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Effect of Blue LED Light on Bioemulsifier Production in Bioreactor by *Aureobasidium pullulans* LB83 in Solid State Fermentation

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Abstract: This study analyzed the impact of LED light on bioemulsifier production by *Aureobasidium pullulans* LB83 in solid-state fermentation (SSF) using pre-treated sugarcane bagasse (PSB). The biomass was subjected to alkaline pre-treatment and conducted fermentations in Erlenmeyer flasks containing 2 g of PSB that were immersed in a humectant solution with a cell concentration of 10^8 cells/mL. The screening involved varying LED light wavelengths (green, red, orange, and blue) over a 7-day period at 28 °C. Notably, under the influence of blue light, the process achieved maximum production, yielding an $EI_{24\%}$ of 63.9% and 45.1% for soybean oil and kerosene, respectively. Prolonged exposure to blue light for 11 days at 28 °C resulted in maximum bioemulsifier production (75%) and cellulolytic enzyme activity (3.67 IU g^{-1} for endoglucanase and 0.41 IU g^{-1} for exoglucanase) with soybean oil and kerosene. Experiments in a bioreactor, with varying light conditions (dark, white light, and blue LED light), demonstrated that the blue LED bioreactor outperformed others, achieving $EI_{24\%}$ values of 55.0% and 45.7% for soybean oil and kerosene, respectively. The scanning electron microscopy (SEM) confirmed yeast growth under these conditions after 9 days. Our findings highlight the significant potential of LED light to enhance bioemulsifier production by *A. pullulans* LB83 from PSB.

Keywords: surface active compounds; yeast; sugarcane bagasse; cellulases; LED light-assisted process

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1. Introduction

Bioemulsifiers (BE) are natural surface-active products of high molecular mass produced mainly by microorganisms. They are structurally diverse and characterized by their amphipathic structure; that is, they have a hydrophilic region (head) and a hydrophobic region (tail) [1–3]. BEs are complex mixtures of heteropolysaccharides, lipopolysaccharides, lipoproteins and high molecular weight proteins. Due to their structural diversity, biodegradability, low toxicity, great stability in extreme conditions of pH, salinity and temperature, and their functional properties (emulsification/demulsification, dispersion and solubilization, among others), they are potential candidates for the replacement of their synthetic counterparts. In addition, bioemulsifiers have recognized thickening, coagulant, antimicrobial and anti-adherent activities that allow their application in the areas of bioremediation, biomedicine, food, pharmaceuticals, and cosmetics, among others [4–6].

These compounds prevent the separation of immiscible substances, which provides stability to an emulsion when used in low concentrations. However, no decrease in the surface and interfacial tension is observed by using these molecules [1,7].

The growing interest in bioemulsifiers can be observed through their application in the oil, food, pharmaceutical and medical industries, among others [8–10]. The global

emulsifiers' market forecast for 2027 is USD 6.1 billion with a compound annual growth rate (CAGR) of 4.8% in the period 2020–2027. Overall, for natural emulsifiers, the estimate is USD 3.3 billion, with a CAGR of 5.4% [11].

However, wide commercialization is still not a reality for most bioemulsifiers due to the high production cost when compared to their synthetic equivalents. Among the factors that contribute to this situation are the type of raw materials and the recovery processes that represent up to 80% of the total cost of production [12,13]. Thus, several strategies have been followed, such as using lignocellulose biomass as raw material. The fact that biomass is a low-cost renewable carbon source allows constant availability and adds value to the microbial synthesis of bioemulsifiers. For example, wheat bran, wheat straw, potato peel, sugarcane bagasse and peanut oil cake, among others, have been studied for this purpose [14–17].

In addition, the choice of the fermentation process is another element that contributes to the reduction in cost. Solid-state fermentation (SSF) is a process that allows microbial growth on the surface of solid materials without excess free water in the system [18,19]. Among their advantages, SSF results in a reduction in the volume of treatment of downstream liquids with non-foaming production, which can complicate the submerged cultivation processes. In addition, the process allows the valorization of biomass using agro-industrial by-products as culture media [20,21].

Bioemulsifiers produced by fungi become particularly interesting because several of these microorganisms have *GRAS* status (Generally Recognized as Safe), which means they can be applied in products for use by humans and animals [22,23]. *Aureobasidium pullulans* is a yeast-like fungus characterized by being a ubiquitous saprophyte. Its habitats encompass the phyllosphere of plants, in fresh and seawater, and even on surfaces such as concrete in urban environments [24–26]. It also inhabits extreme environments such as salt marshes and arctic soil. Due to its metabolic versatility, *A. pullulans* synthesizes a variety of bioproducts that are of industrial interest [27], including poly (β -L-malic acid), enzymes (amylases, cellulases, lipases, proteases) xylanases, β-fructofuranosidases, maltosyltransferases, mannanases and laccases [28]. Another metabolite produced by this yeast is pullulan, an extracellular homopolysaccharide with a wide range of applications. In addition, these microorganisms can produce liamocins, which are recognized as biosurfactants with great potential for use as antibacterial and anticancer agents. These activities allow their use in a wide range of applications in the biotechnology, agro-industry and food industry sectors [29–31]. From the structural viewpoint, liamocins are identified as glycolipids that contain a single sugar alcohol head group, which can include mannitol, arabitol, glycerol, and xylitol, linked to three, four or six 3,5-dihydroxydecanoic ester tail groups.

Microbial processes using *A. pullulans* can be influenced by light of different wavelengths, depending on the strain. For instance, ref. [32] demonstrated that the cultivation of *A. pullulans* strain under the influence of monochromatic blue light LED decreased the coproduction of melanin by 90.2%. They also observed that the pullulan production improved by 34.2% compared to the process under normal laboratory light, demonstrating that the effect of light can modify the metabolic pathway of the yeast. In this context, this study investigated the effect of LED lights with different wavelengths on bioemulsifier production by *A. pullulans* LB83. Subsequently, the wavelength that allowed a higher production was selected for verification of the production of BEs and cellulolytic enzymes as a function of process time. Also, a cultivation in a packed-bed bioreactor under different illumination conditions was performed. To the best of our knowledge, this is the first report on the effects of different wavelengths of light in bioemulsifier production by *A. pullulans* in SSF.

2. Materials and Methods

2.1. Microorganism and Inoculum Preparation

Aureobasidium pullulans LB83 is available in the culture stock of the Laboratory of Sustainable Bioproducts at the Engineering School of Lorena-University of São Paulo

(Lorena, São Paulo, Brazil). The cells are stored at 4 °C in slants and in test tubes containing yeast malt extract solid medium (YMA) (glucose 10 g/L, yeast extract 3 g/L, peptone 5 g/L, malt extract 3 g/L and 20 g/L agar) [33].

For inoculum preparation, *Aureobasidium pullulans* LB83 cells were transferred to 125 mL Erlenmeyer flasks previously filled with the culture medium proposed by [34] and modified by [24]: 0.6 g/L peptone, 0.4 g/L yeast extract, 1 g/L NaCl, 5 g/L K_2HPO_4 and 0.4 g/L $MgSO_4$, added with 50 g/L sucrose. The flasks were incubated at 200 rpm, 28 °C for 48 h in a rotary shaker (New Brunswick Scientific—Excella E24, Hamburg, Germany). Subsequently, the cultures were separated by centrifugation at 2930× *g* for 15 min, resuspended in 0.9% saline solution (m/V) and counted in an Agasse-Lafont-R chamber (Optik Labor, Lancing, United Kingdom), which contains reticles of 0.0025 mm^2 and a depth of 0.100 mm, to adjust the initial cell concentration to 10^8 cells/mL [33]. The previously adjusted moistening solution (mixture of [34]), medium without sucrose at a cell concentration of 10^8 cells/mL was used to humidify 2 g of sterilized pre-treated sugarcane bagasse (PSB) for SSF.

2.2. Alkaline Pretreatment of Sugarcane Bagasse

Sugarcane bagasse (SCB) was provided by Ipiranga Agroindustrial (Descalvado, São Paulo, Brazil). For storage, it was dried under sunlight until the moisture content decreased to about 10% (m/m), as determined in an infrared moisture balance Marte ID-50 (Marte Científica, Santa Rita Do Sapucaí, Brazil). Thus, the biomass was classified until it passed through a 14-mesh standard sieve.

SCB was pretreated in 1 L Erlenmeyer flasks, each one containing 30 g of biomass with 450 mL of NaOH solution. The flasks were autoclaved at 1 atm for 12 min at 121 °C. After that, the biomass was separated from the liquid fraction using a cloth strainer. The solid fraction was washed with water until it had a neutral pH and dried at 65 °C for 24 h. The composition of in natura bagasse and PSB in terms of macromolecular fractions was determined according to the National Renewable Energy Laboratory (NREL) protocol NREL/TP-510-42619 [35].

2.3. Evaluation of Different LED Light Wavelengths on SSF Bioemulsifier Production in Erlenmeyer Flasks

The influence of different LED lights on bioemulsifier production SSF was evaluated by the cultivation in Erlenmeyer flasks of 125 mL, containing 2 g of PSB. Erlenmeyer flasks were sterilized at 121 °C for 15 min, cooled and inoculated with 6 mL of moistening solution (sterile-modified Kitamoto [34] medium containing a cell concentration of 10^8 cells/mL, as described above in the section "Microorganism and inoculum preparation").

Triplicates were incubated without agitation in a microbiological oven (Quimis—Q316M4, Diadema, Brazil) maintained at 28 ± 2 °C, under 80% relative humidity (maintained with the aid of textile fibers) for 7 days under the influence of LED lights in different periods, separately. Lights were placed on the walls of the microbial incubator and lighting was continuous throughout the period. The photon flux intensity was measured at three different points (left, center and right) for each LED light inside the incubator and reported as the average of the measurements.

The Erlenmeyers were kept in the center, and the photon flux at the surface of the flasks was 2.8 ± 0.0 $\mu mol.m^{-2}.s^{-1}$; 5.2 ± 0.1 $\mu mol.m^{-2}.s^{-1}$, 8.1 ± 0.4 $\mu mol.m^{-2}.s^{-1}$ and 2.9 ± 0.2 $\mu mol.m^{-2}.s^{-1}$ for the LED lights with wavelengths red (620 to 630 nm), green (515 to 535 nm), blue (450 to 470 nm) and orange (585 to 595 nm), respectively. Erlenmeyer flasks were placed under a dark environment (flasks were covered with aluminum foil) and in the presence of white LED light (4.5 $\mu mol.m^{-2}. s^{-1}$; 400 to 700 nm) as controls for the experiments. After the fermentation finished, the flasks were removed to analyze the bioemulsifier production.

2.4. Solid-State Fermentation in Erlenmeyer Flasks under Blue LED Light

SSF was performed in Erlenmeyer flasks with a volume of 125 mL containing 2 g of PSB and inoculated with the moistening solution in a microbiological oven (Quimis—Q316M4, Diadema, Brazil) at 28 ± 2 °C under 80% humidity for 11 days. This was under blue LED light at conditions similar to the above described. Triplicates were drawn at 0, 3, 5, 7, 9 and 11 days, to analyze the emulsification index and enzymatic activities (endoglucanase and exoglucanase).

2.5. Evaluation of Light Influence on Solid-State Fermentation in Packed Bed Column

The SSF assays were carried out in a jacketed glass bioreactor with a diameter of 8 cm in the inner region, and a total length of 37 cm. In the inner region, glass spheres of 3 mm in diameter were placed, occupying 5 cm in height and used to distribute the air along the column (Figure 1). The inside of the reactor was previously washed with 70% alcohol and placed in UV light for 1 h 30 min together with the rod of the temperature recording system. An amount of 35 g of previously sterilized pre-treated sugarcane bagasse and 240 mL of the wetting solution were mixed to a final humidity of 70%. After preparation, the bagasse was placed inside the bioreactor over the spheres. The aeration system consisted of a Kitasato flask with a hose inserted in the upper part for an air inlet and a hose connected in the lateral region for a humidified air inlet into the bioreactor. Spheres of 3 mm in diameter were placed inside the flask, occupying a height of 5 cm, and covered with sterile water. The air at the outlet of the Kitasato flask was filtered through a 22 µm millipore filter (K18-230–KASVI, Paraná, Brazil), and at the inlet of the bioreactor, the flow was adjusted to 0.5 min^{-1}. The control of the operating temperature of the bioreactor jacket was carried out with a thermostat bath (7lab SSDu-10L, Piracicaba, Brazil). The bed temperature measurement rod was inserted in the central position at the top and was coupled to a data acquisition system type K (TES 1310, TES 1310, Rui Guang, Taiwan). To analyze the influence of light on the production of the bioemulsifier, fermentations were carried out in the dark (bioreactor isolated with aluminum foil), in the presence of white light (4.5 µmol.m^{-2}. s^{-1}) and also blue light. For this, after inoculation of the bioreactor, the system was assembled, and an LED light strip was placed around the jacket. The wavelength was adjusted and then the bioreactor was covered with aluminum foil. The determination of the emulsification index was determined after 9 days, as well as the activities of cellulolytic enzymes.

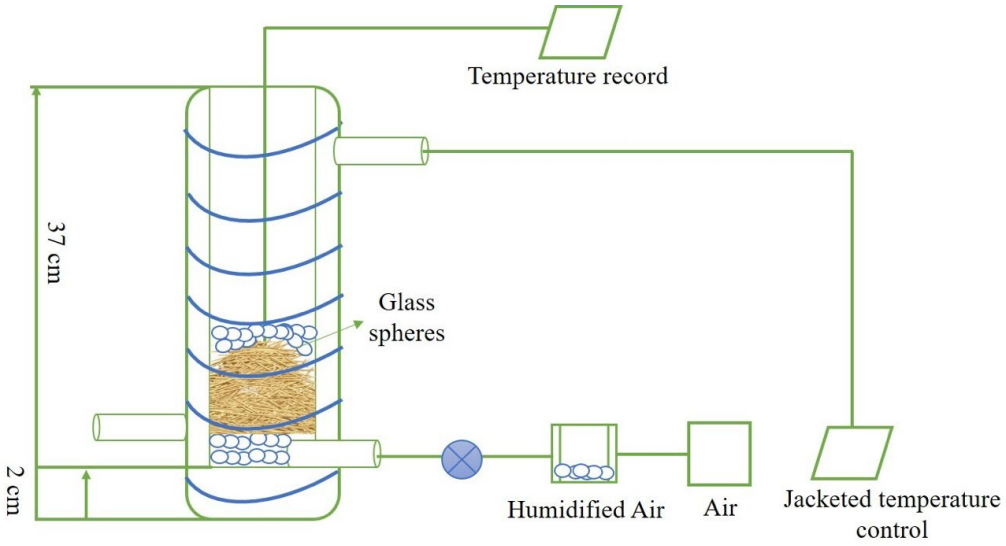

Figure 1. Diagram of packed bed bioreactor system with blue LED light for bioemulsifier production by *A. pullulans* LB83.

2.6. Extraction and Recovery of Biosurfactant

In all fermentations, sampling was performed by using sacrifice flaks. For each sampling, 3 flasks were withdrawn for evaluation of the emulsification index, and 3 for evaluation of the produced enzymes. The crude fermentation liquid was extracted with the addition of 5 mL of water per gram of bagasse, for analysis of the emulsification index, or 5 mL of 0.05 M acetate buffer, pH 4.8, per gram of bagasse, for analysis of the enzymatic activities. For extraction, the flasks were incubated on a rotary shaker (New Brunswick Scientific—Excella E24, Hamburg, Germany) at 200 rpm and 28 °C for 1 h. The liquid was separated from the biomass by pressing, and centrifugation (Novatécnica—NT 810, Piracicaba, Brazil) for 15 min at 2198× *g* was performed to separate the cells. The extraction protocol was repeated three times during the time course of the experiment to ensure the maximum recovery of BE. The bioemulsifier yield was determined gravimetrically and defined as grams of BE per gram (dry weight).

2.7. Emulsification Index

The determination of the emulsification index ($EI_{24\%}$) was carried out according to [36]. The crude fermentation liquid was mixed in test tubes with screw caps with 1:1 kerosene or soybean oil and homogenized in a vortex (Scientific Industries—Vortex Genie 2, Bohemia, NY, USA), at maximum speed, for 1 min. The emulsification index was calculated after 24 h by dividing the height of the emulsified layer (EL) by the total height of the mixture (HTM) and multiplying by 100 (Equation (1)). The measurements were carried out in triplicate.

$$EI_{24\%} = EL/HTM \times 100 \quad (1)$$

2.8. Enzymatic Activities

2.8.1. Analysis of Endoglucanase Activity

The endo-1,4-β-glucanase activity was determined according to [37]. Test tubes were prepared with 0.9 mL of 0.44% carboxymethylcellulose (SIGMA®) and 0.1 mL of enzyme extract. The mixture was incubated for 10 min at 50 °C. After that, 1.5 mL of 3,5-dinitrosalicylic acid (DNS) was added, and the tubes were boiled at 100 °C for 5 min. The absorbance reading was carried out at 540 nm. Absorbance was converted to glucose concentration according to a standard curve prepared (2.7; 2.2; 1.6; 1.1; 0.5 μmol/mL).

2.8.2. Analysis of Exoglucanase Activity

The exo-1,4-β-glucanase activity was determined using the protocol of [38]. Test tubes were prepared with 0.9 mL of Avicel 1% (SIGMA®) and 0.1 mL of enzymatic extract. The mixture was maintained for 60 min at 50 °C. After that, 1.5 mL of DNS was added, and the tubes were boiled at 100 °C for 5 min. The test tubes were centrifuged at 2564× *g* for 20 min, to separate the insoluble fraction. The supernatant was read at 540 nm and the absorbance was converted to glucose concentration according to a standard curve prepared (2.7; 2.2; 1.6; 1.1; 0.5 μmol/mL).

2.9. Scanning Electron Micrograph

PSB samples were examined by Scanning Electron Micrograph (SEM) at time 0 (before inoculation) and at the end of the fermentation process (9 days). For image processing, the dry samples were placed on carbon tape pre-fixed to a metal stump and coated with a thin layer of gold. Samples were observed at 500X magnification on the Hitachi TM3000 microscope at 15 kV.

2.10. Statistical Analysis

Obtained results were expressed as average ± standard deviation (SD) from triplicate experiments. Data were statistically analyzed using the Statistica Software (version 7.0) (StatSoft. Inc., Tulsa, OK, USA), which was used to analyze the data using the one-way

procedure, followed by a one-way linear analysis of variance (ANOVA). Tukey's test was used to perform multiple comparisons between means with a confidence level of 95%.

3. Results

3.1. Screening of LED Lights for Bioemulsifier Production by A. pullulans LB83 in Erlenmeyer Flasks

The compositional analysis of the used sugarcane bagasse showed fractions of cellulose, hemicellulose and lignin, in percentage, respectively, of: 42.0 ± 2.4; 28.8 ± 1.7; 20.5 ± 0.5 for in natura bagasse; 59.7 ± 0.6; 30.7 ± 0.4; 8.6 ± 0.6 for pre-treated bagasse. The process allowed, under the established conditions, the decrease of 74.7% of lignin content in biomass. The removal of lignin is an interesting approach to favor the use of biomass as a carbon source in SSF, considering that the fraction impairs the access of the sugar fraction by microorganisms [39,40].

Subsequently, the PSB was used in SSF to evaluate the ability of bioemulsifier production by *A. pullulans* LB83 under the influence of LED lights with different wavelengths.

As illustrated in Figure 2, under all conditions evaluated, kerosene and soybean oil were emulsified by the cell-free metabolic liquid obtained from different fermentations. This result is interesting, as it indicates that bagasse was used as a carbon source for the growth and production of the bioemulsifier. In addition, this biomass is a residue from the sugar and ethanol industry, which contributes to making the production of the bioemulsifier a more economically and environmentally sustainable process. In this scenario, the production of this biomolecule through SSF can be inserted into the circular economy system.

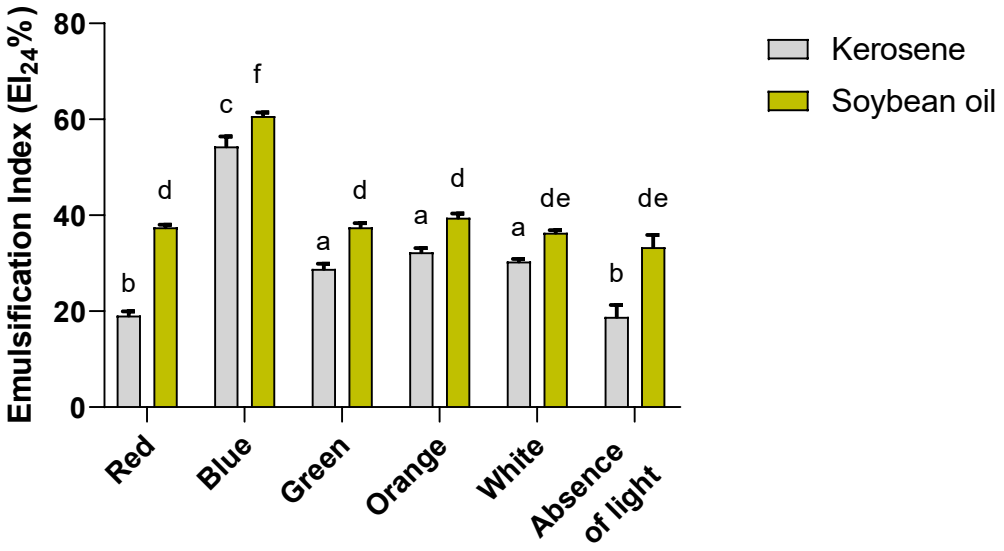

Figure 2. Emulsification index of cell-free broths obtained from SSF under the influence of different wavelengths; a, b, c, d, e, f: Equal letters indicated non-statistically significant differences at $p < 0.05$.

In general, the differences observed for kerosene and soybean oil (Figure 2) can be attributed to the chemical composition of hydrophobic substrates. In this sense, soybean oil is a mixture of fatty acids with one, two and three double bonds, respectively, while kerosene is composed of about ten different hydrocarbons with a size of C16–C20, most of them aromatic [41]. The produced BE showed a greater affinity for soybean oil, probably due to the presence of the lipid portion in its structure.

Most of the studies in the literature report the production of surface-active compounds by *A. pullulans* strains in submerged fermentation. For example, ref. [25] selected *A. pullulans* L3-GPY in a study where the yeast was grown in a synthetic medium and produced a glycerol-liamocin that reduced the surface tension to 31 mN/m. The same authors isolated another *A. pullulans* strain A11211-4-57 which synthesizes pullusurfactans A–E that were

identified as myo-inositol lipids. The novel compounds reduced the surface tension to a range between 22.90 mN/m and 32.28 mN/m, thus suggesting their potential industrial application. In other work, ref. [42] reported the production of several different surface-active compounds by *A. pullulans* YTP6-14 when supplemented with both hydrophobic and hydrophilic carbon sources into the culture media. However, the main active product was reported as a fragrant biosurfactant named massoia lactone, which showed a surface tension reduction capacity of 43.3 mN/m. The study of [33] also informed the production of biosurfactant from sucrose in a stirred tank reactor by the *A. pullulans* strain. The presence of the compound was detected through the oil spread test, with a value of 8.05 cm. However, the present study reports, for the first time, the production of a bioemulsifier in SSF from *A. pullulans* grown on lignocellulosic biomass.

As also shown in Figure 2, the use of blue LED light resulted in the highest values of $EI_{24\%}$, which means 45.1% and 63.9% for kerosene and soybean oil, respectively.

To the best of our knowledge, the increased production of bioemulsifiers by using an LED-light assisted process was not previously reported in the literature for non-photosynthetic organisms. Light and light regimes condition cell reproduction and therefore the production of extracellular polysaccharides by microalgal cells [43,44]. In addition, the emulsifying properties of the polysaccharides produced by cyanobacteria have been confirmed due to the presence of hydrophobic groups in the composition of the molecules [45,46].

Fattom and Shilo [47,48], for example, reported that the benthic cyanobacterium *Phormidium* J-1 A produced an exopolysaccharide in its ability to emulsify, called emulcyan. The process was activated by increasing the availability of light in the water column through the flocculation of suspended clay particles. Muñoz et al. [49] analyzed the effect of light intensity, among other factors, on pollutant removal by a *Chlorella sorokiniana-Pseudomonas migulae* consortium. They noted that high light intensity did not appear to affect the efficiency of the process, as algal activity increases up to a certain light saturation level, beyond which it becomes constant. The authors also observed a greater emulsifying activity in systems where algae were present, which suggested that algal activity also increased pollutant transfer and degradation rates.

You and Barnett [50] observed an increase in light intensity in the range from 39 to 70 $\mu Em^{-2}.s^{-1}$, influenced the photosynthetic capacity of *Porphyridium cruentum* cells and, consequently, increased the synthesis of EPS. The authors reported that the best EPS production results corresponded to blue LED light (465–468 nm) when compared to LED wavelengths of red (620–625 nm) and green (520–52 nm). In addition, it was highlighted that the synthesis of the compound decreased from the photon flux saturation point. Similarly, ref. [51] found that light intensity of 80 $\mu m^{-2}.s^{-1}$ caused an increase in EPS production by *Porphyridium cruentum* up to the point of light saturation, above which the synthesis of the molecule was not recorded. Clément-Larosière [52] showed that successive increases in light intensity (50, 120, 180 $\mu mol\ m^{-2}.s^{-1}$) in a cylindroconical bubble-column photobioreactor reduced the production of exopolysaccharides by *C. vulgaris.*

The strategy of the use of light in cultures of *A. pullulans* has been tested in another work to decrease the content of melanin in pullulan production. The authors of [32] applied different wavelengths of LED light and evaluated the behavior of *A. pullulans* LB83 in a medium containing glucose. The results obtained showed that blue LED light promoted the maximum production of pullulan (15.77 g/L) with low melanin content (4.46 UA540 mm/g of pullulan). The authors observed that under white light, the strain produced 11.75 g/L pullulan with high melanin content (45.70 AU 540 mm/g pullulan). However, in the current study, the blue LED light upgraded both the bioemulsifier and melanin production, as observed by a dark coloration in the fermented broth. Indeed, among all samples, those that were incubated with blue LED light exhibited the darkest coloration. An observation in the present study was carried out with the lights available in the laboratory, with photo flux from 2.8 to 8.1 $\mu mol.m^{-2}.s^{-1}$; however, future studies are necessary to determine the influence of the photon flux in the process.

This behavior can be related to the use, in our work, of conditions to favor the liamocin production. Melanin biosynthesis occurs via the dihydroxynaphthalene (DHN) pathway [53]. The PKS1 gene, which is part of this pathway, encodes polyketide synthase (Pks), which is a key enzyme in this process. At the same time, Pks is responsible for the biosynthesis of 3,5-dihydroxydecanoic acid, which is an intermediate in the liamocin synthesis pathway within the cAMP-PKA signaling pathway [54,55].

The expression of the PKS1 gene is regulated by the specific transcriptional activator Ga11. The analysis of some genes including Gal1 in the genome of *A. melanogenum* has shown that the promoters of these genes contain specific sequences that can be linked by the transcriptional activator Msn2 [49], which is controlled by a variation in glucose concentrations [56,57].

Furthermore, the activity of Msn2 has been reported as regulated signaling pathways, such as cAMP-PKA, HOG1, TORC1 and SNF1 [58]. Thus, in the current study, the presence of a high concentration of glucose resulting from the degradation of PSB, could have been detected by the transcriptional activator Msn2 that could have activated the PKS1 gene. Consequently, this cascade would activate the dihydroxynaphthalene (DHN) and cAMP-PKA signaling pathways involved in the synthesis of melanin and liamocin. However, regarding the effect of LED-light wavelength in the process specifically, there is no hypothesis to explain the observed behavior until now, and more studies are necessary to understand the mechanics of the action of light in the metabolic pathway.

3.2. Time Course of BE Production by A. pullulans on PSB under the Influence of Blue LED Light in Erlenmeyer Flasks

Blue LED light was selected in the screening experiments and an 11-day SSF was performed to analyze the influence of light on bioemulsifier production. The yield of the biomolecule was determined after the fermentation period. In addition, the $EI_{24\%}$ was also investigated using soybean oil and kerosene as hydrophobic substrates. Significant results of increased compound production were observed from the third day for those of the tested substrates (Figure 3).

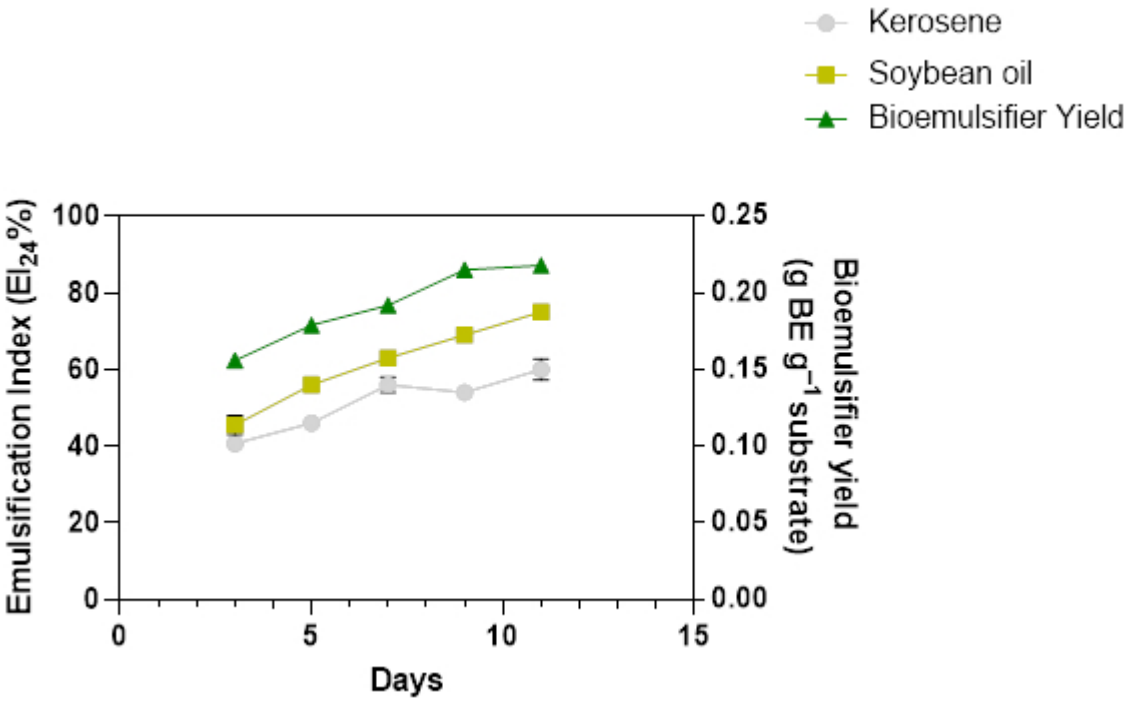

Figure 3. Time course of bioemulsifier production by *A. pullulan* LB83 under the influence of blue LED light.

This fact may be due to the yeast adaptation process at the beginning of growth, along with the reduction in water activity in the SSF [53]. After 7 days, no great increase in $EI_{24\%}$ was observed for kerosene (with a maximum of around 60%), but the value of $EI_{24\%}$ observed for soybean oil was increasing until 11 days, reaching 75%. A comparable trend was observed for the BE yield, which kept increasing over time and reached its maximum on day 11 with 0.23 g/g substrate. Similar results were observed by [59], who analyzed the production of sophorolipids by *Starmerella bombicola* from stearic acid (C18:0) and molasses in SSF. Polyurethane foams (PUF) were used as an inert support and the maximum yield was detected at 13 days with 0.211 g of SLs per g of substrates.

In the literature, several studies report the use of lignocellulosic biomass as support to produce biosurfactants/bioemulsifiers by fungi [55,56] but not for *A. pullulans*. Velioglu and Urek [60] investigated the concomitant production of biosurfactant and lignolytic enzymes (laccase, manganese peroxidase and lignin peroxidase) in SSF by different strains of *Pleurotus* spp. Sunflower seed husks were used as a solid substrate for support, and the results revealed that *P. djamore* produced the most active biosurfactant with 29.79 $\pm$ 0.5 mN/m, 35.29 $\pm$ 2.6% and 3.5 $\pm$ 0.3 cm of surface tension, emulsification index and diameter of the clear zone, respectively. In addition, the lignin peroxidase activity was 5832.26 $\pm$ 102 UL^{-1}. In more recent work, ref. [61] studied lignocellulosic residues and inert materials as support in SSF to produce sophorolipids from *Starmerella bombicola* ATCC 22214. They observed that, among lignocellulosic biomass, rice husk and wheat straw encouraged higher yields with 0.15 g and 0.20 g of SLs per g of dry matter, respectively. Interestingly, ref. [62] evaluated the liamocins production from different agricultural biomass by *A. pullulans* strain NRRL 62031 in submerged fermentation. The authors revealed that pretreated wheat straw allowed a significant liamocin production at lower concentrations but with melanin residue. Furthermore, maximum biomolecule production yield was observed on day 11 with 0.220 g BE per g of substrates (Figure 3). Similarly, ref. [59] reported a yield of 0.211 g sophorolipids per g of substrates from *Starmerella bombicola* SSF grown on polyurethane foam, which functioned as the inert support.

3.3. Determination of the Enzymatic Activity from SSF under the Influence of Blue LED Light in Erlenmeyer Flasks

The influence of blue LED light on enzyme synthesis was also evaluated during the SSF process. It was observed that enzyme production followed a similar trend regarding the emulsification index. In this sense, the detection of cellulolytic enzymes was a confirmation of the use of PSB as a nutrient to support yeast-like fungus growth. This is possible due to the reduction of the lignin content and the availability of fermentable sugars in the pretreated biomass. In general, it was observed that the enzyme production of the raw extracts increased during the fermentation. However, among the two enzymes determined, the highest values corresponded to endoglucanase (Figure 4).

The enzymatic activity of the raw extract obtained varied during the fermentation process. However, the highest values corresponded to endoglucanase with a maximum of 3.67 $\pm$ 0.03 IU g^{-1} on the eleventh day. For exoglucanase, the maximum value was also detected on the same day with 0.41 $\pm$ 0.01 IU g^{-1}. In this sense, the sharper increase in exoglucanases can be explained by its general mechanism. These enzymes attack the ends of the crystalline cellulose, and cellobiose molecules (glucose dimers) are released. As the metabolic demand of the microorganism increases, enzyme action is required to continue cleaving cellobiose units until a minimum length of cellulose microfibrils is reached [63,64].

The detection of cellulolytic enzymes has been widely documented for fungi, but, for *A. pullulans*, there is less information. For instance, ref. [63] performed a screening of *Aureobasidium pullulans* isolates from temperate regions for cellulase production. In the study, a minimal salts medium supplemented with sawdust was used, and endoglucanase and exoglucanase activities were observed ranging from 2375 to 12,884 µmol of glucose (mg of protein)$^{-1}h^{-1}$, and 0.293 to 22.442 µmol of glucose (mg protein)$^{-1}$ day^{-1}, respectively. Leite et al. [65] investigated the synthesis of cellulases by *Aureobasidium pullulans* ER-16

in solid-state fermentation using wheat bran, soybean meal, soybean hulls and corn cob. The results indicated that wheat bran encouraged the highest cellulase production with 1.05 U/mL of endoglucanase and 1.3 U/mL of β-glucosidase. Vieira et al. [66] evaluated cellulase production under submerged fermentation conditions with the strain used in this work. In the study, different lignocellulosic biomasses such as corn cobs, sugarcane bagasse and sugarcane straw were submitted to alkaline pretreatment for use as carbon sources. The authors reported a maximum cellulase activity of 7.42 U/mL.

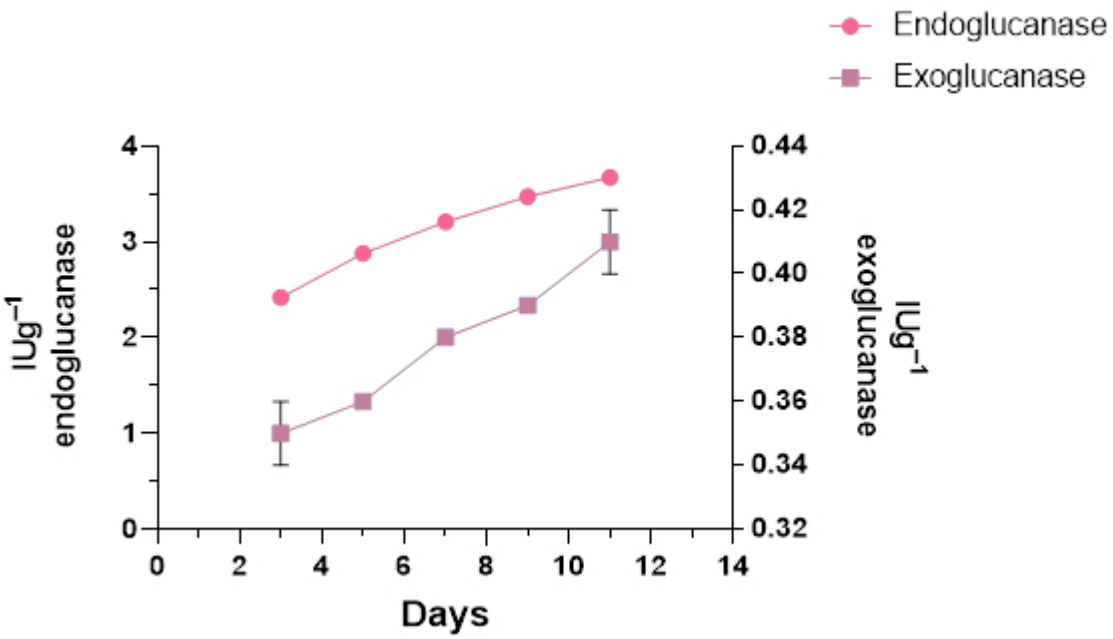

Figure 4. Time course of cellulolytic enzyme production by *A. pullulans* LB83 under the influence of blue LED light.

3.4. Bioemulsifier Production by A. pullulans on PSB under the Light Influence in Packed Bed Bioreactor

The emulsification index, BE yield and production of cellulosic enzymes in the packed bed bioreactor were evaluated after 9 days of cultivation, and the results are shown in Table 1.

Table 1. Different configuration of packed bed bioreactor for emulsification index, BE yield and production of cellulosic enzymes after 9 days of the process (results are shown as average ± standard deviation).

Bioreactor	$EI_{24\%}$ Kerosene	$EI_{24\%}$ Soybean Oil	BE Yield (g BE g^{-1} Substrate)	Endoglucanase (Ug^{-1})	Exoglucanase (Ug^{-1})
Without LED light	29.5 ± 0.6 [a]	36.9 ± 0.8 [a]	0.09 ± 0.6 [a]	2.94 ± 0.02 [a]	0.45 ± 0.00 [a]
White LED light	32.5 ± 0.7 [b]	41.1 ± 0.8 [b]	0.13 ± 0.5 [b]	3.25 ± 0.02 [b]	0.48 ± 0.00 [b]
Blue LED light	45.7 ± 0.4 [c]	55.0 ± 0.9 [c]	0.20 ± 0.3 [c]	3.87 ± 0.02 [c]	0.53 ± 0.00 [c]

a, b, c: Equal letters indicated non-statistically significant differences at $p < 0.05$.

The six-column, four-row table shows the emulsification index ($EI_{24\%}$) values with kerosene and soybean oil, the yield in grams of biomass per gram of dry substrate, and the endoglucanase and exoglucanase enzymatic activities, for three bioreactor configurations (in the dark, with white LED light and with blue LED light).

To complement the ANOVA results, Tukey's test was used to evaluate statistically significant differences ($p \leq 0.05$) between different packed-bed bioreactor configurations in BE production. It was observed that, among the alternatives tested, the presence of blue

LED light promoted the highest values of the emulsification index. Similar to the results observed in the emulsification index in bottles, soybean oil showed better performance when compared to kerosene. In addition, the blue LED light enhanced 2.2 and 2.5 times the BE yield when compared to the configurations without and with white LED. In the case of enzymes, the enzymatic activity of endoglucanase was favored in the presence of blue light when compared to exoglucanase. However, the emulsification index and enzymatic activity values were lower than the results obtained in the same condition, in flasks. This fact may be related to aeration, which was probably impaired in a packed bed composed of bagasse. As shown in the work of [33] with *A. pullulans* in a stirred-tank reactor, higher aeration rates led to higher biosurfactant production. Thus, it would be interesting to carry out studies by varying the aeration rate to establish the appropriate conditions for the packed bed bioreactor process.

Scanning electron microscopy (SEM) allowed for observing the morphology of the surface of pre-treated sugarcane bagasse to compare the structural differences that occurred at the beginning and end of the fermentation process. In Figure 5A, some micropores can be observed on the surface of pre-treated sugarcane bagasse at time 0 (before inoculation) due to the partial removal of hemicelluloses and lignin, which increases the surface area available for enzyme access.

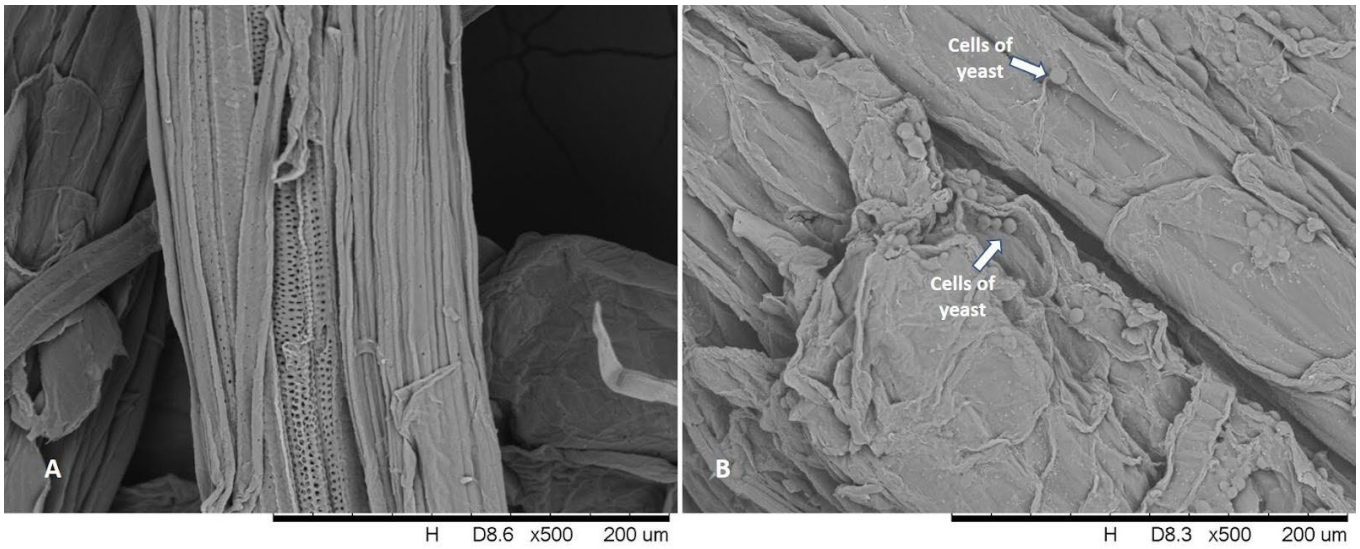

Figure 5. Scanning electron microscopy (SEM): (**A**) PSB at time 0 (before inoculation) with some micropores can be observed on the surface of pre-treated biomass due to partial removal of hemicelluloses and lignin; (**B**) PSB at the end of the fermentation process (9 days) with the presence of yeast cells on the surface of the biomass.

In this sense, some studies have shown that when pores have a larger internal area, cellulase enzymes can become trapped in these cavities [67,68]. On the other hand, Figure 5B shows the presence of yeast cells attached to the surface after 9 days of SSF. Some structural modifications such as holes and cracks may appear on the surface of the biomass due to the degradation of polysaccharides in the cell wall of the plant cell [69]. This result reinforces the use of bagasse as the sole source of carbon by *A. pullulans* LB83 for growth and bioemulsifier production.

4. Conclusions

The results showed the feasibility of using lignocellulosic biomass as a carbon source by *A. pullulans* LB83 to produce high-added-value products such as bioemulsifiers. The detection of enzymatic activity in the crude extracts indicated that PSB was used as a support and carbon source in SSF. Likewise, it was observed that the blue LED light increased the production of bioemulsifier. Among the different alternatives tested in the

bioreactor, blue light showed the best results, although a decrease was observed when compared to the results in Erlenmeyer flasks submitted to the same conditions. This situation could be improved by testing different airflows or aeration modes in the bioreactor.

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