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Volumetric Oxygen Transfer Coefficient Effect on Biomass, Bioactive Compounds Production, and Kinetic Behavior of *G. lucidum* in Submerged Culture Using a Complex Medium.

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HIGHLIGHTS

- *G. lucidum* is able to grow in a low-cost complex medium in a stirred tank bioreactor.
- KLa values between 17.3 - 32.8 h⁻¹ are suitable to scale up bioprocess using complex media.
- Oxygen availability in the bioreactor affects the biomass and biomolecules production.

Abstract: The effect of volumetric oxygen mass transfer coefficient (KLa) on the biomass and the commercial interest biomolecules production, was studied in the *Ganoderma lucidum* submerged culture using a low-cost complex medium. The batch culture was carried out in a 14 L stirred tank bioreactor, a KLa initial value set at 5.3 h⁻¹ had a positive effect on exopolysaccharides production, achieving a maximum concentration of 1.15 g/L, under this condition, biomass production was negatively affected. An increase in the KLa initial values between 17.3 - 32.8 h⁻¹, allowed us to obtain a culture with better oxygen and nutrients transfer conditions. These values improved the kinetic carbohydrate uptake, fungal growth, and enabled the adequate supply of oxygen for a long time, achieving a maximum biomass concentration of 9.8 g/L. Under these conditions, the average production of exopolysaccharides is 0.46 g/L, and crude protein extract production was around 0.3 g/L. The complex medium (made from barley flour) used in this study is a low-cost medium that showed great potential for the replacement of synthetic high-cost media that are conventionally used in *Ganoderma lucidum* submerged cultures in a stirred tank bioreactor.

Keywords: bioreactor; *Ganoderma lucidum*; volumetric oxygen mass transfer coefficient (KLa); biomolecules; complex medium.

INTRODUCTION

Ganoderma lucidum is a basidiomycete mushroom traditionally used in the medicinal field due to its high therapeutic potential. Scientific studies demonstrated convincing evidence regarding the antitumor, anti-HIV, immunomodulatory, and neuroprotective activities of the bioactive compounds (polysaccharides, alkaloids, and triterpenes). The above-mentioned has been related to its pharmacological activities and its profound impact on human health [1]. Despite most of the investigations being focused on *G. lucidum*'s medicinal properties, bioactive molecules have shown outstanding applications in agriculture, specifically for the biocontrol of plant pathogens. Zhang and coauthors (2019), suggested that *G. lucidum* polysaccharides might induce systemic resistance to the cotton plant against wilt, caused by the soil-borne fungus *Fusarium oxysporum* [2]. In another case, the methanolic extract obtained from the fruiting body showed an antifungal effect against *Trichoderma viride*, an extract minimum inhibitory concentration (MIC) of 0.005 mg/mL was effective compared to the MIC reported for commercial fungicides such as bifonazole (0.15 mg/mL) and ketoconazole (1 mg/mL) [3]. Previous studies have reported the presence of enzymes with protease, chitinase, and glucanase activities in the crude protein extract of *G. lucidum*. These enzymes were linked to cell wall degradation and growth inhibition of *Pseudocercospora fijiensis*, a phytopathogen fungus that causes Black Sigatoka disease in Musaceae crops [4, 5]. The use of these biomolecules as biological control agents in plants could help reduce or replace the pesticide amount applied in agriculture uses, contributing to the reduction of the environmental impact provoked by chemical agents conventionally used in the countryside.

The *G. lucidum* products demand is increasing worldwide: it has been estimated that its sales generate more than USD 2.5 billion/year in Asian countries, representing an important economic asset. Additional efforts are needed and justified to improve biomass production and optimize the culture conditions of this mushroom [6]. Traditionally, *G. lucidum* culture has been carried out in solid-state fermentation (SSF) to obtain the fruiting bodies, this cultivation process takes between two and eight months. SSF presents low biomass yields and makes both the monitoring culture process and the monitoring quality of the biotechnological products difficult. The submerged fermentation culture has been conceived as an alternative to increase the biomass yield and reduce the cultivation time from months to days.

It is common to include mono-disaccharides, yeast extract, peptone, and vitamins in the media composition for submerged fermentation cultures of *G. lucidum*, which are expensive components and represent a significant cost during the scale-up process [7, 8]. There is a growing interest to find alternative low-cost raw materials that allow the replacement of high-cost carbon and nitrogen sources currently used in biotechnological processes [9]. Zapata and coauthors (2009) proposed the use of cereal flours as the main carbon and energy source which decreases production costs [10, 11]. Specifically, the culture medium composed of barley flour supplemented with macro and micronutrients has been successfully used on basidiomycetes submerged cultures such as *Ganoderma lucidum*, *Pleurotus* spp, and *Grifola frondosa*. This culture medium showed great potential for fungal biomass and bioactive compounds production in Erlenmeyer flasks [4, 12]. There are no studies that report the use of this complex medium for *G. lucidum* culture in stirred tank bioreactors, and its impacts on fungal growth behavior, carbohydrate and oxygen uptake kinetics, pH variation during culture, and bioactive compounds production.

Unlike flask cultures, a bioreactor enables the control of operational parameters such as temperature, pH, agitation, and airflow, this allows continuous monitoring and standardization of culture parameters and automates the process, reducing the product variability batch by batch [13, 14]. In bioreactor cultures, oxygen is considered an essential nutrient in aerobic biotechnological processes, it has a high influence on fungal growth, morphology, metabolic activity, nutrient uptake, and biomolecules production. Oxygen control during the culture process constitutes a strategy for fungal metabolic channeling that allows to optimize growth conditions and address the culture to increase biomolecules production [15, 16]. The KLa coefficient is used to characterize the oxygen transfer capacity from the gas phase to the liquid phase inside of a bioreactor, also considered one of the most used criteria in aerobic cultures to scale up the fermentation processes [15, 17]. KLa variation depends partly on the stirring speed and partly on the airflow inside the bioreactor. It is well known that a high stirring rate can lead to cell damage since fungal mycelium is considered sensitive to shear stress, it is important to take into account the impeller tip speed (V_{tip}) to quantify the maximum speed that generates shear stress in the mycelium [15]. Pollard (2006) pointed out that KLa conservation was the most effective criterion to scale-up *Glarea lozoyensis* submerged culture for antifungal-Pneumocandin production process from pilot-scale (0.07, 0.8, and 19 m³) to commercial scale (57 m³) [18].

This study aims to evaluate the effect of oxygen supply in a stirred tank bioreactor, through the variation of KLa initial value, on *Ganoderma lucidum* growth, exopolysaccharides production, and crude protein extract obtention [5]. The behavior of the fungus culture is shown through the kinetics of growth and the uptake of nutrients in a stirred tank bioreactor, operating in discontinuous mode and using a low-cost complex culture

medium. The information provided will be useful for future scale-up processes using complex media based on cereal flours.

MATERIAL AND METHODS

Strain, inoculum, and culture maintenance

Ganoderma lucidum strain was provided by Laboratory of Plant Biotechnology collection center of the Universidad de Antioquia (Medellín, Colombia). It was maintained and preserved on a complex solid culture, consisting of the following components: barley flour 30 g/L, sucrose 5 g/L, yeast extract 3 g/L, and agar-agar 8 g/L at 4 °C [19]. The mycelium was picked monthly and transferred to a complex solid medium, the culture was incubated at 25 °C to obtain fresh inoculum available to perform precultures.

Preculture

For the first stage of the culture, 250 mL Erlenmeyer flasks with 62 mL of liquid complex medium (barley flour 30 g/L, NaNO₃ 80 mg/L, KH₂PO₄ 30 mg/L, MgSO₄·7H₂O 20 mg/L, KCl 10 mg/L) at pH = 5.6 ± 0.1, were inoculated with five mycelial discs (Ø 1 cm) and incubated at 25 °C in a rotatory shaker at 100 rpm for 7 days. For the second stage of the culture, 5 g of wet biomass from the first stage of the culture were transferred to 1 L Erlenmeyer flask with 0.4 L of liquid complex medium and incubated under the same conditions.

Bioreactor submerged cultivation

A 14 L stirred tank bioreactor (New Brunswick™ BioFlo® 115) with a working volume of 10 L was used in this study. The culture process was performed in a batch system and the bioreactor was equipped with baffles and two Rushton six-bladed disk turbine impellers. Aeration was done through a ring sparger, which was located 2 cm above the reactor's bottom. To facilitate substrate degradation by the fungus, barley flour was previously sifted (Ø 300 µm) to homogenize and reduce the particle size. The culture medium used in this study was modified from the complex medium reported by Zapata and coauthors (2009) [10]. Barley flour concentration was fixed at 20 g/L, olive oil at 1 mL/L was added to control foam, the concentration of macro and micronutrients used was previously described. The bioreactor was inoculated with 0.4 L of fresh biomass obtained from the second stage of the culture, then the fermentation process was conducted at room temperature (23 °C-25 °C) for 7 days in continuous exposure to blue light, provided by a light-emitting diode (LED) module (BIOINN). This light condition was implemented because it was previously reported that blue light exposition induces an increase in fungal biomass production [10].

Evaluation of KLa initial value effect

To calculate different initial oxygen mass transfer coefficients (KLa), the airflow rate was set at 0.5 vvm and the stirred speed was fixed at 100, 200, 300, 400, and 600 rpm to obtain the desired KLa initial values (Table 1). The KLa initial value was determined using the dynamic method with the cell-free bioreactor according to equation 1 and the impeller tip speed (V_{tip}) was calculated according to Equation 2.

$$\frac{dc}{dt} = KLa (C_{eq} - C) \quad (1)$$

$$V_{tip} = \pi N_i D_i \quad (2)$$

Table 1. Initial KLa and impeller V_{tip} speed values evaluated.

Stirrer speed (rpm)	KLa (h ⁻¹)	V _{tip} (m/s)
100	5.3 ± 0.27	0.3
200	8.5 ± 0.86	0.6
300	17.3 ± 1.39	0.9
400	32.8 ± 3.35	1.3
600	61.0 ± 1.33	1.9

Where, C_{eq} is the oxygen saturation concentration in the bulk liquid in equilibrium to the bulk gas phase; C correspond to the dissolved oxygen concentration in the bulk liquid; N_i Stirrer speed and D_i diameter of impeller.

Polarographic dissolved oxygen (DO) tension probe and pH sensor (Mettler Toledo) were used to monitor the DO and pH during the culture. At the end of the fermentation process, dry biomass (g/L), exopolysaccharides (g/L), and crude protein extract (g/L) production were determined.

Dry weight and soluble carbohydrates measure

The gravimetric method was used to follow up biomass production and insoluble substrate during the culture. Daily, a sample of about 6-10 mL was collected and centrifuged at 11700 g-forces for 10 min, and the pellet dried in the oven at 70 °C until it obtained a constant weight. The supernatant was recovered and the total soluble carbohydrates were quantified by the methodology proposed by Dubois (1956) [20].

KLa condition of 32.8 h⁻¹ was selected to carry out the fungus cultivation process and to determine the percentage of insoluble material (lignocellulosic material) that remains at the end of the fermentation process. Since lignocellulosic material is denser than biomass, separation was performed by centrifugation. For this, 50 mL of culture were centrifuged at 15100 g-forces for 30 min, then the remnant lignocellulosic material was carefully recovered from the bottom and dried out at 70°C until obtaining a constant weight. The percentage of insoluble solids (% IS) was determined using equation 3. Where X_{b+IS} is the weight of biomass plus insoluble solids and X_{IS} corresponds to the weight of insoluble solids.

$$\%IS = \frac{(X_{IS} \cdot 100)}{X_{b+IS}} \quad (3)$$

Exopolysaccharides measurement

The extracellular medium was separated from biomass by centrifugation at 15100 g-forces for 10 min. The supernatant (extracellular medium) was used to quantify the exopolysaccharides, the biomass was stored at -20 °C until used for protein extraction. Obtained exopolysaccharides resulted from mixing the supernatant with four volumes of 95% ethanol and later precipitated overnight at 4°C [21]. Precipitated polysaccharides were collected by centrifugation at 11700 g-forces for 5 min. The pellet was suspended in 1 M NaOH at 60 °C for 1 h and the quantification was carried out by the phenol-sulfuric method [20].

Extraction and measurement of crude protein extract

The biomass was thawed at 4 °C overnight, then, it was twice washed to clean and remove remnants of the culture medium with a 12 mM PBS buffer at pH 7.4. Mechanical cellular lysis was induced by alternating homogenization cycles with cooling ice baths for 1 min each, for ten repetitions. The resulting product was centrifuged at 4 °C and 11700 g-forces for 10 min. The recovered supernatant was treated with triton x-100 and polyvinylpyrrolidone (PVP) at 1% in the final concentration. The mixture was homogenized in a vortex and stored at 4 °C for 20 min. Then, cold acetone was added to the supernatant in a 1:1 ratio and incubated for 1 hour at 4 °C. Finally, centrifugation was carried out at 4 °C and 11700 g-forces for 15 min. The obtained pellet was washed with cold acetone, and a strong vortex was applied for the dispersion in 5 mL of 12 mM PBS pH 7.4 and 0.5 mM sodium deoxycholate [5]. The crude protein extract was dried by lyophilization and measured by the gravimetric method.

Statistical analysis

All experiments were performed in triplicate and the results were reported with mean values and the standard deviation. The parameters evaluated were subjected to one-way Anova analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) test, a p-value < 0.05 was considered to denote a statistically significant difference.

RESULTS

G. lucidum culture behavior in a complex medium under different KLa initial values

At the beginning of the fungal culture process, it was determined that: from 20 g/L barley flour added to the medium, approximately 11.73 ± 0.63 g/L of solids remained insoluble (solids are mainly composed of insoluble starch and lignocellulose) and 7.56 ± 1.5 g/L of total carbohydrates were soluble in the medium

(carbohydrates were free sugars and soluble starch). The solubilization of these carbohydrates was due to the gelatinization process associated with the leaching of amylose and, it was induced by the high temperature reached during the culture media sterilization. The adaptation phase of the microorganism during the culture processes could not be identified due to the difficulty of separating the biomass from the insoluble substrate (B+IS). Figure 1A shows the behavior of dry weight B+IS during the culture process in the bioreactor. In a batch culture with soluble substrates, the microbial growth curve followed a trend of increasing weight until reaching the stationary phase [22]. In this particular case, the figure shows a decrease in the B+IS dry weight for the first two days (48 h) of culture, after this period, there is an increase of weight associated with the growth phase and fungal biomass production. A decrease of B+IS dry weight during the first 48 h of culture may be the result of the degradation of the insoluble substrate by the fungus. *G. lucidum* can produce lignocellulolytic and amylolytic enzymes. These enzymes can hydrolyze and degrade the soluble starch and insoluble particles of barley flour to its basic units of monosaccharides and short-chain polysaccharides. These carbohydrates can be taken directly by the fungus or released into the culture medium for later consumption [8]. This hypothesis is supported by the soluble carbohydrate uptake kinetics graph (Figure 1B), this figure shows an increase of the soluble carbohydrate concentration in the medium during the first 48 h, followed by a characteristic carbohydrate uptake kinetic in batch culture.

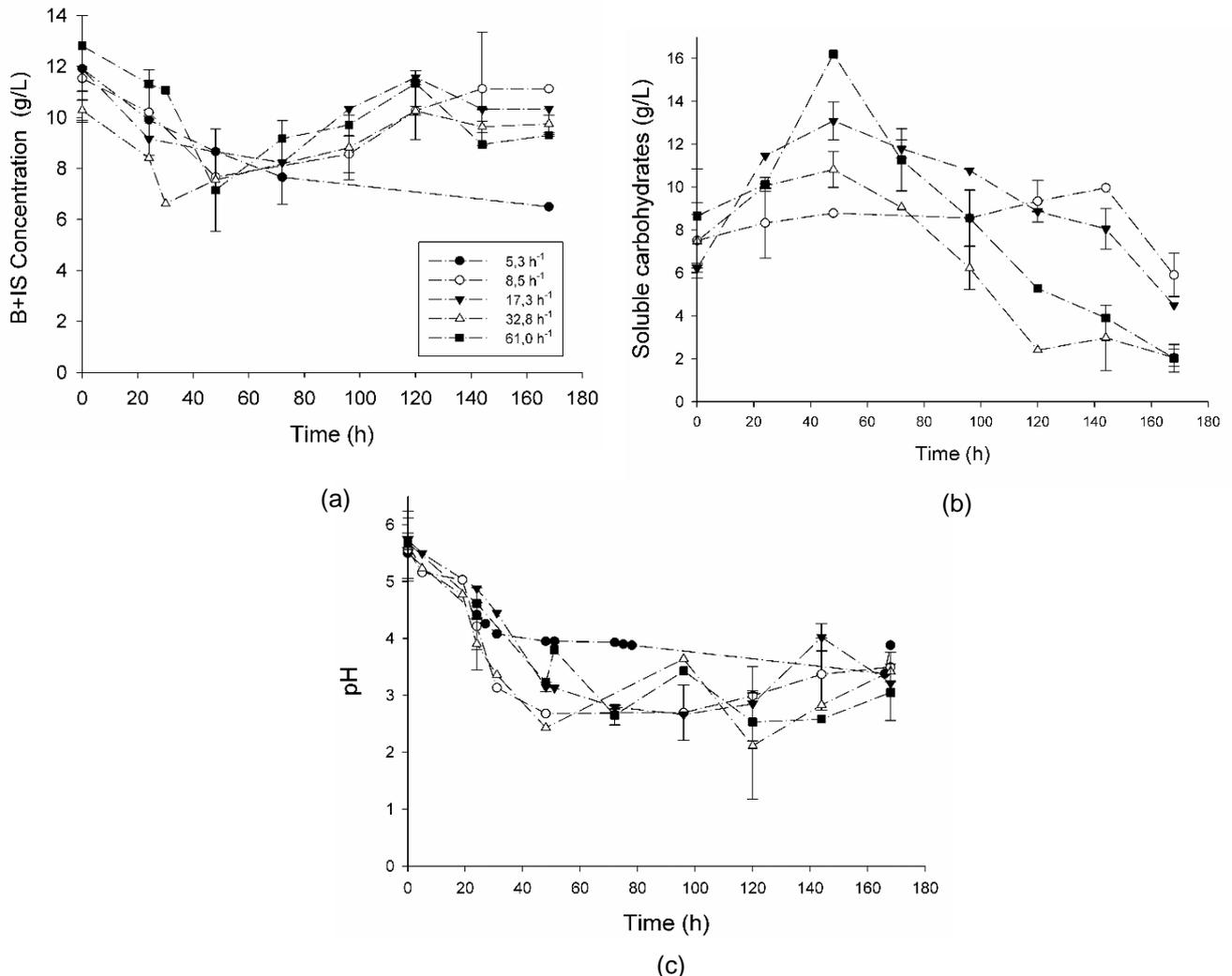


Figure 1. Growth curve (a); carbohydrates uptake kinetics (b) and pH behavior (c) in *Ganoderma lucidum* cultures with complex medium under different KLa conditions.

Cultures with KLa initial values of 8.5 h⁻¹, 17.3 h⁻¹, 32.8 h⁻¹, and 61.0 h⁻¹ had a kinetic growth following the same trend. On the contrary, under a KLa condition of 5.3 h⁻¹, similar behavior is observed during the first 48 h of the culture, however, after this period the kinetic growth changed its trend compared to higher values of KLa. This evidences the lack of biomass production directly proportional to the oxygen amount in the medium.

The carbohydrate uptake kinetics showed that an increase in oxygen supply affected positively the carbohydrates uptake rate, this phenomenon could be observed in Figure 1B. Cultures with KLa higher than 32.8 h^{-1} and 61 h^{-1} exhibited that all the available carbohydrates (free sugars and soluble starch), were consumed around 120 h (5 d). This might suggest a change in cellular metabolism from the exponential phase to the stationary phase. On other KLa conditions studied, the carbohydrates uptake kinetics showed that substrate consumption rate was negatively affected because, after seven days of cultivation, soluble carbohydrates remaining were found.

Regarding pH behavior during culture, in Figure 1C it is observed that independently from the KLa values evaluated, there is a rapid decrease in pH values from 5.6 (initial pH) to acidic conditions close to pH 2 during the first 48 hours. This condition could be possible because the fungus has the metabolic capacity to produce organic acids [23]. During monitoring of this culture parameter, it showed that *G. lucidum* submerged cultures were able to regulate pH between 2 and 4. This behavior was more evident for high KLa values of 32.8 h^{-1} and 61 h^{-1} , related to the highest oxygen availability under these conditions. The pH self-regulation of *G. lucidum* submerged cultures is a desirable characteristic during the fermentation process, because eliminates the necessity to adjust the pH and reduces the risk of contamination with other microorganisms [24].

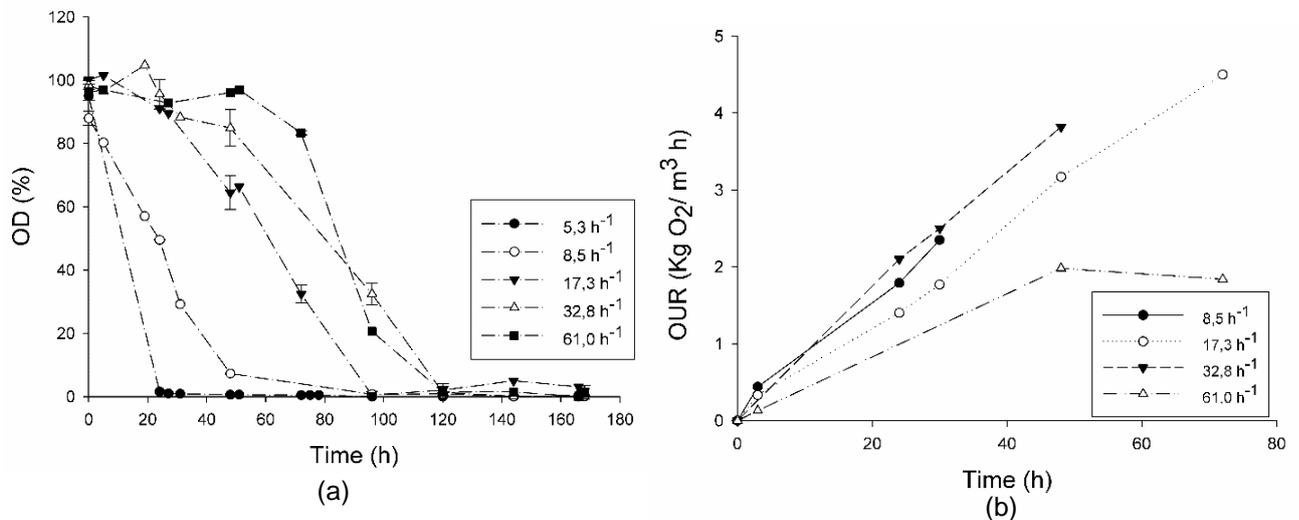


Figure 2. Monitoring of dissolved oxygen tension (a) and the oxygen uptake rate (b) during *Ganoderma lucidum* culture under different KLa initial values.

Changes in the percentage of dissolved oxygen tension during the culture period are depicted in Figure 2. As expected when KLa increases, the time in which the culture is in adequate dissolved oxygen conditions also increases (DO above 20%) [13]. When KLa initial values were 32.8 h^{-1} and 61 h^{-1} , oxygen limitation started after 5 days of culture. This might suggest that KLa values in this range avoid prolonged exposure to anoxia of the aerobic fungal mycelium during culture time. By contrast, in batches run with KLa initial values of 5.3 or 8.5 h^{-1} , oxygen limitation begins prematurely. In both cases, at the end of the culture period, a total oxygen depletion takes place.

The oxygen consumption rate ($\text{Kg O}_2/\text{m}^3\text{h}$) or respiration rate was estimated during culture time while dissolved oxygen was not limited. In cultures with KLa values of 8.5 h^{-1} , 17.3 h^{-1} and 32.8 h^{-1} , the consumption rate increased linearly during this time. Similar respiration rates were observed independently for these KLa values (Figure 2B). The lowest respiration rate was observed for a KLa initial value of 61 h^{-1} , which could be related to shear stress in fungal cells induced by the high-speed agitation ($V_{\text{tip}}=1.9 \text{ m/s}$). Under this operation condition, it is suggested that fungal mycelium needed a longer adaptation time. It is important to consider that the specific oxygen consumption could not be calculated due to the difficulty to determine the solid-free biomass during the first days of culture, since barley flour is an insoluble substrate.

Biomass production

In a batch culture of seven days, the maximum biomass concentration obtained was $9.8 \pm 0.8 \text{ g/L}$. Significant differences in biomass production between KLa initial values ranging from 8.5 to 61.0 h^{-1} were not found. A lower concentration of biomass was observed for a KLa initial value of 5.3 h^{-1} , corresponding to $7 \text{ g/L} \pm 0.38$ (Figure 3). Furthermore, an agitation speed equal to or greater than 300 rpm (KLa value of 17.3 h^{-1} , $V_{\text{tip}} 0.9 \text{ m/s}$) made it possible to maintain a homogeneous mixture of culture medium into a bioreactor, which avoided cells precipitation, fungal agglomerates, and/or adherence of cells to the bioreactor

parts. Substrate-biomass conversion yields (Y_{xs}) were negatively affected by the increase in KLa value, decreasing from 0.65 g/g to 0.52 g/g. This proved that during lower KLa conditions there is higher efficiency in substrate transformation consumed by the fungus for biomass production compared to higher KLa values. When KLa values are high, despite having higher substrate consumption, a part of this substrate is targeted for cellular maintenance, consequently, Y_{xs} observed values are lower. It is important to note that values of final biomass and yields presented correspond to B+IS, although, there is a good transformation of the culture medium to biomass, at the end of the fermentation, small amounts of the exocarp of the barley grains (lignocellulosic material) remained, these small amounts were weighed together with the biomass. During the fermentation process under an initial KLa value of 32.8 h^{-1} , it was determined that the percentage of insoluble solids at the end of the fermentation process did not exceed $4.8 \pm 0.3\%$ of the total biomass concentration obtained (B+IS).

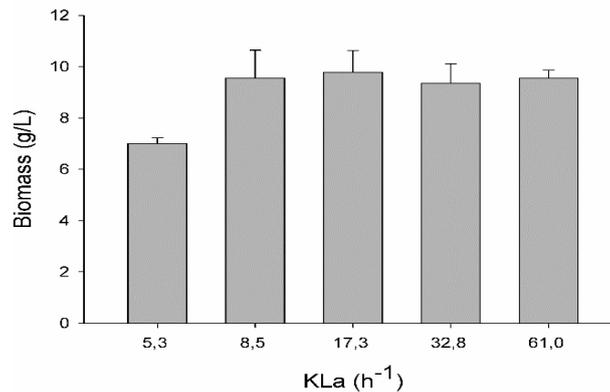


Figure 3. Effect of the initial KLa value on biomass production of *G. lucidum* in a stirred tank reactor using a complex medium based on barley flour.

Figure 4A depicts the appearance of the culture medium with barley flour after the sterilization process, an opaque and dark brown medium is observed. Figure 4B shows the insoluble substrate that was recovered after the centrifugation process of the culture medium before to star the *G. lucidum* culture. In the pellet obtained, two phases can be observed: one white phase on the top corresponding to the insoluble starch and the bottom phase composed of rich fiber particles. In Figure 4C, the biomass and the remaining insoluble particles, obtained at the end of the fermentation, can be differentiated after a centrifugation process is performed. The Figure 4D is the dehydrated biomass washed with PBS buffer, before the protein extraction. Figure 4E shows the color of the extracellular medium after the fermentation process, a clear yellow medium suggests a substrate transformation by the fungus.

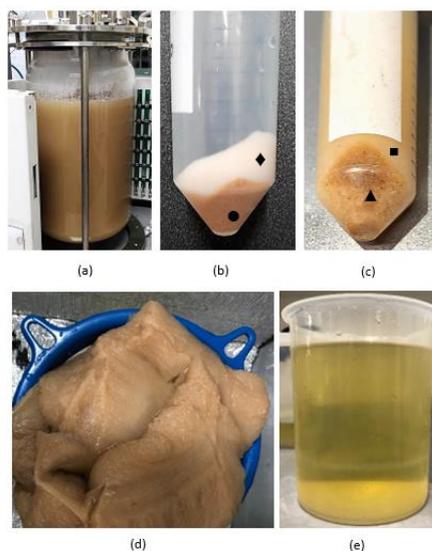


Figure 4. Photograph of the complex culture medium used for *G. lucidum* fermentation (a); Insoluble substrate recovered by centrifugation before starting the process: ♦ Insoluble starch ● Insoluble substrate rich in cellulose and lignocellulose (b); Mycelial biomass recovered by centrifugation at the end of the process: ▲ Remnant lignocellulosic material ■ Fungal biomass (c); Dehydrated biomass washed with PBS buffer (d); Depleted medium obtained at the end of the process (extracellular medium) (e).

Exopolysaccharides production

The effect of KLa initial value on the exopolysaccharides production is shown in Figure 5A. Concentrations of 1.08 ± 0.17 , 0.87 ± 0.06 , 0.48 ± 0.04 , 0.43 ± 0.04 and 0.59 ± 0.08 g/L of exopolysaccharides were obtained from cultures with an initial KLa of 5.3, 8.5, 17.3, 32.8 and 61 h^{-1} respectively at the end of the culture. In cultures with KLa initial values ranging from 5.3 to 17.3 h^{-1} , it is exhibited that an increase in oxygen supply significantly reduced the production of the exopolysaccharides. Unlike other studies reported in the literature, in this work-study, it was not possible to follow the production of the exopolysaccharides during the fermentation process due to the presence of exopolysaccharides and soluble starch in the supernatant, mainly in the first days of culture, co-precipitating with ethanol, consequently making the quantification not reliable.

Crude protein extract production

It was previously reported that the intracellular extract of *G. lucidum* contains proteins with antifungal activity that could be used as a control agent against the phytopathogenic fungus *Pseudocercospora fijiensis* that affects plantain and banana crops [4, 5]. For this reason, this study considers the production, extraction, and quantification of crude protein extract at the bioreactor level. Consequently, protein extracts could have new applications as active metabolites from *G. lucidum*. In Figure 5B the crude protein extract concentration obtained under different culture conditions is shown. According to these results, there are no statistical differences in crude protein extract production, independently of the KLa value studied, it was achieving an average concentration of 0.3 ± 0.04 g/L.

The protein production results were not as expected, in the KLa conditions in which a greater amount of biomass was obtained, it was expected to obtain a higher recovery rate of crude protein extract because proteins are primary metabolites. However, it is important to consider that the crude protein extract is a result of a biomass disruption process in which different kinds of fungal intracellular biopolymers are released, including carbohydrates (intrapolysaccharides, glycoproteins), nucleic acids, and proteins that might co-precipitate with acetone. This could happen due to the variation in the initial value of KLa that would affect the production of other components or metabolites present in the crude protein extract.

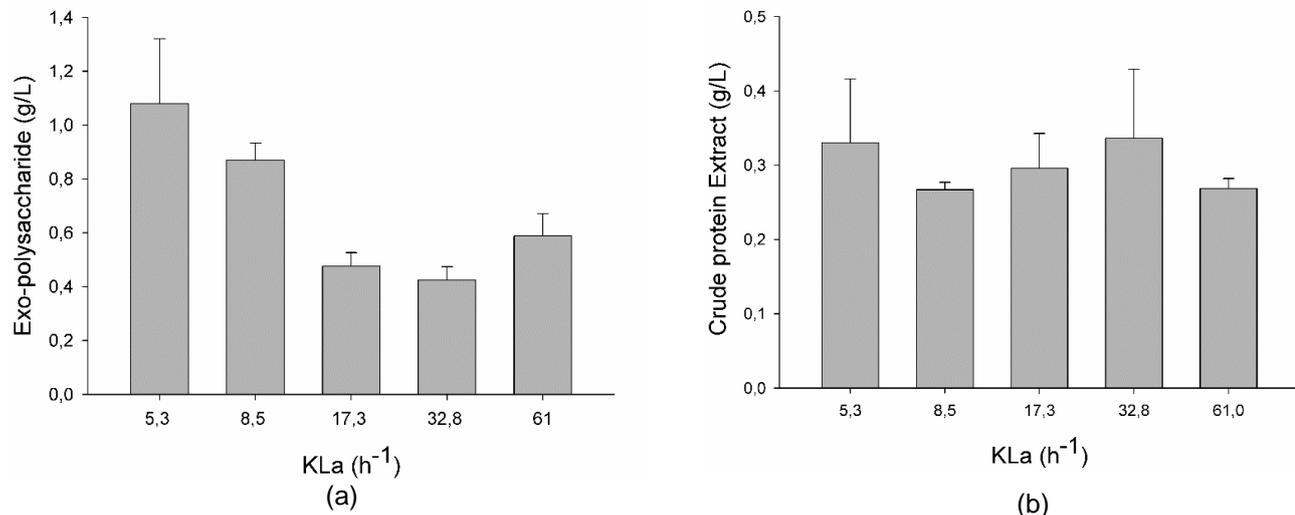


Figure 5. Effect of KLa initial values on the exopolysaccharides (a) and crude protein extract (b) production by *G. lucidum* in batch culture with a complex medium.

DISCUSSION

Media composition and biomass production

Usually, the media for submerged *Ganoderma lucidum* culture, reported in different articles and patents are made with completely soluble nutrients. Sugars such as glucose, sucrose, maltose, xylose, fructose, and lactose have been used for the development of submerged culture media [25–27]. Lactose, glucose, and maltose are the preferred carbon sources, while peptone and yeast extract remains the main nitrogen sources to generate the greatest amount of *G. lucidum* biomass [27, 28]. Barley flour is a complete raw material, in addition to its high carbohydrate content, it has an availability of protein (organic nitrogen) close to 10% which

improves the growth rate of basidiomycetes fungi. It is known that these organisms cannot synthesize essential amino acids from inorganic nitrogen sources [8]. Barley flour contains B vitamins including thiamine, riboflavin, niacin, and micronutrients such as calcium, iron, potassium, magnesium, phosphorous, zinc, among others that are considered desirable components in basidiomycetes culture media [29, 30]. Table 2 compares the costs of different culture media used for *G. lucidum* submerged culture, with relation to the one used in this work-study. For the culture media reported in the literature, preparation costs range from 4 to 16 USD/L [31–34], being carbon and nitrogen sources responsible for more than 70% of the total culture medium cost. The proposed medium based on barley flour has a total preparation cost of 0.14 USD/L, which represents a saving of more than 97% in raw material.

Table 2. Relationship of cost per liter in USD of the culture media used in the *G. lucidum* submerged culture.

Medium Composition	Cost (USD/L)				
	Tang Medium (2011)	Hu Medium (2018)	Fazenda Medium (2010)	Hanafiah Medium (2019)	This Paper
Lactose	6.83				
Glucose		8.52	9.94	2.84	
Barley Flour					0.03
Peptone	2.56		2.56		
Yeast Extract	0.75		1.5	0.3	
KH ₂ PO ₄	2.32	6.96	2.32	1.16	0.07
MgSO ₄ 7H ₂ O	0.09	0.26	0.09	0.09	
NH ₄ Cl				0.45	
NaNO ₃					0.02
KCl					0.02
Vitamin B1	0.19				
TOTAL (USD/L)	12.74	15.74	16.41	4.84	0.14

Regarding other carbon sources such as lactose, it has been reported that initial concentrations exceeding 35 g/L significantly reduce the biomass/substrate yield from 0.4 to 0.28 g/g, due to an inhibition of the mycelium growth by an excess of initial substrate concentration [28]. Fed-batch systems have been used as a culture strategy to achieve higher biomass concentrations using submerged cultures of *G. lucidum* with lactose. For instance, the maximum biomass production using this system was reported in the work carried out by Tang and coauthors (2011), in which a concentration of 25 g/L dry biomass was obtained after 15 days of cultivation, managing to overcome the difficulty of inhibition due to excess substrate [7, 31]. Wei and coauthors (2016) reported a biomass production greater than 25 g/L using the fed-batch strategy mixing glucose and sucrose to start the culture, once the initial substrate had been consumed, sucrose was used to feed the system [25].

Specifically, using barley flour as fermentation substrate, no studies have been reported at the bioreactor level, however, in cultures at the Erlenmeyer level Zapata and coauthors (2012) reported an increase in biomass production from 17.1 g/L to 23.5 g/L with the increase in cereal flour concentration from 30 g/L to 50 g/L in batch culture systems in which no inhibition was observed due to the high initial substrate concentration [11]. According to the behavior of carbohydrates uptake kinetics observed in this work-study, it can be argued, that due to the low solubility of the substrate, greater amounts of barley flour can be added. To metabolize the nutrients present in the medium, *G. lucidum* must hydrolyze the substrate in parallel to the assimilation of nutrients. This avoids a fungal growth inhibition related to the high initial concentrations of free sugar in the culture medium [35]. The hydrolysis of nutrients found in the substrate is associated with the presence of hydrolytic enzymes. Arias and coauthors (2019) carried out a proteomic analysis by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) and suggested that approximately 71.72% of the enzymes extracted from *G. lucidum* submerged cultures (based on barley flour) are glucanase and glycoside hydrolases including α -amylase responsible for substrate degradation and starch hydrolysis [5].

The foregoing opens the possibility to use this substrate at higher concentrations on a bioreactor scale, accompanied by proper management of the foam generation that occurs at the beginning of the process, to increase biomass production without the need to resort to a fed-batch system. Regarding the submerged

culture of *G. lucidum* on alternative nutritional sources, Berovic and coauthors (2003) performed a fermentation process using potato filtrate and olive oil, this mixture was supplemented with 20 g/L of glucose to obtain 9 g/L of fungal biomass in batch cultures [35]. In a different study, Xu and coauthors (2008) performed an optimization of the culture medium at the bioreactor level using glucose, cornflour, and soybean to achieve a maximum biomass production of 21.53 g/L [8]. The obtained yields in this work-study are similar to those obtained by Tang and coauthors (2011), Zhang and coauthors (2014), and Tang and coauthors (2003) of 0.47 g/g, 0.57-0.67 g/g, and 0.44 g/g, respectively; and higher than those reported by Zhou and coauthors (2014), which achieved yields of 0.37 g/g [22, 31, 36, 37].

***G. lucidum* culture behavior in a complex medium under different KLa initial values**

The results of carbohydrate uptake kinetics obtained, are in concordance with Tang and Zhong (2003), who reported that KLa values of 60, 78, and 90 h⁻¹ led to a higher lactose consumption rate and higher biomass production than a lower KLa value of 16 h⁻¹ in a *G. lucidum* submerged culture [37]. Michelin and coauthors (2013) also reported that the use of slight values of KLa with low agitation and aeration is not enough to supply the necessary oxygen to support a good fungal growth [24]. As has been previously mentioned, in all our experiments the airflow rate was set at 2.5 vvm, while the agitation speed was adjusted from 100 to 600 rpm to get KLa initial values between 5.3 h⁻¹ and 61 h⁻¹. Tang and Zhong (2003) tested KLa values between 16 h⁻¹ to 96 h⁻¹ fixing the agitation speed at 200 rpm and increasing the airflow rate from 0.03 to 1 vvm [37]. Several studies have been shown that variations in agitation speed have a greater effect raising KLa values, even more than when increasing airflow, during this study it was not possible to obtain a bigger increase in the KLa, even when operating conditions were more aggressive than those proposed by Tang and Zhong (2003). It is important to consider that oxygen solubility closely depends on the culture medium composition. Based on this, the complexity of the medium used might contribute to the reduction of oxygen solubility compared to the media based on soluble components commonly reported for *G. lucidum* submerged culture [13].

Related to the shear stress effect, Tang and coauthors (2011) studied the effect of this variable on *Ganoderma lucidum* culture in feed batch systems, varying the Vtip from 1.234 m/s to 2.161 m/s, and setting stirring speeds between 400 rpm and 700 rpm [31]. This study showed that a Vtip of 1.234 m/s allows a good mix of the culture medium and generates the best production of biomass and ganoderic acids. When speeds at the impeller tip were higher than 1.543 m/s the effect of shear stress was considered critical and was related to a gradual decrease in maximum biomass production, which fell from 22 g/L with a Vtip of 1.234 m/s up to 11.11 g/L with a Vtip of 2.161 m/s. In addition, the authors observed that nutrient consumption decreased when a Vtip of 2.161 m/s was used, resulting in cell death. In another study, Gong and Zhong (2005) obtained the maximum biomass production using a Vtip of 0.51 m/s [38]. Despite the fact that the value of Vtip was not calculated, Berovic and coauthors (2003) showed that an agitation speed over 300 rpm damages the mycelial agglomerates and hinders hyphal growth, leading to significant reductions in biomass production [35]. The results described above agree with the results observed in this work-study; Vtip values higher than 0.9 m/s (300 rpm, KLa 17.3 h⁻¹) generate good mixing inside the bioreactor. Concerning biomass production, our results differ because when a Vtip of 1.8 m/s (600 rpm, KLa 60 h⁻¹) was used, no significant decrease in biomass production or nutrients consumption was observed. Under this culture condition, a decrease in oxygen consumption was observed during the first days, which could be related to a longer adaptation time of the cells to the culture. Following the above, for future scaling processes, it is recommended to maintain the initial KLa between 17.3 – 32.8 h⁻¹ with a Vtip between 0.9 and 1.3 m/s to reduce the risk of inhibition of fungal growth due to shear stress.

pH effect

Fang and Zhong (2002) evaluated the effect of the initial pH on the production of biomass and metabolites such as ganoderic acids and exopolysaccharides. Regardless of the pH evaluated, the authors observed a rapid drop in pH to values of 3.2, which remained constant during the first 10 days of culture. After this time, an increase in the pH value to 7 was observed, until the end of the culture [23]. The drop in pH was attributed to the secretion of organic acids (including ganoderic and lucid acids) to the extracellular medium when there is a high rate of substrate consumption. The increase in pH was evidenced when glucose levels were below 5 g/L, limiting the consumption of the substrate and the metabolic activity of the fungus [23, 39]. Our results demonstrated that pH fell rapidly and remained acidic throughout the culture, even when the measurement of soluble carbohydrates was below 5 g/L at 5 d of culture for the KLa initial value at 32 h⁻¹ and 60 h⁻¹. The increase in pH was not evidenced in this work-study, this fact could be related to the total depletion of the nutrients.

Exopolysaccharides production

The polysaccharides of *G. lucidum* are highly desired, these metabolites have been associated with antioxidant, antimicrobial, and antitumor properties. The major group of polysaccharides in *G. lucidum* are compounds of (1-3), (1-6) - β -glucans, glycoproteins, and water-soluble heteropolysaccharides [40, 41]. The chemical structure of these compounds determines their bioactivity. As for medicinal mushroom biomass production, there is limited information available on the use of complex substrates in submerged culture for exopolysaccharides production. Berovic and coauthors (2003) used potato starch as a medium's component for the *G. lucidum* submerged culture and carried out a purification by ion-exchange chromatography and affinity chromatography of the exo and intra polysaccharides, obtained at the end of fermentation [35]. The authors found that the exopolysaccharides obtained are mainly composed of β -D glucans. This report suggests that at the end of the fermentation process, by using starch as a substrate, the extracted polysaccharides mostly correspond to those generated by the fungus.

Tang and Zhong (2003) suggested that there are no significant differences in exopolysaccharides production between several KLa initial values studied in *G. lucidum* submerged cultures with oxygen control set up at 25% during the fermentation process. Nonetheless, these authors found that carrying out the culture with dissolved oxygen control over 10%, instead of 25%, generated higher exopolysaccharides, intrapolysaccharides, and ganoderic acids production [37]. Lee and coauthors (2007), reported that the production of exopolysaccharides varied between 1-1,5 g/L, in a *G. applanatum* batch culture without oxygen control and under different ratios of C/N in the culture medium [40]. These results agree with those obtained in this work since no oxygen control was performed during batch cultures. Under the lowest initial KLa values tested (5.3 and 8.6 h⁻¹), the fungus depletes oxygen in the medium quickly and remains in an anoxic condition longer time. According to reports found, these conditions favor the production of the exopolysaccharides in *G. lucidum* submerged batch cultures. The exopolysaccharides concentrations obtained in the present study agree with Wagner and coauthors (2003) who reported in his bibliographic review exopolysaccharides production ranging from 0.43 to 1.1 g/L for the submerged cultivation of *G. lucidum* [7].

Crude protein extract production

Arias and coauthors (2019) identified 46 potential enzymes present in the crude protein extract of *G. lucidum*, that may be related to antifungal activity, including chitinases, proteases, glucanases, and deoxyribonucleases [5]. No studies have been reported yet about crude protein extract production and quantification obtained from the submerged *G. lucidum* culture, under different conditions of KLa initial values in a stirred tank bioreactor. Generally, the protein extraction from *G. lucidum* is carried out to verify changes in protein profiles by polyacrylamide gel electrophoresis when different culture conditions are analyzed. This method is also carried out to protein identification by Liquid Chromatography-Mass Spectrometry in proteomics studies or towards the specific protein purification in the pharmaceutical industry applications [42, 43]. Concerning the effect of KLa on the protein production in a fungal submerged culture, Michelin and coauthors (2013) reported that oxygen supply influences the production of xylanases by *Aspergillus niger* in the extracellular medium, increasing its activity from 3000 U/L to 9000 U/L when the fermentation process was performed with KLa values of 12 h⁻¹ and 30 h⁻¹ respectively [24]. Singh and coauthors (2000) reported a relationship between the agitation speed and dissolved oxygen, to produce different types of enzymes generated by *Thermomyces lanuginosus*. The authors determining that high agitation conditions favor the production of xylosidase, arabinofuranosidase, and glucosidase, while the use of low agitation favors the production of xylanases [44]. Also determined that the development of the culture without dissolved oxygen control generates an oxygen deficiency which favored the production of the xylanase enzyme while negatively affecting the biomass production. In comparison, Burkert and coauthors (2005) suggested that the production of the lipase enzyme is linked directly related to the production of biomass, by the fungus *Geotrichum candidum* in submerged cultures [45]. In this work, a significant change in the total production of the crude protein extract was not shown under the different KLa conditions evaluated, it could be related to the crude protein extract composition. As mentioned above, in addition to proteins, the protein extract also contains other biopolymers whose production might be affected by culture conditions. Therefore, in future works, it would be necessary to implement a methodology to quantify each of these components to determine the oxygen effect on metabolite composition in the crude protein extract.

This study aims to contribute to the existing knowledge on fungal biomass production of *G. lucidum* in submerged batch culture using a complex, insoluble and low-cost medium that could be exploited for different commercial applications. The information presented here about growth and uptake nutrients kinetics, and the study of the oxygen transfer effect on biomass and bioactive molecules production is valuable for upcoming studies focus on the scale-up process, optimization of the fermentation cultures, and the search of new raw

materials such as by-products and agro-industrial waste, that can be availed through biotechnological processes for fungal submerged cultures.

CONCLUSION

The high potential of a complex medium based on barley flour for the submerged *G. lucidum* culture in a stirred tank bioreactor was demonstrated. Despite the low solubility of this medium, *G. lucidum* was able to produce and use its hydrolytic enzymes to degrade insoluble particles and consume this substrate. Biomass production of 9.8 g/L was achieved during seven days of the fermentation process, substrate-biomass yields between 0.64 and 0.54 g/g for KLa initial values between 8.8 and 61.0 h⁻¹ were obtained. This low-cost complex medium supplies the fungal nutritional requirements and may be considered as a valuable alternative to replacing conventional media used in commercially submerged *G. lucidum* cultures, reducing raw material costs by 97%.

The KLa initial value selection depends on the culture's purpose. The lowest KLa initial value (5.3 h⁻¹) induced exopolysaccharide production but negatively affected biomass production. Considering the culture behavior, the biomass production, and the production of crude protein extract; the KLa conditions between 17 and 61 h⁻¹ are the most suitable to develop the culture. Under these conditions, allowing to satisfy the cellular oxygen requirements for a longer time. Stimulating a rapid consumption of carbohydrates and generating a greater production of biomass and crude protein extract.

Finally, taking into consideration the KLa initial value as a scale-up criterion for upcoming *Ganoderma lucidum* cultures in a stirred tank bioreactor, it is recommended to keep the KLa initial value between 17.3 h⁻¹ (Vtip=0.9 m/s) and 32.8 h⁻¹ (Vtip =1.3 m/s) to avoid possible growth inhibition due to shear stress when a complex medium based on barley flour is used.

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