Rheological Analysis of Biopolymer Produced by Aureobasidium Pullulans in Different Sources of Nitrogen

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Summary: In this study, the rheological behaviors of media fermented by two *Aureobasidium pullulans* strains (IOC 3467 and IOC 3011) were evaluated in different nutritional conditions. The media consisted of crystal sugar (sucrose), as the main carbon source, and different nitrogen sources (ammonium sulfate, sodium nitrate, ammonium nitrate, urea or residual brewery yeast - RBY). Viscosity measurements were performed on cell-free supernatants, from 48-hour fermentation assays, at 25 °C. Shear rates ranged between 0.1000 and 500 s^{-1}. All samples showed pseudoplastic behavior. Nevertheless, the viscosimetric profile of each one varied according to the nitrogen source, its concentration and the strain used. The maximum viscosity of 0.06 Pa.s. was achieved at 15.6 s^{-1} for IOC 3011 strain grown on RBY as nitrogen source.

Keywords: biopolymer; brewery yeast; industrial waste; pullulan; rheology

Introduction

Most polymers are industrially produced from petroleum-based raw materials. Thus, these products, when disposed in the environment, can resist to degradation over long periods of time.[1] As a consequence, the large amounts of synthetic polymers, that are consumed worldwide each year, make even more serious the disposal problems. Therefore, the replacement of conventional petrochemical polymers with biodegradable ones is of great interest.

The polymers synthesized by microorganisms are gaining prominence mainly due to advantages such as biodegradability, physicochemical properties and can be produced from renewable raw materials. Among microbial biopolymers, pullulan emerges because of its importance in food applications, in particular to improve adhesion and gloss of foods, without changing its caloric value.[9,11] Nevertheless, is already recognized its use for more noble purposes such as a prebiotic, since pullulan can promote the selective growth of *Bifidobacterium* spp. in the human gut.[10,11] Also, the pharmaceutical and biomedical industries are showing interest in pullulan, since its derivatives may be use as non-toxic conjugates in the production of vaccines.[4,12] Studies have demonstrated that pullulan can act a blood plasma substitutes[4,13,14] and even increase the effect of interferon, a protein that is being used effectively to treat some viral diseases such as hepatitis.[4]

Pullulan is a linear a-D-glucan built of maltotriose subunits, connected by (1→6)-α-D-glucosidic linkages.[4,8,9] The regular alternation of (1→4)-α and (1→6)-α bonds influences two distinctive properties: structural flexibility and enhanced solubility.[10] Such properties make pullulan useful in food production and packaging.[3,4]

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The production of pullulan by *Aureobasidium pullulans*, a polymorphic fungus that has been isolated from different ecosystems, has been studied under many nutritional and physico-chemical conditions[2–7] as the structure and composition of microbial polysaccharides depend on strain, medium composition and fermentation conditions (pH, temperature, oxygen concentration).[15] This work aimed to study the rheological behavior of pullulan biopolymers produced by two isolated *A. pullulans* strains in fermentation broth consisting of crystal sugar as main carbon source, a raw material commercially available at low price in Brazil, and various nitrogen sources - sodium nitrate, ammonium sulfate, ammonium nitrate, urea and residual brewing yeast - in different concentrations.

Experimental Part

Microorganism

Two strains of *Aureobasidium pullulans*, IOC 3011 and IOC 3467, were used. Both strains were provided from the Laboratory of Taxonomy, Biochemistry and Bioprospecting of Fungi, Instituto Oswaldo Cruz, RJ, Brazil. The cultures were maintained in Sabouraud medium (Merck, Germany) at 4 °C, subcultured monthly.

Inoculum

The stock culture was reactivated through growth in 100 mL of Sabouraud medium in Erlenmeyer flask of 500 mL capacity, incubated at 28 °C for 48 h on a rotary shaker (Controlled Environmental Incubator Shaker, New Brunswick Scientific Co, USA) at 150 rpm.

Biopolymer Production

The experiments were performed in triplicate on 500-mL Erlenmeyer flasks containing 100 mL medium composed of (g/L): crystal sugar (30.0), yeast extract (0.4), potassium phosphate (5.0), magnesium sulfate (0.2) and sodium chloride (1.0), based on literature.[16,21,22] Five nitrogen sources were tested: $NaNO_3$, $(NH_4)_2SO_4$, NH_4NO_3, urea and residual brewery yeast (RBY), added in order to establish carbon/nitrogen (C/N) ratios of 5 and 150 g/g. Except the industrial waste (RBY), that was provided by a brewery company (AMBEV, Brazil), all reagents were analytical grade (Merck). The pH was adjusted to 6.0 by adding 1M NaOH and the media were sterilized at 121 °C for 15 minutes. An initial cellular concentration of 5.0×10^5 cells/mL was used, which corresponds to 0.084 and 0.075 g/L of dry cell weight, for strains IOC 3467 and IOC 3011, respectively. After inoculation, the fermentation media were incubated under the same conditions mentioned for preparation of inoculum.

Viscosimetric Measurements

After 48 hours, the fermented medium was heated to 100 °C for 15 minutes to promote cells inactivation.[16,23] Then, the cells were harvested by centrifugation (SORVALL RC 26 Plus refrigerated centrifuge) at 12,000 g for 15 min at 4 °C and the cell-free supernatant obtained was used for viscosity analysis. The viscosity of samples was measured using a controlled stress/shear rheometer (Advanced Rheometer, AR 2000, TA Instruments Inc., Leatherhead, UK) cone-plate geometry, diameter 60 mm and angle of 1°, at 25 °C. Viscosity readings were made in shear rates ranging from 0.1000 s^{-1} to 500 s^{-1} for 5 minutes and were reported in Pa.s.

Biopolymer Recovery

The cell-free fermentation broth was mixed with two volumes of 95% ethanol to precipitate the crude biopolymer. Afterwards, the precipitate was separated by filtration, through 0.2 μm Millipore membrane, and rewashed with ethanol. The biopolymer recovered was vacuum-dried for 3 days at room temperature until constant weight.

Critical Concentration

The range of the critical concentration (c^*) was analyzed measuring the viscosities of biopolymer recovered solutions and of standard pullulan (Sigma-Aldrich) solutions. The solutionś concentrations were 0.05, 0.1, 0.3 and 0.5 g/L at 10, 25 and 35 °C. The shear rate was corresponded to the plate Newtonian (613 s^{-1}).

Results and Discussion

The amount of biopolymer produced and the dry cell weight for both *Aureobasidium pullulans* strains used (IOC 3467 and IOC 3011), determined for each nitrogen source at the end of the process, are shown in Table 1. The variation of the nitrogen source as the variation of the strain produced different quantity of dry cells and biopolymer. In general, the strain IOC 3467 generated more dry cells then the strain IOC 3011, while the first one produced more biopolymer then the second one. The nitrogen sources that allowed the higher production of the biopolymer were the residual brewery yeast (C/N 150) for the strain IOC 3467 and ammonium sulfate (C/N 5) for the strain IOC 3011.

The Figure 1A shows the viscosity of the medium fermented by *A. pullulans* IOC 3467 strain. For all conditions, it was observed a decrease in viscosity with increasing shear rate, suggesting that all samples presented pseudoplastic behavior. The same behavior was previously related by Seviour and coworkers.[24] 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.[25] 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.[26] In turn, this property favor the use of pullulan as a thickener in food products and viscous liquid.

The maximum viscosity of 0.02 Pa.s was determined at the lowest shear rate (15.9 s^{-1}), when using ammonium sulfate and RBY, both at the C/N ratio 150. On the other hand, low viscosity values were observed for media containing ammonium sulfate at the C/N ratio 5, regardless of shear rate (Figure 1A and B).

For ammonium sulfate (C/N 150) and RBY (C/N 150) it was observed the higher decrease of viscosity with the increase of shear rate, while the viscosity for ammonium sulfate (C/N 5) was the lowest (Figure 1A). According to Shingel,[12] the nitrogen source has a positive effect on the molecular weight of pullulan. Therefore, the different behavior of fermented broth of this study may be related to the nitrogen source.

It is known that the viscosity is dependent on the structure and concentration of the polymer, its molecular weight and distribution, the conformation of macro-

Table 1.
The amount of biopolymer produced and the dry cell weight for different nitrogen sources and strains.

Nitrogen Source	IOC 3467		IOC 3011	
	Dry cell (g/L)	Biopolymer produced (g/L)	Dry cell (g/L)	Biopolymer produced (g/L)
Ammonium sulfate (C/N 5)	1.6	4.3	3.7	8.1
Ammonium sulfate (C/N 150)	2.3	3.8	0.6	1.0
Sodium nitrate (C/N 5)	3.3	1.7	2.7	6.0
Sodium nitrate (C/N 150)	0.4	1.0	1.8	5.9
Ammonium nitrate (C/N 5)	0.7	1.3	2.6	3.1
Ammonium nitrate (C/N 150)	1.8	2.7	1.7	7.2
Urea (C/N 5)	0.7	2.3	0.7	2.1
Urea (C/N 150)	2.2	0.9	0.6	0.8
Residual brewery yeast (C/N 5)	9.9	2.4	6.0	1.9
Residual brewery yeast (C/N 150)	1.7	6.3	1.0	6.9

C/N: carbon/nitrogen ratio.

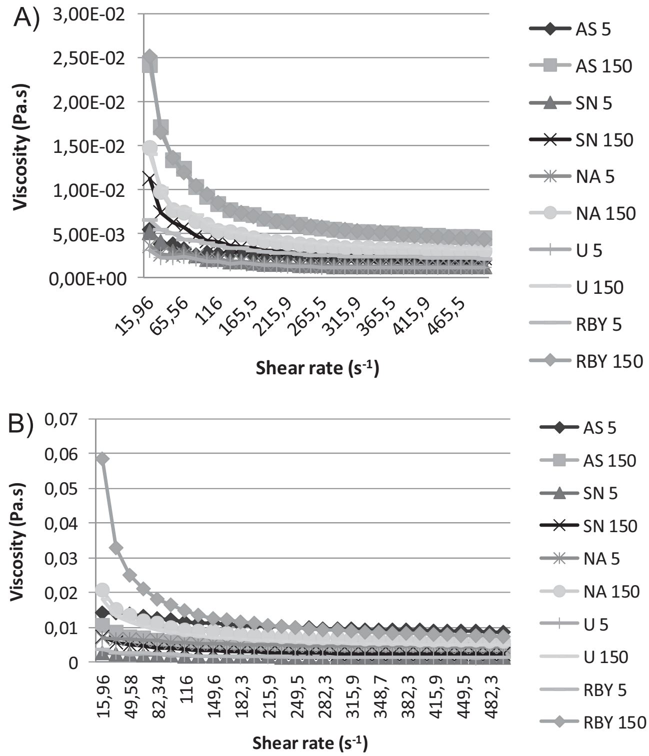

Figure 1.
Viscometric profiles of different media compositions fermented with IOC 3467 (A) and IOC 3011 (B) strains [AS - ammonium sulfate, SN - sodium nitrate, AN - ammonium nitrate, U - urea; RBY - residual brewery yeast, carbon/nitrogen 5 and 150; Advanced Rheometer 2000, 25 °C; initial viscosity of different media: approximately 1.33×10^{-3} Pa.s. (Newtonian behavior)].

molecules in solution and its interaction with solvents, the type of intermolecular and intramolecular aggregation, and also the flexibility of the chains with temperature.[28–30] Lin and coworkers[27] observed variations of molar mass polymers according to the producer strain and nutritional conditions, which was correlated to the nitrogen source as the main factor. Ammonium ion induced the microbial production of pullulan of higher molecular weight than nitrate ion.

Figure 1B shows the viscometric profiles for IOC 3011 strain grown on different nitrogen sources and C/N ratios. Similarly to that observed for IOC 3467 strain, the variation of viscometric changes was related to the nitrogen source and its concentration. The highest viscometric values (0.014, 0.003, 0.007, 0.02 and 0.06 Pa.s) were determined at the lowest shear rate ($15.9\,s^{-1}$) for, respectively, ammonium sulfate (C/N 5), sodium nitrate (C/N 5), sodium nitrate (C/N 150), ammonium nitrate (C/N 150) and RBY (C/N 150).

After observing that the highest viscosity values were obtained using strain IOC 3011, and nitrogen in RBY C/N ratio 150 as a source of, a new study was done comparing the viscometric results to the strain IOC 3011 using RBY with C/N ratio of 50, 100, 200 e 250 (data not shown). From this study it was concluded that the best C/N ratio was 100. Thus, the critical concentration analyses were made from the polymers obtained in this condition.

The viscosity *versus* polymer concentration of samples of the biopolymer obtained

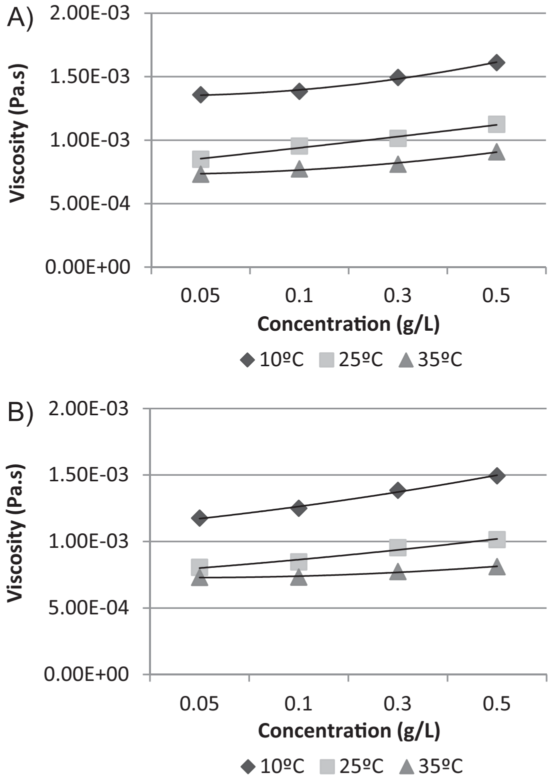

Figure 2.
Viscosity behavior of the biopolymer produced by *Aureobasidium pullulans* IOC 3011 strain, using brewery residual yeast as nitrogen source at C/N ratio of 100 (A), and pullulan Sigma-Aldrich standard (B) at different concentrations in solution at a rate of shear of 613 s^{-1} (Advanced Rheometer 2000, 10, 25 and 35 °C) [Initial viscosity of different media: approximately 1.33×10^{-3} Pa.s. (Newtonian behavior)].

and of the standard pullulan at different temperatures are given in Figure 2 (A and B). These results indicate that there is a linear trend for all samples at temperatures and concentrations studied. This conclusion is based on the values of correlation index (R^2) close to unity (0.924 to 0.999). Thus, from the equation of straight line obtained is possible to predict the viscosity in the range 0.05 to 0.5 g/L. As expected, the increase in viscosity is a function of increasing concentration and decreasing temperature.

Based on the studies developed in this work can be concluded that under the conditions examined, it was not possible to obtain the critical concentration of pullulan.

The values of critical concentrations vary greatly between biopolymers. For κ-carrageenan (0.1 M NaCl, pH 9), Croguennoc and colleagues found a value of 4.5 g/L.[31] Morris and colleagues observed values of 2.2 g/L for guar gum, 10 g/L for alginate (0.2 M NaCl) and 80 g/L for dextran.[32] Loret and colleagues observed a value of 170 g/L for aqueous solutions of maltodextrin.[33] In 1989, Lopes observed critical concentration of guar gum produced by Hercules Inc. equal to 1.78 g/L. Can be found in the literature for samples of xanthan gum M_W of magnitude of 2×106 dissolved in distilled water, values of critical concentration in the range of 0.6 to 1 g/L.[29]

Lazaridou and co-workers analyzed the effect of molecular weight on the rheology of pullulan. Therefore, analyzing four samples of this polymer: P100, P260, P360 and P560 with molecular weights of 9.9×10^4, 2.63×10^5, 3.61×10^5 and 5.64×10^5 and 10^5 Da, respectively, for which were determined the following values for c^*: 31 (P100), 21 (P260), 18 (P360) and 14 (P560) g/L. The c^* depends on the volume occupied by each molecule. Therefore, differences in the value of c^* for different pullulan samples studied are related to differences in molecular size, in other words, the value of c^* progressively increased with decreasing molecular weight (Mw) of the polysaccharide. Izydorczyk and Biliaderis[35] reported a similar trend for the values of c^* estimated for wheat arabinoxylan fractions that varied in the range of 2.6 to 3.8 g/L. Kasapi and Morris[36], found that entanglement does not occur in a chain of capsular polysaccharide in microbial solutions to a relatively high concentration (~2.0 w/v) because of its low hydrodynamic volume. Moreover, the value c^* depends on the polymer structure, which can affect the chain stiffness and thus the flow characteristics.

Conclusion

The media fermented by *Aureobasidium pullulans* strains, IOC 3011 and IOC 3467, showed pseudoplastic behavior. In fact, both strains were able to produce biopolymer when grown in media constituted of crystal sugar and different nitrogen sources. The higher values of viscosity were obtained for residual brewery yeast (RBY) at the C/N ratio of 150. Those results are of great interest as the use of an industrial waste reduces the process cost, rendering the biopolymer more commercially competitive. In addition, the use of RBY benefits the brewing industry by reducing the costs of waste treatment. Finally, it may also be concluded that both the production and the quality of the polymer were dependent on the nitrogen source and the strain used.

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