Low Cost Production of Pullulan Obtained From Granulated Sugar and Different Nitrogen Sources

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Abstract—Pullulan production using white granulated sugar as carbon and energy source by two Aureobasidium pullulans strains (IOC 3467 and IOC 3011) was studied aiming cost reduction and maximization of the process yield. For this purpose, different sources of nitrogen – NaNO\textsubscript{3}, (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, NH\textsubscript{4}NO\textsubscript{3}, urea and residual brewery yeast – were added to the medium in different concentrations to a carbon/nitrogen ratio of 5 and 150. All nitrogen sources in the proportions tested were capable to promote cell growth and biopolymer production by both strains. However, for both stains fermentations, the amount produced and broth viscosity were dependent on the tested conditions. Overall, the best results were observed for the IOC 3011 strain. Residual brewery yeast was the nitrogen source that provided the greatest pullulan yield (0.48 and 0.55 g/g, strains IOC 3467 and IOC 3011, respectively) and the highest values of fermented broth viscosity for both strains. The results showed that the use of white granulated sugar and residual brewery yeast as carbon and nitrogen source, respectively, increased the pullulan production. The use of these carbon and nitrogen sources allow the cost reduction of pullulan production since they are renewable feedstock, low-cost, abundant and available worldwide.

Index Term—Biopolymer, Industrial waste, Nitrogen source, Pullulan, Rheology

I. INTRODUCTION

PULLULAN is an exopolysaccharide produced by yeast-like fungus Aureobasidium pullulans. It is a polymer consisting of linear maltooliose repeating units interconnected by α-(1→6) glycosidic bonds. This important biopolymer has applications in several industrial sectors like pharmaceutical, food, and cosmetic industries [1]. Pullulan can form oil resistant and impermeable to oxygen thin transparent films. Therefore, it can be used as coating and packaging material, sizing agent for paper, starch replacer in low-calorie food formulations, cosmetic emulsions, and industrial applications [2]-[6]. Recently, pullulan has been widely used in pharmaceutical industry as a biomaterial [7]-[12]. Many works have studied the modification of pullulan for new applications [13]-[16].

Published reports indicate that components used in the media represent significant production cost and it may even reach up to 30% of the total production cost [17]. Therefore, it is important to find low-cost substrates for pullulan production, which will make the process economically feasible. Reference [18] reported that the pullulan production cost is about three times higher than that of other polysaccharides. So, the development of economical processes for the production of expolysaccharides should be focused [17], [19]. Various approaches have been adopted to reduce pullulan production cost, which include engineering innovations, improved strains [20] and identification of cheaper and effective carbon and nitrogen sources [21].

Most fermentation processes employ glucose and sucrose as carbon sources on the development of the fermentation medium [22]. Various other carbon sources are already studied for the production of pullulan, including: grape pulp [23]; peat hydrolyzate [24],[25], the bioethanol production stillage [26], beet molasses [23], [27], locust bean [27], soybean oil [28]; potato starch hydrolyzate [28], [29], [21], brown sugar [30], jackfruit seed powder [31], cassava starch, wheat bran and rice [32], corn syrup and cashew juice [33], sweet potato [34], sugar crystal [35]; among others. The number of studies related to the issue, involving different groups of researchers, confirms the importance of developing technologies for the industrial production of pullulan. In industrial scale production, pullulan is made by the fermentation of hydrolyzed starch with Aureobasidium pullulans strain non-genetically modified [36], [37]. However, our country has other raw materials, relatively cheap, with good availability, and directly fermentable. White granulated sugar, whose the substrate is sucrose, is a carbon and energy source. The sugar produced in crystalline form without refining is widely used in the food industry for making beverages, biscuits and confectionery, among others. In Brazil, sugar crystal is already being used as a raw material for different companies for microbial production of high value-added products, such as lactic acid and citric acid [38]. Additionally, it is a renewable, low-cost,
abundant and available feedstock in Brazil [39]. Therefore, the crystal sugar appears as an interesting substrate to obtain pullulan.

Further, several kinetics parameters can affect the ratio of pullulan content in relation to the total exopolysaccharide produced. The quantity and quality of the produced pullulan can be affected by both type and concentration of the nitrogen source in the production medium [40]-[42]. This is due to the fact that nitrogen source affects the growth and the metabolic activities of the microorganism, including their morphology [33]. Most studies used as inorganic nitrogen source ammonium sulfate, sodium nitrate, sodium nitrite, potassium nitrate and ammonium nitrate, and as organic sources, yeast extract, casein hydrolyzed, peptone, glutamate, L-asparagine and ammonium succinate [40], [43]-[44].

Taking this into consideration, the aim of this investigation was to examine the possible utilization of white granulated sugar as a carbon source, and five different concentrations of different nitrogen sources for pullulan production by two *Aureobasidium pullulans* strains.

II. MATERIALS AND METHODS

A. Microorganisms

*Aureobasidium pullulans* IOC 3467 and IOC 3011 were obtained from Laboratory of Taxonomy, Biochemistry and Bioprospecting of Fungi, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. The microorganisms were maintained in PDA agar slant (Oxoid) at 4°C, monthly subcultured.

For inoculation, cells were activated through growth in PDA agar slant at 28°C for 48 h, then were transferred to 500 mL flask containing 100 mL of Sabouraud medium (Oxoid). The flask was incubated at 28°C ±1°C in a rotary shaker incubator (Controlled Enviromental Incubator Shaker, New Brunswick Scientific Co, EUA) at 150 rpm. This seed culture was used to inoculate the production medium.

B. Fermentation conditions

All of the components used in the media were p.a. quality except the carbon source, which was commercial granular sugar. Per liter of distilled water, the standard media contained: 30 g of white granulated sugar (sucrose), 5.0 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, and 1.0 g NaCl 1.0. The influence of nitrogen was evaluated using different sources and concentrations: NaNO₃, NH₄NO₃, urea and residual brewery yeast (RBY), from AMBEV, Brazil. The amount of each nitrogen source added resulted in different carbon/nitrogen ratios (C/N): 5 and 150 g/g. The pH value was adjusted to 6.0 with 1 M NaOH and, subsequently, the medium was sterilized at 121°C for 15 minutes.

A volume of the seed culture prepared as described above was transferred to 500 mL flasks containing 100 mL of each production medium in order to establish an initial cell concentration of about 5.0 x 10⁵ cells/mL. The flasks were incubated at 150 rpm with controlled temperature at 28°C ±1°C. After 120 h incubation, the fermentation broths were heated at 100°C for 15 min in water bath for cell inactivation, then centrifuged at 12000 x g for 20 min for cell removal. The substrates were analyzed on the supernatant.

C. Recovery and purification of pullulan

The supernatants were treated with 95% ethanol (1:2) to precipitate the produced biopolymer. After recovery, pullulan was consecutively washed with 70, 80, and 90% (v/v) p.a. ethanol. The biopolymers were dried in desiccator at room temperature (around 25°C ±1°C) until constant weight.

D. Analytical methods

Residual sugar concentrations in the supernatant were determined using Somogyi colorimetric method [45], after hydrolysis with 2 N HCl to 65-70°C/10 minutes, as described by reference [46]. Viscosity measurements of fermented broth were carried out with an Advanced Rheometer, model AR-2000 (TA Instruments, USA), cone-plate geometry. The results related to the polymer concentration were used to calculate the conversion factor Yₚₛ, which indicates the conversion of substrate to product (yield).

III. RESULTS AND DISCUSSION

In general, ammonium sulfate is usually indicated as nitrogen source for pullulan production by *A. pullulans*. The usual nitrogen source was replaced by others compounds to maximize the biopolymer synthesis and to reduce the production cost. The inorganic sources (ammonium nitrate and sodium nitrate) and organic (urea and residual brewing yeast) also have been evaluated in different amounts, establishing carbon/nitrogen ratio (C/N) of 5 and 150.

A. Polymer production

Fig. 1 shows the values of obtained biomass for the two strains of *Aureobasidium pullulans*, IOC 3467 and IOC 3011, after 48 hours of cultivation in different production media.

All nitrogen sources used enabled growth of both strains, though in different concentrations. In general, there was a large variation in the final concentration of cells, since the minimum
and maximum values were, respectively, 2.5 x 107 and 5.9 x 108 cells/mL.

Between the tested nitrogen sources, the residual brewery yeast (RBY) was the one that provided similar profiles of cell growth. In this case, the cell concentration was directly proportional to the amount of nitrogen available in the medium. The maximum biomass values obtained by IOC 3467 and IOC 3011 were 5.9 x 108 cells/mL and 4.0 x 108 cells/mL, respectively, in the C/N ratio of 5.

To the other sources of nitrogen, different behaviors were observed: in some cases, increasing the amount of nitrogen slightly interfered with cell growth (IOC 3467 strain/ammonium sulfate, IOC 3011 strain/nitrate sodium). In others, it was indirectly proportional to the growth (strain IOC 3467/ammonium nitrate and urea). The maximum biomass values were hit for intermediate concentrations of nitrogen (strain IOC 3011/ammonium nitrate and urea).

The biopolymer production by \textit{A. pullulans} IOC 3467 and IOC 3011 strains after 48 h of fermentation in media consisting of white granulated sugar and different nitrogen sources is shown in Fig. 2.

![Fig. 2. Biopolymer produced by \textit{Aureobasidium pullulans} IOC 3467 and IOC 3011 strains from white granulated sugar and different sources and amounts of nitrogen (AS – ammonium sulfate; SN – sodium nitrate; AN – ammonium nitrate; U – urea; RBY – residual brewery yeast; carbon/nitrogen ratio of 5 and 150).](image)

The biopolymer production (Fig. 2) varied in function of strain, nitrogen source and concentration. Note that the production increased with time, behavior similar to that seen in other studies [47]-[48]. However, the conditions that were most favorable to the cell growth and biopolymer synthesis were not the same. Indeed, particularly for \textit{A. pullulans} IOC 3467 strain, the industrial waste (RBY) was the most appropriate nitrogen source for growth and biopolymer production, although in different nitrogen ratio. In contrast, to the stain IOC 3011, all the nitrogen sources used, except urea, have shown appropriate to the microorganism growth.

The production of pullulan varies depending on the strain, the medium composition, conditions and manner of conducting the process. Numerous studies have shown that the sources of carbon and nitrogen play important roles in the production of exopolysaccharides by \textit{A. pullulans} [49]-[50].

In general, polysaccharide production by microorganisms is induced by limitation of an essential nutrient. According to some authors, the production of microbial polysaccharides is usually influenced by the type and amount of the nitrogen source used [46], [49].

The nitrogen source, generally ammonium ion (NH$_4^+$), plays a significant role in pullulan production by \textit{A. pullulans} [50], [51], [52]. References [53], [54] shows that the nitrogen depletion is interpreted by \textit{A. pullulans} as a signal for the exopolysaccharide formation. Reference [55] examined the nitrogen limitation effect to produce pullulan by \textit{A. pullulans}. The results indicated that, with similar ratios of carbon, the polysaccharide production is dependent on the ammonium ion concentration. Additionally, reference [42] demonstrate that the excess of nitrogen supply could contribute to the higher level of biomass but not enhance polysaccharide production. It was reported that a 10:1 carbon nitrogen ratio is the most favorable for the exopolysaccharide production [56], [57], in contrast to the present study.

The use of organic nitrogen sources often results in a higher specific growth ratio and exopolysaccharide production, which might be due the addition of growth factors in trace amounts [58]. Although, in generally, the organic and inorganic nitrogen sources induced the growth and biopolymer production in this study.

Fig. 2 shows the largest biopolymer amounts was from 6.9 to 8.1 g/L. These results were gotten by the \textit{A. pullulans} IOC 3011 strain in media containing ammonium sulfate (C/N = 5), sodium nitrate (C/N= 25), ammonium nitrate (C/N = 150), urea (C/N = 25) and residual yeast and brewer (C/N = 150). Therefore, unlike what is described in literature, the restriction of the nitrogen source does not necessarily induce the polymer synthesis.

Reference [59] evaluated five strains of \textit{A. pullulans} in medium with 50 g/L sucrose, and three different nitrogen sources [(NH$_4$)$_2$SO$_4$, NaNO$_3$ and peptone]. On their study, the minimal (6.3 g/L) and maximum (25.2 g/L) production were obtained on 168 hours of cultivation using ammonium sulfate and peptone, respectively. Reference [5] used a combination of 75 g/L of sucrose, 3g/L of yeast extract and 5 g/L of ammonium sulfate to produce 25.8 g/L of pullulan after 168 hours in a bioreactor using \textit{A. pullulans} ATCC 201253 strain.

The result for the biopolymer concentration (Fig. 2) was used to calculate the conversion $Y_{PS}$ factor, which indicates the conversion of substrate to product (Fig. 3). The determinations were done using the concentration of consumed substrate (data not shown).
Table I shows that the yield varied depending on the strain and the nutritional condition. Particularly for RBY, the increase of the amount of nitrogen resulted in lower biopolymer synthesis, while decreasing its quantity was directly proportional to the yield, and it was similar for the two strains. Probably the substrate conversion was into biomass, and not into biopolymer. Through the analysis of Table I, it can be seen that the maximum values of 0.48 and 0.55 g/g for the strains IOC 3011 and IOC 3647, respectively, were achieved under the same conditions (RBY and C/N = 150). These results are very promising, since other studies have shown values from 0.01 to 0.38 g/g (19, 8).

Reference [5] obtained after 168 h YPS 0.3, 0.21, 0.19, 0.18 and 0.14 g/g using 1, 3, 5, 7.5 and 10 g/L of yeast extract, respectively. Reference [51] determined YPS maximum of 0.33 g/g for batch cultivation of A. pullulans ATCC 42023 in medium containing glucose (50 g/L) and ammonium sulfate (0.6 g/L) with pH control at 6.5. Reference [50] studied cultures of three isolates of forest soils using glucose as carbon source and two nitrogen sources, (NH4)2SO4 and bactopeptone (Difco) as nitrogen source. For ammonium sulfate, the authors obtained for A. pullulans PR and A. pullulans CU after 120 hours, a yield of 0.23 g/g and, for A. pullulans SU after 96 h, 0.16 g/g. The use of peptone as nitrogen source yielded only 0.17 g/g (A. pullulans PR), 0.131 g/g (A. pullulans CU) and 0.03 g/g (A. pullulans SU), for the same periods (96h).

Since this work is still in an initial stage, the results appear promising, since, in order to obtain pullulan, it was used a low-cost substrate (white granulated sugar) in quantities equal or lower than the used in reported in literature. The results also showed better performance when it was used as a nitrogen source an industrial reject (RBY).

### B. pH values

pH analysis was also done after fermentation (Table 2). Except for media consisting of organic nitrogen sources (urea and RBY), in general it was noticed that there was a decrease in pH. Compared to all nitrogen sources, the use of ammonium sulfate and ammonium nitrate caused the highest reduction of pH for both strains.

### Table II

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Final pH IOC 3467</th>
<th>Final pH IOC 3011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate (C/N = 5)</td>
<td>3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Ammonium sulfate (C/N = 150)</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Sodium nitrate (C/N = 5)</td>
<td>6.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Sodium nitrate (C/N = 150)</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Ammonium nitrate (C/N = 5)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Ammonium nitrate (C/N = 150)</td>
<td>4.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Urea (C/N = 5)</td>
<td>8.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Urea (C/N = 150)</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Residual brewery yeast (C/N = 5)</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Residual brewery yeast (C/N = 150)</td>
<td>4.0</td>
<td>4.5</td>
</tr>
</tbody>
</table>

In these cases, the decrease in pH was inversely proportional to the amount of nitrogen source added to the medium. For example, the cultivation of the IOC 3011 strain in medium with ammonium sulfate (C/N = 5) reduced the pH to 2.5. Reference [51] also showed that the decrease in pH from 6.5 to 4.5 after 24 hours of fermentation of A. pullulans ATCC 42023 strain in medium consisting of glucose and ammonium sulfate. Similarly, reference [60] reported that the initial pH of 7.0 drops to 5.0 in the first 48 hours of fermentation of beet molasses and ammonium sulfate. However, during the fermentation process, there is a gradual increase until 8.0. The authors attribute the increased acidity to the formation of inorganic acid (sulfuric acid) and the excess of sulfate in the medium. The depletion of this nitrogen source induces the use of some amino acids in molasses, whose demineralization could justify the subsequent rise in pH. The gradual decrease in pH during the fermentation can also be attributed to the formation of organic acids as result of microbial metabolism. Regardless of the origin of the acidity, the fact is that its increase may adversely affect the production of polysaccharide [46]. The pH of fermentation broth can influence the morphology of the A. pullulans, which may subsequently influence cell growth and pullulan production [61]. Reference [62] obtained that lower pH of the medium benefits cell growth, while higher pH of the culture medium supports mass pullulan production.

The behavior of IOC 3011 strain can be noted for its ability...
to efficiently convert white granulated sugar in pullulan in the presence of all nitrogen sources tested in this work. However, depending on the nitrogen source, the Y_DS varied depending on the amount added to the culture medium. Probably, this behavior is due to pH variations observed in media by the end of fermentation as a function of the nitrogen source added.

C. Media viscosity

Figs. 3 and 4 show the viscometric profiles of both strains broth whose yield reached values superior of 0.27 g/L, which corresponds to half of the maximum yield value obtained in all tests (0.55 g/g – obtained by IOC 3011 using RBY as nitrogen source (C/N 150).

By the analysis of Fig. 3, it can be seen that the maximum value of viscosity (0.02 Pa.s) was obtained in lower shear rate (16 s⁻¹), when used as nitrogen source ammonium sulfate and RBY, both in C/N = 150. On the other hand, in the medium containing ammonium sulphate in the ratio C/N = 5, low viscosity values were determined, regardless of shear rate.

As shown in Fig. 4, the highest value of viscosity to broth from IOC 3011 strain was obtained by the use of RBY ration C/N = 150.

![Viscometric profile of fermented broth using A. pullulans IOC 3011 strain (AS - ammonium sulfate, SN - sodium nitrate, AN - ammonium nitrate, U - urea; RBY - residual brewery yeast, carbon / nitrogen ratio of 5 and 150) (Advanced Rheometer 2000, 25°C).](image)

The viscosities determined in this nutritional condition were 0.06 and 0.008 Pa.s, respectively, in the lower (15.6 s⁻¹) and higher (415 s⁻¹) shear rates. The use of sodium nitrate as nitrogen source detected the lower viscosity values.

For all conditions, it was observed a decrease in viscosity with the increase of shear rate, suggesting that all samples presented a pseudoplastic behavior. Reference [49] related previously the same behavior. According to them, pullulan presents pseudoplastic properties in aqueous solution, due to the orientation of the molecules in the flow direction, leading to lower resistance to flow [63]. The change in the form of flexible molecules with the variation of shear rate, and the disruption of intermolecular interactions by flow rate may also contribute to the pseudoplastic propriety [64]. This property favors the use of pullulan as a thickener in food products and viscous liquid. The viscosity results were discussed in more details in a previously work [35].

IV. CONCLUSION

Both strains of Aureobasidium pullulans were able to produce biopolymer from white granulated sugar and different nitrogen sources. The IOC 3011 strain produced pullulan more satisfactorily than IOC 3467, reaching a yield of 0.55 g/g when grown on medium with white granulated sugar and residual beer yeast on C/N ratio of 150. In general, the pH of fermented broth decreased when it was used organic nitrogen source and increased when it was used inorganic nitrogen source. The results are of great interest, once the use of an industrial waste as nitrogen source and a low-cost carbon source (white granulated sugar) reduce the process cost, making the biopolymer more commercially competitive.

ACKNOWLEDGMENT

This work was financially supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors wish to thank Dra. Maria Inez de Moura Sarquis (Instituto Oswaldo Cruz, RJ, Brazil) who isolated and kindly provided the microorganisms and MsC. Juliana Cruz and MsC. Patrícia Sardela for all supply.

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