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Study of the Molecular Weight of Pullulan Produced by *Aureobasidium pullulans* from Industrial Waste

Victor R. L. Oliveira*[a]**, ***Victória V. Coelho*[b]**, ***Eliana F. C. Sérvulo*[a]**, ***Verônica M. de A. Calado*[a]**

[a]*Universidade Federal do Rio de Janeiro, Centro de Tecnologia, Escola de Química, Programa de Pós-graduação em Engenharia de Processos Químicos e Bioquímicos, 21941-909, Rio de Janeiro, RJ, Brasil.*
[b]*Universidade Federal do Rio de Janeiro, Centro de Tecnologia, Escola de Química, 21941-909, Rio de Janeiro, RJ, Brasil.*

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The objective of this research was the sustainable development of pullulan and its structural and rheological characterization. In addition, the cultivation of the *A. pullulans* Y2092 strain by submerged fermentation on a laboratory scale was evaluated, valuing the use of industrial waste (residual brewery's yeast), RBY, and a low-cost renewable raw material VHP (very high polarization sugar). A 2^2 factorial statistical design of experiments was used to analyze the molecular weight of the pullulans produced. The maximum value of 1955 kDa was determined for the pullulan recovered after 96 h of fermentation in the mineral medium added with 70 g/L of VHP and 50 g/L of RBY. The FTIR analysis for the pullulan obtained was similar to the commercial pullulan. The rheological behavior of pullulan solutions is shown, evidencing the relationship between this property and M_w. The results from all analyses indicate that the pullulan obtained is suitable for the future production of biopolymeric films.

Keywords: *pullulan, molecular weight, intrinsic viscosity, food packaging.*

1. Introduction

Notably, the use of plastics has been increasing in contemporary society, particularly in the food sector, where packaging is expanding strongly[1]. The use of packaging has been an effective strategy to ensure the quality of foods such as meat, fruits, and vegetables for a longer time throughout the supply chain, covering storage, processing, transport, and distribution to the final consumer. Packages for contact with food ensure their structural integrity, protecting them against environmental factors such as light, humidity, oxygen, chemicals, and microorganisms. However, they do not prevent mechanisms of chemical deterioration and physiological disturbances from establishing themselves later on[2].

A study recently carried out by the Getúlio Vargas Foundation (FGV), reported by ABRE (Brazilian Packaging Association) points out that the physical production of packaging in Brazil in 2019 reached a gross value of R$ 80.2 billion, which represents an increase of 6.5% compared to the previous year[3]. However, the exacerbated use of non-biodegradable plastic packaging is one of the leading causes of severe environmental problems[4,5].

Among the possible strategies is the development of films or coatings based on consumable polymers to cover the food completely, not requiring its removal before consumption[6]. These are colorless thin films, invisible to the naked eye, which act as barriers against exposure to light, loss of moisture and gas exchange, reducing metabolic disturbances and microbial action, which results in the preservation of food freshness and guarantees its quality for longer[3,7].

Some authors have shown that pullulan, a neutral homopolysaccharide, mainly synthesized by strains of the fungal species *Aureobasidium pullulans*, can be used in the preparation of edible films and packaging for food, which are colorless, odorless, non-toxic and have excellent physicochemical and mechanical properties[8-12]. However, the cost of producing microbial biopolymers is high, often up to 50% higher than the commercial value of synthetic polymers. For this reason, the cost is considered the main obstacle to implementing the bioprocess on a large scale. An alternative is the replacement of carbon and nitrogen sources with abundant and low-cost raw materials, especially industrial waste, which is generally polluting, which would imply some form of treatment to adjust its disposal to the standards established by environmental legislation[4].

However, both nutritional and environmental variations in culture conditions can lead to changes in the molecular structure of macromolecules synthesized by microbial pathways affecting the mechanical properties, such as flexibility or rigidity of the films[6]. A biopolymer's molecular weight (M_w) can vary from 50 kDa to 2000 kDa, with M_w values close to the upper limit generally being the most indicated when the biopolymer is intended to be applied in the formation of biopolymeric films.

The present research had the purpose of reducing the cost of producing pullulan by carrying out the fermentation of the *A. pullulans* strain in a medium consisting of different amounts of industrial residue RBY (Residual Yeast Extract, dry biomass from the brewery) and VHP sugar (Very High Polarization sugar), abundant and relatively low-cost raw

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*e-mail: vrafaeloliveira@eq.ufrj.br

material in Brazil, derived from the sugar industry[13]. The pullulan samples generated under the different nutritional conditions tested were recovered from the fermented media by precipitation with ethanol. Then they characterized in terms of structural, rheological conditions, and mainly molecular weight, with a view to the future sustainable production of biopolymeric films for application in the protection of fruits.

2. Materials and Methods

2.1. Microorganism and growth

The strain *A. pullulans* Y 2092, provided by the Center for Taxonomic Collections of the Institute of Biological Sciences - Federal University of Minas Gerais - CM-UFMG (WDCM 1029), was used. In the laboratory, stock cultures were prepared in 1 mL of aqueous glycerol solution (25%), maintained by cryopreservation in an ultra-freezer at -80ºC. To prepare the inoculum, the contents of a cryotube, after rapid thawing, were transferred to 100 mL of Sabouraud broth in 500 mL Erlenmeyer flasks, followed by incubation at a temperature of 28°C, in a rotary shaker (Shaker Incubadora Environmentally Controlled – Tecnal, TE-420), at 150 rpm, overnight.

2.2. Biopolymer production and recovery

Fermentation tests were carried out in 100 mL of a liquid medium composed of (g/L): 0.2 $MgSO_4$; 1.0 NaCl; 0.6 $(NH_4)2SO_4$, added with VHP and RBY, in the amounts outlined in the 2^2 full factorial design with triplicate at the central point (Table 1), so that the statistical analysis of the data allowed verifying the influence of the primary sources of carbon and nitrogen on the molecular weight of pullulan. The media were adjusted to 6.0 before autoclaving at 121°C for 15 minutes. In the exponential phase of growth, the media were inoculated with the growing volume of the stock culture in the Sabouraud medium to establish the initial concentration of $5x10^5$ cells. Then, the cultures were incubated in a rotary shaker at 28ºC and 150 rpm. After 96 h, the fermented media were heated to 100°C to inactivate the cells, followed by centrifugation at 4000 rpm and 5°C (BioVera refrigerated centrifuge) for 20 min. For biopolymer precipitation, each cell-free fermented medium was added with ethanol P.A. in the proportion of 1:3 (cell-free fermented medium:ethanol). Then, the precipitate was washed in series with 70%, 80%, and 90% ethanol solutions and absolute ethanol and filtered under vacuum. The biopolymer was dried in Petri dishes and placed in a desiccator overnight at room temperature. The biopolymer was characterized by Fourier transform infrared spectroscopy, rheological behavior, and molecular weight distribution. For comparison, the same analyses were performed on commercial food-grade pullulan purchased from Baoding Faithful Industry Co. Ltd.

A software called Statistica 13.1, by Statsoft, was used.

Table 1. Full factorial design 2^2.

Component	Maximum (+1)	Center point (0)	Minimum (-1)
VHP (g/L)	50	40	30
RBY (g/L)	200	125	50

2.3. Characterization of the biopolymer

For FTIR analysis, the Shimadzu IRAffinity Spectrometer (32 scans) was used in a range of 4000-500 cm^{-1} at a resolution of 4 cm^{-1} over the KBr pellet.

The apparent viscosity of the cell-free fermented medium was determined with an advanced rheometer, model Physica MCR 501 - (Anton Paar, USA), cone-plate geometry, diameter 60 mm. The viscosimetric analyses of the samples were performed at 25ºC at different shear rates, ranging from 0.1 to 500 s^{-1}. A shear rate close to 0 must be adopted to calculate the intrinsic viscosity. The ASTM D4603 standard test method will perform the remaining viscosity measurements. The relative viscosity was analyzed from the ratio between the solution's viscosity and the solvent's specific viscosity (Equation 1). In this research, water was used as a solvent.

$$\eta_r = \frac{\eta}{\eta_0} \tag{1}$$

where

η= dynamic viscosity of the solution (Pa.s);

η_0= dynamic viscosity of the solvent (Pa.s).

Intrinsic viscosity is a very relevant variable to describe the viscous behavior of a polymeric solution, as it provides the valid viscosity-increasing properties of a polymer regardless of its concentration in the solution[14]. Intrinsic viscosity can be evaluated from the reduced viscosity limit extrapolated to a zero concentration. The intersection with the y-axis gives zero shear intrinsic viscosity. It is denoted by [η] and was calculated using the Huggins equation:

$$\left[\eta\right] = \lim_{\eta_{sp}\to 0} \frac{\eta_{sp}}{c} \tag{2}$$

Parameter "c" corresponds to the concentration value of the biopolymeric solution.

Solutions with 10%, 15%, and 20% of biopolymer concentrations were prepared and analyzed in an advanced rheometer, model Physica MCR 501 - (Anton Paar, USA), cone-plate geometry, diameter 60mm. The analysis was performed at 25ºC at different shear rates, ranging from 0.1 to 500 s^{-1}.

The average molecular weight of pullulan was based on Debye's plotting method and the one described in the studies by Spatareanu et al.[15], using the Zetasizer Nano ZS series model from Malvern Instruments Ltd. In this analysis, the scattered light intensity was measured from five concentrations of biopolymers prepared using an aqueous solution of 0.1 M $NaNO_3$ (0.01 g/L, 0.03 g/L, 0.05 g/L, 0.08 g/L, and 0.1 g/L).

3. Results and Discussion

3.1. Full factorial design

Table 2 shows the pullulan samples' molecular weight response variable values for the tests defined by the matrix model for a 2^2 full factorial design.

As seen in Table 2, molecular weight ranged from 1173 kDa to 1955 kDa, depending on nutritional conditions. There was an increase in the molecular weight of the pullulan when a higher amount of carbon source and a smaller amount of

Table 2. Design Matrix for the molecular weight (response variable).

Experiment	[RBY, g/L] (X_1)	[VHP, g/L] (X_2)	Molecular weight (kDa)
1	50	30	1440
2	200	30	1173
3	50	70	1955
4	200	70	1398
5	125	50	1512
6	125	50	1536
7	125	50	1531

nitrogen source were provided. The maximum value obtained (experiment 3) corresponded to an increase of more than 65% compared to the lowest value (experiment 2).

Hernandez-Tenorio and Giraldo-Estrada[16] used *A. pullulans* ATCC 15233 size-exclusion chromatography using $NaNO_3$ 0.1 N to prepare polymeric solutions and obtained a molecular weight of 93.57 kDa, which includes M_W between 100 and 200 kDa. They showed that forming polymeric films requires obtaining pullulan with higher molecular weight, decreasing the degree of acetylation, adding other substances such as plasticizers, or mixing with other biopolymers. This work aimed at providing consistent molecular weight data for obtaining polymeric films.

Le et al.[17] used pullulan purchased from Carbosynth, Germany. They were obtained from solutions prepared with 0.1 M $NaNO_3$ solution and using the scattering light intensity method of at least five different polymer concentration Mw values around 200 kDa to 400 kDa. They observed that the pullulan obtained by polyamine modification had a higher molecular weight (3500 kDa). They attributed this increase to better adhesion of the low-molecular-weight polyamine graft to the pullulan backbone. This adhesion was performed after the activation of hydroxy groups by mesyl chloride without opening the ring of glucose subunits, which leads to excellent swelling properties and increased dynamic viscosity. These properties are essential in the sustained release of the drug.

The analysis of the Pareto diagram (Figure 1) for M_w data from the experimental design (Table 2) also shows that the RBY concentration had the most significant influence on the molecular structure of the biopolymer. The dotted line precisely provides the point at which the effect estimates are statistically significant at 5% ($p \leq 0.05$). The negative RBY signal indicates that lower concentrations promote polymer chain enlargement. The positive signal for the VHP shows that an increase in response occurs when this variable goes from level −1 to +1.

The decrease of RBY in the medium can be beneficial for the increase of the molecular weight because, according to Manitchotpisit and collaborators[7], high amounts of yeast extract can induce the synthesis of α-amylase in the stationary phase, which, in turn, can lead to hydrolysis of secondary maltotetraose, a subunit of the biopolymer, with an abrupt decrease in molecular weight[7]. Wang et al.[18] also evaluated pullulan production by an experimental design using the response surface method. They identified that pullulan M_w depends on the activities of enzymes involved in both its biosynthesis and degradation. Among the enzymes responsible for pullulan degradation using *A. pullulans*, α-amylase has been reported as the primary enzyme impacting M_w values.

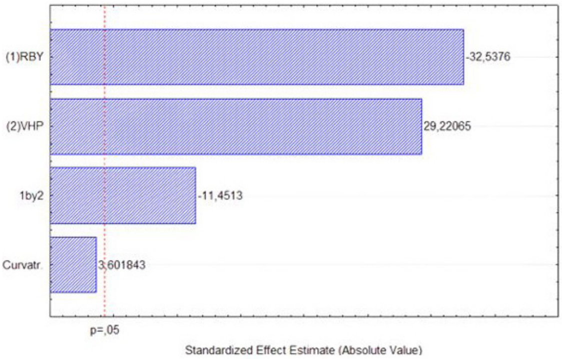


Figure 1. Pareto diagram.

A different behavior was observed for VHP sugar as this variable significantly influenced the response variable ($p \leq 0.05$). An increase in the concentration of VHP significantly increases the molecular weight as indicated by the positive sign. Therefore, the molecular mass was higher when a higher concentration of VHP (70 g/L) was used. That may be related to the polarization of the VHP, which varies from 99.00 to 99.49%, a percentage that expresses the mass of sucrose in this sugar[13,19]. Moreover, as evidenced by Prasongsuk et al.[9], the yeast cultures tested showed the synthesis of pullulans with higher M_w in a medium consisting of sucrose, with low activity of α-amylase and endoamylase, enzymes involved in the "breaking" of bonds between monomers, which, as already discussed, cause a reduction in the molecular weight of the macromolecule.

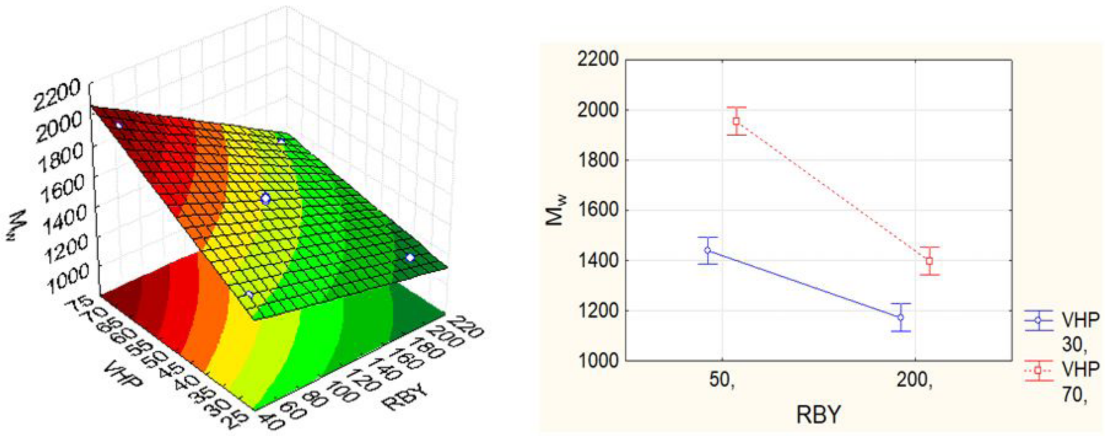
It can be observed that a decrease in RBY or an increase in VHP provides higher molecular weight values of pullulan when analyzing the response surface (Figure 2a) and the graph of marginal means (Figure 2b). From this planning, the highest molecular mass obtained of 1955 kDa was reached using 70 g/L of VHP and 50 g/L of RBY. An increase in molecular weight is ideal for the objective proposed in this research as an increase in molecular weight represents an improvement in the mechanical properties of the biopolymer, evidenced by the increase in flexibility in how it facilitates the handling and packaging of food[20]. Vijayendra and Shamala[6] and Wang et al.[21] also reported that increasing molecular weight improved the mechanical properties of the biopolymer. However, studies by Madera-Santana et al.[22] stated that a sharp increase in molecular weight makes it challenging to prepare biopolymeric films using the casting method.

The interaction between carbon and nitrogen sources also significantly influenced the molecular weight response variable ($p \leq 0.05$), like RBY, indicated by the negative sign. Therefore, a more negligible interaction between the factors has a significant favorable effect ($p \leq 0.05$) on the increase in molecular weight.

In Table 3, it is possible to verify the estimated effects of the two investigated factors, model coefficients, and statistical parameters for the molecular weight response variable.

The molecular weight can be estimated as a function of the factors by using Equation 3.

$$M_W(kDa) = 1070,25 - (0.33 * X1) + (15.29 * X2) - 0.05 * X1 * X2) + 34.83 \tag{3}$$



(a) response surface

(b) graph of marginal means

Figure 2. (a) response surface; (b) graph of marginal means.

Table 3. Estimated effects of the two investigated factors, model coefficientss and statistical parameters for the molecular weight response.

Parameter	Effect	Model coefficients	Standard deviation	*t*-Test[2]	*p*-value
Average	1491,50	1070,25	33.134	235.58	0.000018
Curvature	69.67	34.83	9.671	3.60	0.069179
RBY (X1)	-412.00	-0.33	0.227	-32.54	0.000943
VHP (X2)	370.00	15.29	0.615	29.22	0.001169
1*2	-145.00	-0.05	0.004	-11.45	0.007540

Structural and rheological analyses were performed on the pullulan obtained in experiment 3, with higher molecular weight. The Y2092 strain produced, in this experiment, 20.6 g/L with an average yield ($Y_{P/S}$) of 0.792 g/g.

3.2. FTIR analysis

Figure 3 shows the configuration of the peaks from the FTIR analysis of the pullulan obtained from the nutritional condition indicated by the experimental design (VHP 70 g/L and RBY 50 g/L). Figure 3 also compares the characteristic absorption frequencies between the commercial and pullulan obtained in this work.

As shown in Figure 3, the obtained biopolymer and commercial pullulan spectra are similar regarding the FTIR analysis. A wide band is evident between 3430 and 3440 cm^{-1}. This range is attributed to –OH stretching[23]. A sharper band, approximately at 2930 cm^{-1}, is characteristic of the symmetric and asymmetric vibrational stretching of CH_2 groups and the vibrational stretching of CH[24]. Two sharper peaks from 1710 cm^{-1} to 1630 cm^{-1} are characteristic of O-C-O bonding and glycosidic bonds[25]. The characteristic peaks around 1410 cm^{-1} are attributed to the bending of C-O-H bonds[26]. The range corresponding to stretching in C-O-C and C-O is observed between 1150 cm^{-1} to 1030 cm^{-1}[27].

The range close to 950 cm^{-1} indicates the presence of α-1,6-type bonds -d-glycosidic[28], and the sharp peak at 617 cm^{-1} corresponds to the presence of α-1,4-d-glycosidic bonds[29]. According to Sapper et al.[30], the ratio of the α-1,4 glycosidic bond to the α-1,6 glycosidic bond in the pullulan structure is 2:1. This structure also has a degree of polymerization of approximately 100–5000 and a molecular weight between 1.5×10^4 and 1.0×10^7. Although the pullulan mechanism is not fully understood, it is known that α-phosphate glucose mutase (PGM), UGP-glucose pyrophosphorylase (UGP), and glycosyltransferase (FKS) are the three main enzymes involved in pullulan biosynthesis, responsible for encoding genes, which can affect molecular weight[31,32].

In the studies by Sugumaran et al.[33], the bands found in the synthesized and commercial pullulan FTIR spectrum are similar to those we found. They claim that the broadband close to 3400 cm^{-1} is affected by intermolecular or intramolecular hydrogen bonds in the O-H stretch and that these bonds are strong, which leads to a lower vibration of the molecules, resulting in a broad band like the one presented in the spectrum.

Finally, from the FTIR data presented on the commercial pullulan by several authors and the one obtained in this work, it is possible to infer that the exopolysaccharide obtained in the presented synthesis is the pullulan[12,34-38].

3.3. Rheological analysis

Figure 4 shows the apparent viscosity behavior of cell-free fermented wort. This figure shows that the highest apparent viscosity value was reached at the lowest shear rate (10 s^{-1}) when 0.027 Pa·s is shown. Soon after, there is a decrease in viscosity with an increase in the shear rate, suggesting that they must have a pseudoplastic behavior[15].

According to studies by Fernandes et al.[39], the change in the rheological behavior of the medium is certainly caused by the production of exopolysaccharides by microorganisms. In this phase, the biopolymer acquires non-Newtonian

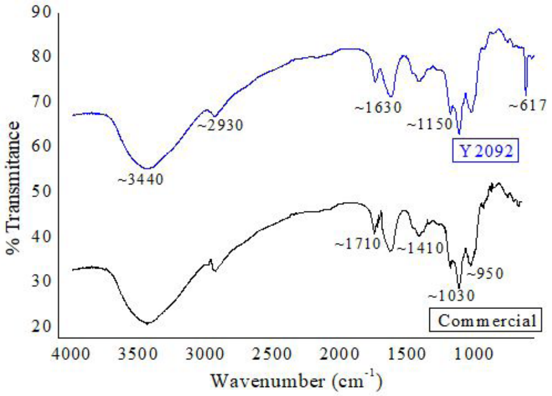


Figure 3. Spectra in the infrared region of the obtained biopolymer, Y2092, and commercial reference pullulan.

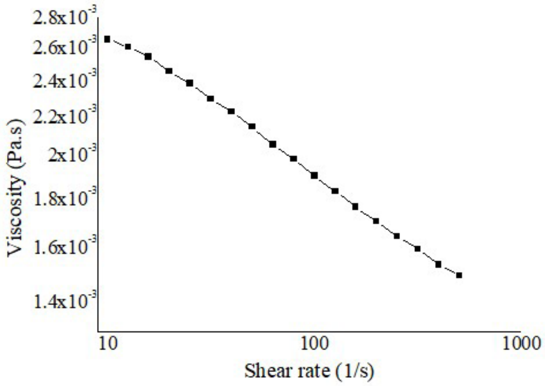


Figure 4. Viscosimetric behavior of the cell-free fermented must after 96h of VHP and RBY fermentation by *A. pullulans* Y2092.

characteristics, and rheological behavior can strongly interfere with the molecular structure and present itself as a pseudoplastic fluid, as visualized.

According to the studies by Lin et al.[40], the production of microbial EPS induces the pseudoplastic property mainly because of the molecule orientation in the solution, which reduces the flow resistance. Moreno Balderrama et al.[41] obtained results similar to this research. They stated that the change in the molecule shape in the solution added to the variation in the shear rate, and the interruption of intermolecular interactions caused by the flow contributed to the presence of pseudo-plasticity in the production of EPS. This behavior is fundamental and favorable for using the biopolymer in post-harvest technology in fruits and vegetables, in the form of biopolymer films and coatings, the objective of the pullulan synthesis in this work[42].

Measuring the intrinsic viscosity is essential to assess which type of application the material obtained is suitable[14]. The reduced viscosity *vs.* concentration is shown in Figure 5.

When observing the intrinsic viscosity result (performed with the biopolymer in solution) (7.81 dL/g), it is noticed that the data corroborate both the must viscosity data and the M_W data presented in Table 2. Data show they must present pseudoplastic fluid, higher molecular weight value, and corresponding intrinsic viscosity.

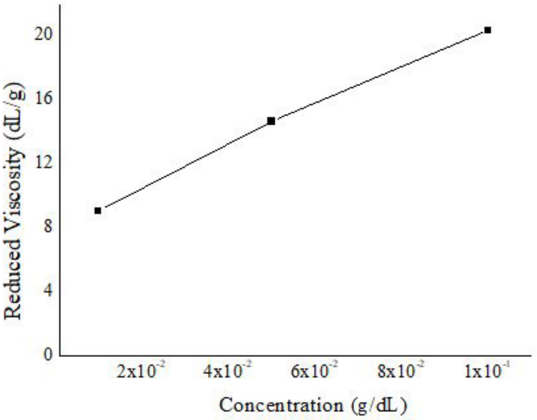


Figure 5. Reduced viscosity vs. concentration of the biopolymeric solution.

The higher the intrinsic viscosity, the higher the M_w. The molecular structure strongly depends on the rheological behavior; for some natural polysaccharides, the authors reported that composition and chain length distribution significantly affect rheological properties. This fact makes the parameter necessary, as the molecular weight is essential in determining the type of biopolymer application. High molecular weights are essential in obtaining biopolymer films[42].

Figure 5 shows a pronounced increase in the viscosity gradient above a specific critical concentration (c∗). It is possible to notice that at low polymer concentrations (more dilute region), where individual polymer molecules are present as isolated spirals, they increase more linearly with increasing concentration. Then, a slight inclination can be noticed in the region with higher concentrations (entangled domain), where the solution is less dilute, and the total hydrodynamic volume of the individual chains exceeds the volume of the solution, presenting higher values[43].

Other authors used intrinsic viscosity as a determinant in obtaining the molecular weight. Le et al.[17] used the static light scattering technique by the Debye plot to determine the molecular weight and relate it to the intrinsic viscosity. Constantin et al.[44,45] used the intrinsic viscosity associated with the Huggins equation, compared to the Marlk-Hauwink method, and found its similarity. Experimental measurements for determining the viscosimetric constants K and "α" are reported by numerical test methods with standard pullulan samples. The studies by Kasaai[46] showed these tests and their applications. They discussed determining viscosimetric parameters in depth, arriving at the following relationship: $[\eta] = 2{,}056\times10^{-4}\times M^{0{,}657}(\text{dL g}^{-1})$ for pullulan samples with M_w up to 1789 kDa.

These facts confirm that the approach used adequately describes the viscometric behavior of pullulan and provides a satisfactory prediction of the M_w of the evaluated biopolymers. Thus, when we have a high-viscosity biopolymer, we have a high M_w biopolymer, enabling various applications, including biopolymer packaging[47].

In Figure 6, it is possible to evaluate the dynamic storage modulus (G') and loss modulus (G″) to analyze the feasibility of obtaining different materials with pullulan as a

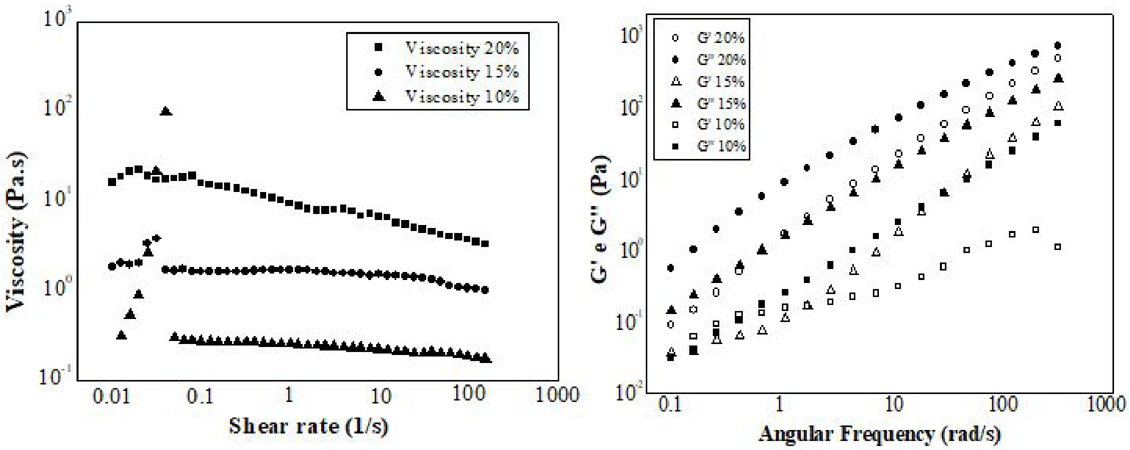


Figure 6. Viscosity vs. shear stress graph for pullulan polymeric solutions; and angular frequency vs. graph. G' and G".

polymeric matrix or support. One of them is the production of packaging films or even nanofibers by electrospinning.

The results in Figure 6 show that the polymeric solutions showed a reduction in viscosity as a function of the increase in the shear rate. This same behavior can be evaluated when observing the curve of complex dynamic viscosity versus the angular frequency curve, which generally decreases with increasing angular frequency.

This phenomenon demonstrates that pullulan-based solutions are non-Newtonian fluids, exhibiting the typical pseudoplastic fluid behavior[17]. Furthermore, G' and G'' showed that solutions prepared with pullulan gradually increased with increasing angular frequency. The higher the angular frequency, the greater the elasticity and solution viscosity. Likewise, when the same angular frequency was adopted, G' and G'' of the polymeric solutions increased with increasing pullulan concentration. This behavior can be observed because, with the increase in pullulan concentration, there is an increase in the density of geometric entanglement between molecular chains, increasing the intermolecular force, which hinders the movement of molecular chains[12,48,49].

In conclusion, the studied polymeric solutions exhibited a more elastic behavior under low shear stresses. These results show that these solutions have strong shape retention abilities with pronounced shear thinning behaviors. These elastic characteristics facilitate the dimensional stability of these solutions and provide adequate rheological properties for a better molecular mass and consequent improvement of the properties of biopolymer packaging, including obtaining fibers by electrospinning and 3D printing[42,50].

4. Conclusions

There was a robust significant influence on the molecular weight of the pullulan produced as a function of VHP sugar and brewery residue (RBY) concentrations. The maximum value of 1955 kDa was reached by fermenting the mineral medium with 70 g/L of VHP and 50 g/L of RBY. The amount of pullulan produced is within the average of pullulan production by the biotechnological route. The FTIR data showed that the pullulan obtained in this work is similar to the commercially available pullulan. The rheological properties of the pullulan obtained from the Y 2092 strain allow evaluation of the excellent capacity of this biopolymer to produce a polymeric matrix able to form biopolymer packages with good mechanical properties.

5. Acknowledgments

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