Bioresource Technology 230 (2017) 76–81

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Low-melanin containing pullulan production from sugarcane bagasse hydrolysate by *Aureobasidium pullulans* in fermentations assisted by light-emitting diode

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HIGHLIGHTS

- Low-melanin containing pullulan production by wild strain of *A. pullulans*.
- Blue LED light inhibits synthesis of melanin in wild strain of *A. pullulans*.
- Blue LED light improves the pullulan production by *A. pullulans*.
- Sugarcane bagasse (SCB) as an important alternative source for pullulan production.
- *A. pullulans* metabolizes different sugars present in SCB hydrolyzed.

ARTICLE INFO

Article history:
Received 10 December 2016
Received in revised form 25 January 2017
Accepted 27 January 2017
Available online 30 January 2017

Keywords:
Pullulan
Exopolysaccharide (EPS)
Melanin pigment
Light-emitting diode
Sugarcane bagasse
Enzymatic hydrolysate

ABSTRACT

Pullulan is a polymer produced by *Aureobasidium pullulans* and the main bottleneck for its industrial production is the presence of melanin pigment. In this study, light-emitting diodes (LEDs) of different wavelengths were used to assist the fermentation process aiming to produce low-melanin containing pullulan by wild strain of *A. pullulans* LB83 with different carbon sources. Under white light using glucose-based medium, 11.75 $g.L^{-1}$ of pullulan with high melanin content (45.70 $UA_{540nm}.g^{-1}$) was obtained, this production improved in process assisted by blue LED light, that resulted in 15.77 $g.L^{-1}$ of pullulan with reduced content of melanin (4.46 $UA_{540nm}.g^{-1}$). By using sugarcane bagasse (SCB) hydrolysate as carbon source, similar concentration of pullulan (about 20 $g.L^{-1}$) was achieved using white and blue LED lights, with lower melanin contents in last. Use of LED light was found as a promising approach to assist biotechnological process for low-melanin containing pullulan production.

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1. Introduction

Polymers, mainly derived from petroleum sources, present excellent mechanical properties, low cost and versatility; however, usually they pose serious environmental problems due to their persistent and non-biodegradable nature (Thompson et al., 2010). In recent years, alternative biopolymers have attracted a lot attention, mainly due to several aspects superior to petroleum-derived polymers as biocompatibility and biodegradable (Mitra et al., 2011). Their specific properties turn possible their applications as medical material, packaging, cosmetics, food additives, biosensors and others (Niaounakis, 2015). These materials can be produced from microbial sources (e.g. xanthan, polyhydroxyalkanoate and pullulan), extracted from animals (e.g. chitosan and collagen) and plants (e.g. starch and gums) or chemically synthetized (e.g. poly (lactic acid)) (Koutinas et al., 2014). Microbial polymers are usually produced by *Pseudomonas*, *Lactobacilli*, *Xanthomonas* and *Aureobasidium* species (Öner, 2013).

Pullulan is one such biopolymer mainly produced by *A. pullulans* (Jiang et al., 2011). It consists of maltotriose repeating units further connected by α-(1→6) linkages. This particular linkage confers it a considerable solubility in water compared to other polysaccharides. Moreover, it can form thin films that are transparent, odorless and almost impermeable to oxygen, enabling its multipurpose applications in packaging materials, as a sizing

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http://dx.doi.org/10.1016/j.biortech.2017.01.052
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agent, and for food industry as a replacement of starch in low-calorie formulation (Wu et al., 2016).

However, *A. pullulans* strains are associated with the formation of a pigment (melanin) unwanted in pullulan production process. This pigment induces a rather darker color to the end product and prompts an additional decolorization process making it an expensive choice (Wu et al., 2009; Ravella et al., 2010). Thus, the production of pullulan specifically with limited melanin formation by wild strain of *A. pullulans* is a technological need.

Previously, various process parameters, as pH, oxygen and temperature, have been studied to investigate their effects on the qualitative production of pullulan. Nevertheless, for its production from *A. pullulans*, the effects of light incidence with different wavelengths were not explored yet, although these have been studied previously for other strains. For example, in *Neurospora crassa*, the blue light regulates the circadian rhythms and processes such as synthesis of carotenoids (pigment photo-protector), spore formation and phototropism (Linden, 2002). In another work, inhibition of mycotoxin production under different spectrum of light by *Aspergillus* was related by Haggblom and Torgny (1979). For extracellular polysaccharide production (EPS), effect of light was related by Han et al. (2014) and Ge et al. (2014) for strains of *Nostoc flagelliforme* and *Nostoc* sp., respectively.

Another key aspect for the pullulan production is the carbon source used in the fermentation process. Several cheaper carbon sources can be assimilated by *A. Pullulans* such as raw potato starch hydrolysate (Wu et al., 2016), sugarcane juice (Vijayendra et al., 2001), rice hull hydrolysate (Wang et al., 2014), cassava starch residue (Ray and Moorthy, 2007) and sugarcane bagasse hemicellulosic hydrolysate (Chen et al., 2014). However, a complex mix of sugars (glucose, xylose, cellobiose and oligomers) from the carbohydrate lignocellulosic fractions can also be a potential carbon source for its production (Terán-Hilares et al., 2016; Terán Hilares et al., 2016; Zhang and Zhu, 2016).

This study deals with the effects of light-emitting diode (LED) wavelengths on the pullulan production by a wild strain of *A. Pullulans* using glucose and SCB hydrolysates as carbon sources, considering also the effect of light incidence in melanin synthesis during process.

2. Material and methods

2.1. Preparation of SCB hydrolysate

To employ SCB hydrolysate as a carbon source, it was prepared according to our previous study (Terán-Hilares et al., 2016). The schematic diagram of this system is shown in the Supplementary material (Fig. S1). Briefly, SCB was pretreated with 0.3 M NaOH in a packed bed flow through column reactor at 70 °C for 4 h, maintaining a recirculating flow ratio of 20 mL.min^{-1} with a peristaltic pump ALITEA-XV (Bioengineering AG-Wald, Switzerland).

The enzymatic hydrolysis was performed in the same reactor maintaining a high solids concentration (18%) with constant recirculation through the reactor of 200 mL of buffer solution containing commercial cellulases Cellic® CTec2 (Novozyme, Brazil) at enzyme loading of 20 FPU.g^{-1} of SCB. The enzymatic hydrolysis was carried out during 48 h at 50 °C and sugars obtained was used for pullulan production (main sugars: 50 g.L^{-1} of glucose and 25 g.L^{-1} of xylose).

2.2. Microorganism and inoculum preparation

A wild strain of *Aureobasidium pullulans* LB83 was kindly donated from Center for Study of Social Insects (CEIS/UNESP-Brazil). This strain was isolated from bitu ant (*Atta sexdens rubropilosa*) collected and identified in the state of São Paulo, Brazil, by Arcuri et al. (2014). Microorganism was stored at 4 °C on yeast malt agar medium, until use. The inoculum was grown in a rotary shaker at 28 °C and 200 rpm during 48 h in a 125 mL Erlenmeyer flasks containing 50 mL of medium composed by 20 g.L^{-1} of glucose, 0.6 g.L^{-1} of $(NH_4)_2SO_4$, 2.5 g.L^{-1} of yeast extract, 2 g.L^{-1} of K_2HPO_4, 1 g.L^{-1} of NaCl and 0.1 g.L^{-1} of $MgSO_4$. After the intended time, the medium was centrifuged and suspension was prepared in sterile distilled water. About 1 mL of cell suspension was inoculated in the fermentation flasks to obtain about 1.0 g.L^{-1} (dry weight) of initial cell concentration.

2.3. Fermentation assisted by different LED lights

Initial fermentation experiments were carried out with *A. pullulans* LB 83 in 125 mL Erlenmeyer Flasks containing pure glucose, 45 g.L^{-1} supplemented with 0.6 g.L^{-1} of $(NH_4)_2SO_4$, 2.5 g.L^{-1} of yeast extract, 2 g.L^{-1} of K_2HPO_4, 1 g.L^{-1} of NaCl and 0.1 g.L^{-1} of $MgSO_4$ (Wang et al., 2014). Erlenmeyer flasks containing fermentation medium were isolated from white light (fluorescent lamps) and kept at about 3 cm of LED light source. LED lights with wavelengths between 620 and 630 nm for red, 585 and 595 nm for orange, 515 and 535 nm for green and 450 and 470 nm for blue were used to illuminate each Erlenmeyer flask during fermentation. Used photon flux density was 440 µmol.m^{-2}.s^{-1}. Control experiments were also performed under dark condition and white light (fluorescent lamps). In order to differentiate among each fermentation condition (incidence of light), Tukey's range test was performed for pullulan production achieved after 96 h of fermentation time, aided by the software STATISTICA 8.0 (StatSoft, Inc., Tulsa, OK, USA). All the experiments were carried out in a rotary shaker at 28 °C and 200 rpm for 96 h. Samples were taken periodically, centrifuged at 2000×*g* for biomass removal and saved in the refrigerator prior to analyzing for the substrate consumption, cell growth, pullulan exopolysaccharide production and melanin presence.

2.4. Recovery and analysis of the product

For pullulan recovery, the centrifuged fermentation broth samples were precipitated with 1 vol of absolute ethanol; the precipitate was dissolved in deionized water (80 °C) and dialyzed at 4 °C for 48 h to remove small molecules and residual sugars. In the end, it was precipitated once more with 1 vol of absolute ethanol for subsequent centrifugation and drying at 80 °C (Wu et al., 2016). The biomass in fermented broths were also recovered by washing and making them dry at 70 °C (Wang et al., 2013). The sugar contents were analyzed by High Performance Liquid Chromatography (HPLC) Agilent 1200 series (Agilent Technologies, Inc., USA) equipped with a Refractive index detector RID-6A and a HPX-87H (300 × 7.8 mm) column (Bio-Rad, USA). Used conditions were the following: 45 °C column temperature, 0.01 N H_2SO_4 as the mobile phase, 0.6 mL/min flow rate, and 20 µL injection volume (Ahmed et al., 2016).

2.5. Pullulan production using SCB hydrolysate as carbon source

The SCB hydrolysate obtained as described in Section 2.1 was used for pullulan production. To this, additional supplements as described in Section 2.3 were also added. Initial sugar concentrations in the medium were 37.29 g.L^{-1} of glucose, 22.5 g.L^{-1} of xylose, 4.49 g.L^{-1} of cellobiose and 2.04 g.L^{-1} of arabinose. The samples were periodically taken and centrifuged at 2000×*g* for biomass removal. Pullulan recovery and determination was carried out according to the same procedure above described (Section 2.4).

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