STUDY OF THE INFLUENCE OF pH ON THE MORPHOLOGY OF *A. PULLULANS* FOR BIOTECHNOLOGY PRODUCTION OF PULLULAN

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ABSTRACT

In order to slow down the damage caused by plastics produced by the oil industry, as they are difficult to decompose and have high toxicity, several studies have been leveraged that point to the production of exopolysaccharides (EPS) instead. That is because of its economic advantage and the possibility of reducing costs using renewable raw materials, mainly agribusiness by-products and industrial waste. Among EPS, pullulan has stood out for presenting excellent mechanical properties and gas exchange restrictions in the production of biopolymer packaging. However, the yield and productivity of the process are strongly affected by several factors, one of the essential being pH. The objective of this research was to evaluate the fermentation process of a low-cost renewable raw material (VHP sugar, very high polarization sugar) and an agro-industry by-product (steep corn liquor) with and without pH control on the behavior of the Aureobasidium pullulans Y2092 strain, to develop an economically more advantageous bioprocess to obtain pullulan of commercial interest. The production medium consisted of KH2PO4, MgSO4.7H2O, and NaCl, plus 30 g/L VHP and 7.0 g/L of steep corn liquor, as sources of carbon and nitrogen, respectively. The tests were performed in triplicate: one assay using phosphate buffer to control pH and the other without buffer. Both assays were started at pH 5.5. After 72 h, the pullulan production of assays 1 and 2 was 23.1 g/L and 8.6 g/L, indicating a reduction higher than 60% in the assay without phosphate buffer. FTIR analyses are shown to prove pullulan obtainment. The thermal stability between the pullulan obtained and the commercial pullulan was similar. The result indicates that the synthesized pullulan is suitable for future biopolymer films, valuing agro-industrial waste and reinforcing the reduction of pollution levels from production to disposal of current plastic packaging.

Keywords: pullulan, exopolysaccharide,biosynthesis, fermentation, biopolymeric films

INTRODUCTION

Biopolymers have become a sustainable alternative to the development of packaging for the food industry, replacing plastics from the petrochemical industry, accumulating and harming various systems in nature. Thus, the study and development of biopolymers have grown over the years. From the immense diversity of biopolymers present in nature, microbial polysaccharides have stood out for their economic advantage and the possibility of reducing costs in the production process as it is possible to use several renewable raw materials, such as agro-industry by-products and industrial residues of the most different natures, as a source of carbon and nitrogen. Among the infinity of microbial polysaccharides, pullulan has been highlighted in the most recent studies. Pullulan can be classified as an exopolysaccharide (EPS), which, in addition to being one of the few to have no ionic charge, has applications in important industrial sectors such as pharmaceuticals and food.[1,2]

Pullulan can be produced by an aerobic fermentation process, by the dimorphic fungus of the *Aureobasidium pullulans* strain in the yeast-like state, although its biosynthesis has not yet been fully elucidated[1]. Pullulan has a particular structural organization that gives it exciting properties, such as mechanical flexibility and restriction in the passage of oxygen, which is essential for the efficient production of biofilms. It is a homopolysaccharide composed of monomeric units of maltotriose, joined together by α-(1→6) glycosidic bonds, although the glucose units in maltotriose are linked by α-(1→4) bonds.[3]

Several studies show that it is essential to define and control the conditions for the production of pullulan as a source of carbon and nitrogen, pH, temperature, and agitation, as changes in these parameters during production can lead to deviations in the microorganism's biosynthesis route. Moreover, it affects production. As mentioned earlier, the fungus *Aureobasidium pullulans* has two microscopic morphologies: unicellular (yeast-shaped) or filamentous, and pullulan synthesis occurs when the fungus presents unicellular development[6]. Thus, it is necessary that factors such as pH, which influence the morphological nature of the fungus, be controlled, as they influence the yield of production and the molecular weight of the polymer[7]. In general, acidic pHs lower than 5.0 reduce the yield of the product by inducing filamentation, while pHs between 5.0 and 7.5 favor yeast cells. In addition, the stress in the reaction medium leads the microorganism to the production of blastospores (spore formation) and melanin, which promotes the undesirable browning of the product.[1,4]

Using agro-industrial residues to produce pullulan is essential to reducing production costs. *A. pullulans* can be synthesized by simple and more complex forms of carbon and nitrogen sources, which facilitates using residues and undesirable co-products from other processes. Potato starch residues from the brewing industry, various agro-industrial processes, and by-products from the sugar industry have been reported by several researchers. The use of such residues in the substrate of fermentation media presents both ecological and economic advantages. However, the purity of the pullulan produced may vary according to the complex substrates used as carbon and nitrogen sources.[3,5,6]

In the present research, the behavior in the production of pullulan via fermentation by *A. pullulans*, in the presence or not of buffer (pH control), was evaluated, aiming for its use as a sustainable raw material in the production of biopolymer films. In addition, corn-steep liquor as a source of nitrogen, an agro-industrial residue, makes the process more ecological and economical. As an alternative to a carbon source, VHP is used. VHP is considered an economically viable and abundant raw material in Brazil. It is the type of sugar most exported by several Brazilian companies, while the world production of VHP evaluated in 2014 was over 170 million tons. Analysts estimate that consumption of this sugar continues to be high because of its low cost (USD 0.40/kg), easy refining, and high availability.[7]

MATERIALSAND METHODS

Microorganism

The strain *A. pullulans* Y2092, provided by the Center for Taxonomic Collections of the Institute of Biological Sciences - Federal University of Minas Gerais, was used and stored in an ultra-freezer at a temperature of -80 °C. To prepare the inoculum, the contents of a cryovial, after rapid thawing, were transferred to 100 mL of Sabouraud broth in 500 mL Erlenmeyer flasks, which were then incubated on a rotary shaker (Shaker Controlled Environmental Incubator –Tecnal, TE- 420), at 150 rpm, overnight.

<u>Pullulan production</u>

A bibliographic review and subsequent tests were carried out to produce the medium's composition, not shown in the present research. The media were constituted by (in g/L): KH2PO4 (5.0), MgSO4.7H2O (0.2), and NaCl (1.0). The mineral medium added 30 g/L VHP and 7.0 g/L of maize as carbon and nitrogen sources, respectively. The tests were performed in triplicate, one using phosphate buffer (PO_4-HPO_4) to control pH and the other without pH control.

Both had their pH adjusted to 5.5 with 1M NaOH, and the flasks were autoclaved at 1 atm for 15 min. Each medium (100 ml) was inoculated with a volume of the Sabouraud broth culture to establish an initial number of 105 cells. The cells' morphological analysis was determined by examining fresh preparations using the methylene blue dye, in a Nikon Eclipse E200 optical microscope, with a 40x magnification. The flasks were then incubated on a rotary shaker at 28°C and 150 rpm.

After 72 h, cells in the fermented media were inactivated by heating at 80 °C with constant stirring for 10 min. After cooling to room temperature, the media were centrifuged (BioVera refrigerated centrifuge) to separate the cells, and ethanol P.A was added to the supernatant at a ratio of 1:3 (medium: ethanol) to precipitate the polymer, which was then vacuum filtered and dried in an oven at 35 °C until constant weight.

<u>Analytical determinations</u>

As described by Wu et al., (2016)[8], the residual concentration of reducing sugars was determined. With the supernatant, hydrolysis was performed with 2M HCl at approximately 70°C for 10 minutes; after cooling, 1M NaOH was added. Then, dinitrosalicylic acid (DNS) was used for quantification by spectrophotometry at a wavelength of 540 nm, using a standard curve of pure glucose (200 mg/mL).

The results related to the biopolymer concentration were used to calculate the conversion factor $Y_{P/S}$, indicating substrate conversion to product (yield). $Y_{P/S}$ can be calculated according to Equation (A):

$$Y_{P/S} = \frac{P_f - P_0}{S_f - S_0} \quad \text{(A)}$$

S and P represent carbon source and product (biopolymer) concentrations;
The f and 0 subscripts represent the final and initial values of the respective variables.

<u>Characterization</u>

Spectrometric (FTIR) analyses, ranging from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}, were made in 32 scans and over a KBr pellet or by attenuated total reflectance. For the thermogravimetric analysis, a TGA device model TA Instruments 5500 TGA was used, in an inert atmosphere (N_2), with a nitrogen flow of 20 mL/min and a heating rate of 10 °C/min.

The analysis will be carried out at a temperature between 25 and 900ºC. Analyses were performed at the Laboratory of Thermoanalysis and Rheology of EQ/UFRJ.

RESULTS AND DISCUSSION

Table 1 shows the values of pullulan production and yield according to whether or not buffer was used in the medium. Both media had their initial pH adjusted to 5.5.

Table 1: Test results for pullulan production.

Media	Phosphate buffer	pH final	Production (g/L)	Yield (g/g)
1	Yes	5.59	23.1	0.859
2	Not	6.29	8.6	0.320

It is noted that the production showed a reduction above 60% when the phosphate buffer was not present in the biopolymer production medium. Considering that the pullulan synthesis has not yet been fully elucidated, it is believed that a change in pH to less than 5 occurred during the pullulan biosynthesis process. The pH may have reached variations below 5 due to the production of by-products after some time, in which part of the yeast cells changed to their filamentous form, not producing EPS. Thus, during this phase, a decrease in production may have occurred, in addition to stress in the reaction medium. This morphological change, followed by stress, led to melanin production, evidenced by the dark brown color in the fermented must and the final product.[1,2]

In addition, as the available nitrogen is in the complex form of corn-steep liquor and a smaller amount of carbon, the microorganism may have consumed the entire carbon source first. It leaves more nitrogen resulting in the production of amines at the end of production, which explains the final increase in the pH of medium 2, as shown in Table 1. The pullulan produced from medium 2 presented a dark brown appearance, notably with many impurities. In contrast, the product of medium 1, after precipitation and drying, presented an appearance of white powder, as shown in Figure 1, which evidenced an appearance similar to commercial pullulan.[9]

Figure 1: Appearance of pullulan produced from medium 1.

In Figure 2, it is possible to verify the FTIR spectrum of the pullulan obtained. The presented spectrum corroborates with several studies obtaining pullulan.[2,8,10]

A wide band is evident at 3440 cm^{-1}. This range is attributed to the elongation - OH. A more accentuated range, approximately at 2920 cm^{-1} and 2250 cm^{-1}, is characteristic of the symmetrical and asymmetrical vibrational stretching of CH_2 groups and the vibrational stretching of CH. A sharper peak of 1630 cm^{-1} is characteristic of the O-C-O and glycosidic bonds. The characteristic peak around 1418 cm^{-1} is attributed to the bending of C-O-H bonds. The range corresponding to stretching at C-O-C and C-O is observed at 1031 cm^{-1}. The band close to 930 cm^{-1} indicates the presence of α-1,6-d-glycosidic bonds.[3,6,10]

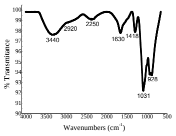

Figure 2: FTIR of the biopolymer produced by strain Y2092.

The thermal stability of the reference pullulan and the obtained pullulan are shown in Figure 3.

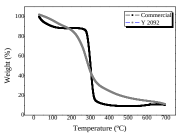

Figure3: Thermogravimetry of biopolymers: commercial pullulan and pullulan obtained.

The maximum peak, which indicates the degradation temperature of the material, that is, the maximum point of thermolysis, occurs at 302.08°C and 279.74°C, respectively, for commercial and synthesized pullulan. That demonstrates a decrease of about 22°C between the materials and a slightly lower thermal stability for the synthesized pullulan. Although these results indicate a slightly lower thermal stability of the biopolymer obtained, possibly associated with the molar mass, presence of aggregates and contaminants (mainly inorganic) in the fermentation medium, the thermal stability of the pullulan obtained in comparison to commercial pullulan was similar to the research of other authors. [3,6,10]

CONCLUSIONS

It is possible to evaluate that the pullulan obtained in this work presents similar characteristics to the commercially available pullulan and with the research of several authors. In this way, it is possible to infer that it is possible to produce pullulan with agro-industrial residues and unwanted by-products from the industry, valuing these products and encouraging the dissemination of the production of biopolymers that can replace polluting plastic packaging.

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