hamedi A, et al. Study on the effects of different culture conditions on the morphology of Agaricus blazei and the relationship between morphology and biomass or EPS production. Ann Microbiol . 2012;62:699-707.
13. Catley BJ. Pullulan, a relationship between molecular weight and fine structure. FEBS Lett 1970;10:190-193.
14. Shu CH and Lung MY. Effect of pH on the production and molecular weight distribution of exopolysaccharide by Antrodia camphorata in batch cultures. Process Biochem. 2004;39:931-937.
15. Shu CH and Xu CJ. Medium optimization for producing bioactive exopolysaccharides by Agaricus brasiliensis S. Wasser et al. (=A. blazei Murrill ss. Heinem) in submerged culture. Food Technol. Biotechnol. 2007;45:327-333.
16. Gern JC. Production and characterization of extra-cellular polysaccharide from submerged fermentation by Agaricus brasiliensis. Tese de mestrado. Universidade Federal do Parana. 2005.
17. Wasser SP, et al. Is a widely cultivated culinary-medicinal Royal Sun Agaricus (the Himematsutake Mushroom) indeed Agaricus blazei Murrill? Int J Med. Mushrooms. 2002;4:267-290.

e-ISSN: 2320-3528
p-ISSN: 2347-2286

18. Pardo-Gimenez A, et al. Adaptability of Brazilian strains of Agaricus subrufescens Peck to fruiting on various casing materials in commercial crops. Rev Iberoam Micol. 2014;31:125-130.

19. Wasser SP, et al. Is a widely cultivated culinary-medicinal Royal Sun Agaricus (champignon do Brazil, or the Himematsutake mushroom) Agaricus brasiliensis S. Wasser et al. indeed a synonym of A. Subsufescens Peck? Int J Med Mushrooms. 2005;7:507-511.

20. Tanaka HS, et al. Cereal grains and glycerol in Agaricus blazei cryopreservation. Bioscience Journal. 2013;29:627-633.

21. Vargas-del-Rio LM, et al. Preserving and maintaining culinary-medicinal Royal Sun mushroom, Agaricus brasiliensis (Agaricomycetes), in sterile distilled water. Int J Med Mushrooms. 2017;19:467-475.

22. Lisiecka J, et al. Almond mushroom Agaricus brasiliensis (Wasser et al.) - properties and culture conditions. ACTA Scientiarum Polonorum Horticulture. 2013;12:27-40.

23. Wu B, et al. A polysaccharide from Agaricus blazei inhibits proliferation and promotes apoptosis of osteosarcoma cells. Int J Biol Macromol. 2012;50:1116–1120.

24. Yue L, et al. A polysaccharide from Agaricus blazei attenuates tumor cell adhesion via inhibiting E-selectin expression. Carbohydr Polym. 2012;88:1326-1333.

25. Yamanaka D, et al. Agaricus brasiliensis-derived β-glucans exert immunoenhancing effects via a dectin-1-dependent pathway. Int Immunopharmacol. 2012;14:311-319.

26. Cardozo FT, et al. Antiherpetic activity of a sulfated polysaccharide from Agaricus brasiliensis mycelia. Antiviral Res. 2011;92:108-114.

27. Sui Z, et al. Chemical analysis of Agaricus blazei polysaccharides and effect of the polysaccharides on IL-1 beta mRNA expression in skin of burn wound-treated rats. Int J Biol Macromol. 2010;47:155-157.

28. Rowan NJ, et al. Medicinal Mushrooms: Their Therapeutic Properties and Current Medical Usage with Special Emphasis on Cancer Treatments. Ed. Aloja Medicinals Inc., Isla de Maui, Hawaii. 2002;256.

29. Mizuno M, et al. Anti-tumor polysaccharide from the mycelium of liquid-cultured Agaricus blazei Mill. Biochem Mol Biol Int. 1999;47:707-714.

30. Angeli JP, et al. Beta-glucan extracted from the medicinal mushroom Agaricus blazei prevents the genotoxic effects of benzo[a]pyrene in the human hepatoma cell line HepG2. Arch Toxicol. 2009;83:81-86.

31. Carvajal AESS, et al. Bioactive of fruiting bodies and submerged culture mycelia of Agaricus brasiliensis (A. blazei) and their antioxidant properties. LWT - Food Sci Technol. 2012;46:493-499.

32. Vincent M, et al. Dietary supplementation with Agaricus blazei Murill extract prevents diet-induced obesity and insulin resistance in rats. Obesity. 2013;21:553-561.

33. Liu GQ and Wang XL. Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by Agaricus blazei. Appl Microbiol Biotechnol. 2007;74:78-83.

34. Kim HH, et al. Effects of dissolved oxygen control on cell growth and exopolysaccharides production in batch culture of Agaricus blazei. Korean J Chem Eng. 2005;22:80-84.

35. Shu CH, et al. Monitoring the polysaccharide quality of Agaricus blazei in submerged culture by examining molecular weight distribution and TNF-α release capability of macrophage cell line RAW 264.7. Biotechnol Lett. 2003;25:2061-2064.

36. Prada R. Alternative for the efficient use of biodegradable waste: the case of residual starch derived from the industrialization of the potato Bogotб, 180-192. Magazine EAN. 2012;72:182-192.

37. Ruales J, et al. Manual de mйtodos de caracterizaciyn de carbohidratos. Ed. Escuela Politйcnica Nacional. Departamento de Ciencia de Alimentos y Biotecnologна, Quito, Ecuador. 2000;113.

38. Noda T, et al. The effect of harvest dates on the starch properties of various potato cultivars. Food Chem. 2004;86:119-125.

39. Mazzeo M, et al. Starch obtainment from post-harvest residues of Dominico Harton plantain (Musa AAB Simmonds). Vector. 2008;3:57-69.

40. Somogyi M. Notes on sugar determination. J Biol Chem. 1952;195:19-23.

e-ISSN: 2320-3528
p-ISSN: 2347-2286

41. Nelson N. A photometric adaptation of the somogyi method for the determination of glucose. J Biol Chem. 1944;153:375-380.

42. Collmer A, et al. Assay Methods for Pectinc Enzymes. Methods in enzymology, Ed. Academic Press, Inc. 1988;333-334.

43. Gonzalez GH and Castellanos OF. Alternatives for modifying the Somogyi-Nelson method for determining reducing sugars by using their thermical possibilities. Revista Ingenieria e Investigacion. 2003;52:5-17.

44. Sadasivam S and Manickam A. Carbohydrates. Biochemical Methods, Ed. New Age International. 1996;1-19.

45. Dubois M, et al. Colorimetric method of determination of sugars and related substances. Anal Chem. 1956;28:350-356.

46. Fan K, et al. Intracellular polysaccharide and its antioxidant activity by Cordyceps militaris SU-08. J Appl Polym. Sci. 2011;120:1744-1751.

47. Montoya S. Obtenciyn de enzimas lignocelulolнticas y polisacбridos a partir de residuos lignocelulysicos del departamento de Caldas empleando macromicetos de pudriciyn blanca por fermentaciyn sumergida y fermentaciyn en estado sylido. Tesis doctoral. Universidad de Caldas. 2012.

48. Medina JA and Salas JC. Morphological characterization of the native starch granule: Appearance, shape, size and distribution. Revista de Ingenieria. 2008;56-62.

49. Belitz HD and Grosch W. Quнmica de los Alimentos. 2da Ed. Editorial Acribia, SA Zaragoza, Espana. 1997;1087.

50. Takaya BT, et al. Degradation of starch granules by alpha-amylases of fungi. Starch. 1978;30:289-293.

51. Thang YJ and Zhong JJ. Exopolysaccharide biosynthesis and related enzyme activities of the medicinal fungus, Ganoderma lucidum, grown on lactose in a bioreactor. Biotechnol Lett. 2002;24:1023-1026.

52. Wilches JC. Valuation and growth of nutraceutical and nutraceutical edible fungi in agroindustrial substrates of Valle del Cauca. University of Manizales. 2014.

53. Chang ST and Miles PG. Mushrooms. Cultivation, Nutritional Value, Medicinal Effect, and Environmental Impact. 2nd Ed. CRC Press LLC, United States. 2004;477.

54. Baars J, et al. Nitrogen assimilating enzymes in the white button mushroom Agaricus bisporus. Microbiology. 1994;140:1161-1168.

55. Meti RS, et al. Enzymes of ammonia assimilation in fungi: An overview. Rec Res Sci Tech. 2011;2:28-38.

56. Xu D, et al. Charaterization and immunomodulatory activities of polysaccharide isolated from Pleurotus eryngii. Int J Biol Macromol. 2016;92:30-36.

57. Sato T, et al. Melting behavior of Schizophyllum commune polysaccharides in mixtures of water and dimethyl sulfoxide. Carbohydr Res 1981;95:195-204.

58. Cui YQ, et al. Effect of agitation intensities on fungal morphology of submerged fermentation. Biotechnol and Bioeng. 1997;55:715-726.