Biomedical Potential of Hyaluronic Acid from *Streptococcus zooepidemicus* Produced in Sugarcane Molasses

Potencial Biomédico do Ácido Hialurônico do *Streptococcus zooepidemicus* Produzido no Melaço de Cana de Açúcar

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ABSTRACT
Hyaluronic acid is a polymer with many biomedical applications with high production costs if obtained by fermentation. However, the use of agroindustry byproducts can improve the commercial viability. The aims of this work were to optimize the production of hyaluronic acid by *Streptococcus zooepidemicus* in sugarcane molasses medium supplemented with nutrients and to analyze the antioxidant and antimicrobial properties of hyaluronic acid. Hyaluronic acid production was greater in molasses (0.710 g·L$^{-1}$) than in glucose (0.469 g·L$^{-1}$). Only glutamine exhibited positive effects on production (0.843 g·L$^{-1}$) in Erlenmeyer flasks. Using a bioreactor under 100 rpm agitation, production was three times greater than in flasks (2.55 g·L$^{-1}$) and no differences were observed at higher agitation rates of 300 rpm. Hyaluronic acid produced presented with approximately 40 % (1 g·L$^{-1}$) of antioxidant activity but did not show antimicrobial activity. Hyaluronic acid produced in molasses could be used in formulations as an antioxidant.

Keywords: Hyaluronic acid, sugarcane molasses, bioreactor, antioxidant, antimicrobial

INTRODUCTION
Hyaluronic acid (HA) is a polysaccharide composed of glucuronic acid and N-acetylglucosamine units [28]. HA is hydrophilic and viscoelastic and shows antioxidant and antimicrobial properties. These properties promote the use of HA in cosmetic, pharmaceutical and biomedical formulations. Due to this wide range of applications, demand for hyaluronic acid has increased in recent years and has concurrently enhanced the development of optimization methodologies. Although hyaluronic acid could be extracted from animal tissues, microbial production is preferred because biochemical compounds derived from animal tissues have inherent bioprocess safety concerns [10].

The commercial competitiveness of hyaluronic acid is limited due to the high (more than 80 %) production costs of fermentation medium [42]. To improve the efficiency of fermentation
processes and reduce production costs, low-cost substrates should be utilized, such as agroindustry byproducts [26,41], which include cheese whey [1], dairy industrial waste [29,34], steep liquor [2,31] and sugarcane molasses [2,32].

Sugarcane molasses is a rich substrate, containing carbohydrates (e.g., sucrose, glucose and fructose), metal ions, vitamins and peptides [41,14]. We previously studied hyaluronic acid production in sugarcane molasses and observed higher production (2,825 g·L⁻¹) when the molasses was pretreated with charcoal [32]. In that work, Pan and co-workers [32] optimized the production of hyaluronic acid in medium containing only yeast extract. Because Streptococcus is a fastidious genus, in this present study, we tested hyaluronic acid production using other culture medium supplementation. The aim of this work was to evaluate the effect of niacin, glutamine, oxalic acid, sodium iodoacetate and sodium acetate on hyaluronic acid production by *Streptococcus zooepidemicus* in sugarcane molasses and glucose media. The antioxidant and antimicrobial properties of the polymer were also determined.

2 MATERIALS AND METHODS

2.1. HYALURONIC ACID PRODUCTION IN ERLENMEYER FLASKS

*Streptococcus equi* subsp. *zooepidemicus* ATCC 39920 was obtained from the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI). The strain was maintained in 37 g·L⁻¹ of Brain Heart Infusion (BHI) media containing 50 % of glycerol and stored at −80 °C. *S. zooepidemicus* was cultivated in BHI 37 g·L⁻¹ with 2 % agar at 37 °C for 24 h. Colonies from plates were transferred to 25 mL pre-inoculum medium (BHI 37 g·L⁻¹) in Erlenmeyer flasks at 37 °C, 150 rpm. After 24 h, 10 % (v/v) of the pre-inoculum were transferred to Erlenmeyer flasks containing 25 mL of inoculum medium (g·L⁻¹): glucose (30.0); yeast extract (30.0); K₂HPO₄ (2.5); NaCl (2.0); and MgSO₄ (1.5) for 6 h, at 150 rpm and 37 °C. After centrifugation (9,956 xg, 4 °C, 15 min), the inoculum was standardized at 0.2 g·L⁻¹ for all fermentations.

2.1.1 Optimization of hyaluronic acid production

The effects of sodium acetate (X₁), glutamine (X₂), niacin (X₃), oxalic acid (X₄) and sodium iodoacetate (X₅) on hyaluronic acid production were verified by Box Hunter × Hunter 2⁵⁻¹ fractional factorial design (Table 1) in glucose or sugarcane molasses media. Molasses was obtained from the Alltech group (São Pedro do Ivaí, PR, Brazil) and pretreated with activated charcoal according to Pan et al. [32]. The fermentation medium contained (g·L⁻¹): glucose or molasses (30.0 of total sugars); yeast extract (30.0); K₂HPO₄ (2.5); NaCl (2.0); and MgSO₄ (1.5). The 125-mL Erlenmeyer flasks
were maintained for 24 h at 37 °C, 100 rpm and an initial pH 8.0. Posterior, triplicate fermentation processes with varying glutamine concentrations (0.0 to 12.0 g·L⁻¹) were carried out using the best carbon source.

2.2 HYALURONIC ACID PRODUCTION IN BIOREACTOR

The effect of orbital shaker agitation speed (100 and 300 rpm) was evaluated under optimized hyaluronic acid production conditions. Fermentations were performed in a 5.0-L bioreactor (FerMac 320 – Electrolab Biotech Ltda.) at pH 8.0 (maintained by NaOH), 0.5 vvm, 37 °C in 3.5 L media (g·L⁻¹): molasses (30.0 of total sugars); yeast extract (30.0); K₂HPO₄ (2.5); NaCl (2.0); MgSO₄ (1.5); and glutamine (8.0). Samples were collected every 2 h for 24 h.

2.3 BIOMASS, HYALURONIC ACID, ORGANIC ACIDS, ETHANOL AND SUGAR CONSUMPTION DETERMINATIONS

Fermentation samples were centrifuged at 9956 x g, 4 °C for 15 min. Biomass was determined by turbidity measurement (λ= 600 nm) and correlated with the biomass curve (g·L⁻¹). Reducing and total sugars were determined from supernatant samples according to Somogyi-Nelson [39,30] and Dubois et al. [18], respectively. To assess hyaluronic acid, lactate, formate, acetate and ethanol levels, the culture supernatant samples were filtered (0.45 µm pore size, Millipore), and 20 µL were injected into an HPLC (Shimadzu Corporation, Kyoto, Japan). The peak elution profile was monitored with a Shimadzu RID – 10A refractive index detector (Shimadzu Corporation, Kyoto, Japan). Hyaluronic acid evaluation was performed in an OHpak SB-806MHQ 80 x 300 mm column (Shodex, Japan) at 40 °C with a 0.1 M NaNO₃ mobile phase and a 1 mL·min⁻¹ flow rate. Sodium hyaluronate (1.5-1.8 x 10³ kDa) (Sigma-Aldrich, Brazil Ltda.) was used as a standard. Organic acids and ethanol were evaluated using an Aminex 7.8 x 300 mm HPX-87H column (Bio-Rad, CA, USA) at 60 °C. The mobile phase was 0.005 mol·L⁻¹ H₂SO₄ solution at 0.7 mL·min⁻¹. Standards (Sigma-Aldrich, Brazil Ltda.) were used for each compound.

2.4 HYALURONIC ACID CHARACTERIZATION

Bioreactor-produced hyaluronic acid was precipitated in ethanol (1:1.5) for 1 h at 4 °C, centrifuged (1.259 x g, 4 °C, 20 min) and solubilized in 0.15 M NaCl. The procedure was repeated (3 times), and the resultant solution was treated with trichloroacetic acid until pH 2.0 [37]. After 1 h, the collected solution was centrifuged (7.744 x g, 4 °C, 30 min), and the supernatant was dialyzed (72
h) and lyophilized to characterize hyaluronic acid. Sodium hyaluronate (1.5-1.8 x 10³ kDa) (Sigma-Aldrich, Brazil Ltda.) was used as standard.

Fourier transform infrared spectroscopy (FTIR) was recorded at wavelengths between 4.000 cm⁻¹ to 400 cm⁻¹ on an IR PRESTIGE-21 (Shimadzu, Kyoto, Japan) spectrophotometer. Thirty-two scans at 4 cm⁻¹ resolution were averaged and referenced against air. The powdered samples were compressed into KBr disks.

The UV-Vis absorption spectrum was assessed using a UV-Vis recording spectrophotometer (Biochrom Libra s22) in the 190-450 nm range. Distilled water was used as a reference and to dilute 1 mg·mL⁻¹ hyaluronic acid. NMR analysis was performed using a Bruker Spectrometer (AVANCE III 400 MHz) operated at 24 °C and a ¹³C frequency of 100.6 MHz, using cross polarization (CP) at the magic angle (MAS) [15].

2.5 BIOMEDICAL POTENTIAL ASSESSMENT

2.5.1 Antioxidant Activity

Antioxidant activity was estimated using 2,2, diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich Brazil Ltda.). Briefly, 300 µL 0.1 mM DPPH in ethanol were added to a 1-mL sample in 0.2-1.0 g·L⁻¹ concentrations. The mixture was left to stand for 30 min at room temperature. The absorbance was measured at 517 nm against a blank (water instead of sample and ethanol instead of DPPH). The scavenging percentage activity was calculated as Equation 1:

\[
(\%) = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100
\]  

where A₀ is the control absorbance (water instead of sample), A₁ is the sample absorbance, and A₂ is the sample absorbance under identical conditions as A₁ with ethanol instead of DPPH solution. Ascorbic acid (Sigma-Aldrich Brazil Ltda.) was used as a standard for the assay.

2.5.2 Antimicrobial Activity

The strains evaluated were *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Salmonella enterica* subsp. *enterica* ATCC 14028, *Enterococcus faecium* ATCC 6569, *Staphylococcus aureus* ATCC 6336, and *Streptococcus mutans* ATCC 25175 following the standards of the Clinical and Laboratory Standards Institute (CLSI) [12].

To determinate the minimum inhibitory concentration (MIC), different concentrations of sodium hyaluronate (Sigma-Aldrich, Brazil Ltda) (0.25 to 10.0 mg·mL⁻¹) and hyaluronic acid were produced in a bioreactor (0.25 to 80.0 mg·mL⁻¹) prepared in 38 g·L⁻¹ Müeller-Hinton Broth (MHB) were transferred to 96-well plates containing 50 µL of 10⁶ CFU/mL to achieve a final bacterial density.
of $5 \times 10^5$ CFU/mL. Sodium hyaluronate standard (10.0 mg·mL$^{-1}$) and produced hyaluronic acid (80.0 mg·mL$^{-1}$) were tested. Negative controls (50 μL of $10^6$ CFU/mL and 50 μL of MHB) were also tested. All plates were incubated at 37 °C for 18-24 h. To the hyaluronic acid standard, plates were analyzed for turbidity [12]. To the produced hyaluronic acid, solutions were diluted (1:1000) in 0.9 % NaCl, and 100 μL were plated in 38 mg·mL$^{-1}$ of Müeller-Hinton agar plates. After 18-24 h, CFU were accounted.

For the agar diffusion test, strains solutions at $10^8$ CFU/mL (0.5 MacFarland scale) were plated in 38 mg·mL$^{-1}$ of Müeller-Hinton agar. Disks with 20 μL hyaluronic acid (0.5; 2.0; 4.0; and 8.0 mg·mL$^{-1}$) were arranged on the plates. After 18-24 h at 37 °C, the formation of inhibition zones was observed around disks and measured (mm).

2.6 STATISTICAL ANALYSIS

Data analysis was performed using Statistica 7.0 for fractional factorial design analysis. Variance analysis and media comparisons by Tukey and t-test were used with $\alpha = 5 \%$. The profiles of hyaluronic acid, biomass, lactate and acetate productions in bioreactor were modelled using the logistic equation (Equation 2):

$$P = P_{\text{max}}/(1 + \exp[(x_0 - x)/k])$$

(2)

where $P_{\text{max}}$ is the maximum biomass, hyaluronic acid, lactate or acetate production (g·L$^{-1}$), $k$ is the numeric scale parameter on the input axis, and $x_0$ is the x value at the inflection point of the curve.

The parameters significance of logistic models and their interactions when applied were tested by variance analysis and t test.

3 RESULTS AND DISCUSSION

3.1 OPTIMIZATION OF HYALURONIC ACID PRODUCTION

High hyaluronic acid production levels were 0.469 g·L$^{-1}$ in glucose and 0.710 g·L$^{-1}$ in molasses media both in the presence of glutamine (4 g·L$^{-1}$) (Table 1, run 3). The production in molasses was 51 % higher than that in glucose because this source is rich in fermentable sugars, amino acids and metallic ions (e.g., Fe$^{2+}$) that could improve hyaluronic acid production, as observed by Choi and co-workers [9]. Treatment with activated charcoal could result in a source with fewer inhibitors to hyaluronic acid production, such as inhibitor protons [35,40]. Pan et al. [32] observed that pre-treated molasses yielded higher hyaluronic acid production than crude molasses.
Interactions among sodium acetate, oxalic acid and iodoacetate and glutamine with sodium acetate, niacin and iodoacetate were significant and presented negative effects on hyaluronic acid production in glucose medium. In the molasses medium, sodium acetate ($p = 0.048647$) niacin ($p = 0.001423$) and iodoacetate ($p = 0.000516$) presented significant negative effects on hyaluronic acid production.

Niacin (vitamin B3) is a coenzymes precursor of NAD$^+$ and NADP$^+$. Amrane and Prigent [3] observed that B-vitamins are the main contributors of yeast extract in the production of hyaluronic acid. The amide forms of these vitamins are considered integral supplements in chemically defined medium (CDM) to group A of *Streptococcus* [24]. However, in this work, niacin did not show any positive effects on HA production because we used vitamin B3 in amine form. During hyaluronic acid production, reactive oxygen species (ROS) could depolymerize the polymer chain and decrease the overall molecular weight (i.e., a crucial parameter for different polymer applications [27]). The addition of oxalic acid did not show any significant effects on hyaluronic acid production in molasses.
medium but presented negative effects in glucose medium. However, the effect of oxalic acid on hyaluronic acid weight was not investigated.

Figure 1 - Profiles to predict values for hyaluronic acid production by *S. zooepidemicus* in glucose (a) and sugarcane molasses (b)
In the glucose and sugarcane molasses media, glutamine exhibited non-significant but positive effects at the tested concentrations (Fig. 1) due to the amino acid acting as the amino donor group to UDP-N-acetylglucosamine formation [10], limiting hyaluronic acid precursor synthesis [38]. The determination coefficient (R²) for fermentation using molasses by fractional factorial design was 0.8485, explaining the 84.85 % response variability. The desirability function was used to find the optimal conditions for hyaluronic acid production. Under this condition (glutamine at 4 g·L⁻¹), the predicted production level of hyaluronic acid was 0.712 g·L⁻¹, which was validated (p = 0.3024) with an observed average production of 0.723 g·L⁻¹.

Lactate, acetate and formate are also produced during Streptococcus fermentation and are responsible for decreasing the pH of the cultures, thereby inhibiting microbial growth and hyaluronic acid production [20,21]. Since sodium acetate can react with acetate to form a buffer system, the addition of sodium acetate to the medium could improve hyaluronic acid production to maintain the pH of cultures near optimum for polymer production, as suggested by Aroskar, Kamat and Kamat [5] using calcium carbonate. As previously optimized by our group [33], the initial pH of fermentations was 8.0, and pH values after 24 h fermentation ranged from 4.85 to 6.40 in glucose and 4.85 to 6.63 in molasses, indicating a decreasing pH due to lactate and acetate production. Variance analysis showed that sodium acetate was significant (p ≤ 0.04) and had positive effects on pH in glucose cultivations, as well glutamine and iodoacetate. Although the variable had negative effects on hyaluronic acid production, suggesting a more complex regulation than expected. In molasses medium, only iodoacetate exhibited positive effects on pH cultures (p = 0.0002).

Streptococcus are lactic acid bacteria that convert most consumed glucose into lactate by glycolysis. Therefore, decreasing lactate production indicates decreasing glycolysis [36]. In fermentations containing iodoacetate, a glyceraldehyde 3-phosphate dehydrogenase enzyme inhibitor, a reduced carbon flux in the glycolytic pathway with decreased biomass formation was observed in runs 4, 10 and 16 (Table 4). Iodoacetate already showed significant effects on organic acid production. Beyond a partially inhibited glycolytic pathway, this compound also showed an inhibitory effect against lactate dehydrogenase enzyme [38], favoring the redirection of pyruvate to ethanol production in glucose (0.273 g·L⁻¹, run 1) and causing decreased lactate production (Table 2). In the presence of iodoacetate, the produced hyaluronic acid had a higher molecular weight [38,36], resulting in greater agglomeration and the creation of an anoxic zone in which alcoholic fermentation is preferred [11]. In molasses medium, ethanol production was not detected.

Acetate was produced in large quantities in presence of sodium acetate (runs 2, 4, 6, 8, 10, 12, 14 and 16, Table 2) in the glucose and molasses media. The variable showed the major effect on the
production of this organic acid. In molasses cultures, sodium acetate also exhibited positive and significant effects ($p = 0.0004$) on lactate production. However, this last effect was strongly and negatively affected by iodoacetate ($p<0.0001$), likely due to the inhibitory action of iodoacetate on lactate dehydrogenase.

Table 2 - Organic acids production by *S. zooepidemicus* in glucose and sugarcane molasses in Erlenmeyer flasks at 37 °C, 100 rpm, 24 h, pH initial 8.0 (Box Hunter x Hunter $2^{5-1}$).

| Runs | Glucose Acetate (g.L$^{-1}$) | Glucose Lactate (g.L$^{-1}$) | Molasses Acetate (g.L$^{-1}$) | Molasses Lactate (g.L$^{-1}$) |
|------|----------------------------|-----------------------------|----------------------------|-----------------------------|
| 1    | 1.046                      | 4.197                       | 1.130                      | 1.968                       |
| 2    | 0.743                      | 2.479                       | 10.147                     | 11.583                      |
| 3    | 0.779                      | 5.750                       | 0.933                      | 7.477                       |
| 4    | 9.561                      | 1.705                       | 9.156                      | 2.014                       |
| 5    | 0.524                      | 5.714                       | 1.538                      | 8.641                       |
| 6    | 9.874                      | 3.434                       | 8.862                      | 5.059                       |
| 7    | 0.543                      | 2.341                       | 0.815                      | 2.106                       |
| 8    | 9.213                      | 6.242                       | 8.282                      | 12.235                      |
| 9    | 0.479                      | 5.088                       | 0.565                      | 7.629                       |
| 10   | 13.159                     | 2.719                       | 9.724                      | 2.358                       |
| 11   | 0.547                      | 1.975                       | 0.828                      | 2.210                       |
| 12   | 9.632                      | 5.916                       | 9.172                      | 12.420                      |
| 13   | 0.437                      | 4.963                       | 1.025                      | 2.298                       |
| 14   | 9.287                      | 6.847                       | 9.719                      | 13.082                      |
| 15   | 0.326                      | 4.586                       | 0.990                      | 7.794                       |
| 16   | 9.114                      | 1.839                       | 9.410                      | 2.655                       |
| 17   | 4.560                      | 3.319                       | 4.432                      | 5.517                       |
| 18   | 4.355                      | 2.853                       | 4.350                      | 5.355                       |
| 19   | 4.018                      | 3.389                       | 4.308                      | 5.561                       |

3.2 HYALURONIC ACID PRODUCTION IN DIFFERENT GLUTAMINE CONCENTRATIONS

Since molasses was the best carbon source for hyaluronic acid production, molasses was used for subsequent fermentations. Increased glutamine concentrations from 0 to 12 g·L$^{-1}$ improved polymer production levels from 0.716 ± 0.025 to 0.843 ± 0.012 g·L$^{-1}$ ($p<0.0001$), likely due to a higher level of UDP-N-acetylglucosamine production from glutamine and fructose-6-P [10]. The observed greater polymer production was like the production obtained in 8 g·L$^{-1}$ glutamine (0.826 g·L$^{-1}$ ± 0.014) ($p = 0.245$). Thus, this concentration was used for bioreactor fermentations.

Glutamine concentration also had a significant effect on microbial growth ($p = 0.021$), which increased at 8, 10 and 12 g·L$^{-1}$ compared with the growths at 0 to 4 g·L$^{-1}$. However, glutamine had no effect on sugar consumption ($p > 0.1$). Culture pH levels were similar, ranging from 4.87 to 4.91, due to the similar lactate and acetate levels ($p = 0.187$ and 0.052, respectively).
3.3 HYALURONIC ACID PRODUCTION IN BIOREACTOR

The proposed hyaluronic acid, biomass, lactate and acetate production models showed similarities between the predicted and observed values, with $R^2$ values higher than 0.9 for all responses. Residual analyses demonstrated normal and similar residual variances. Furthermore, the p-values in the Fisher F-test were significant ($p<0.001$) for all responses, indicating model consistency in describing experimental data.

Hyaluronic acid production was improved approximately 3 times in the bioreactor when compared with flask production under the same conditions. Although agitation speed showed some effects on microbial growth, hyaluronic acid production levels were similar at 100 and 300 rpm. At 100 rpm, the specific growth rate was 0.171 h$^{-1}$ with a maximum polymer production level of 2.55 g·L$^{-1}$ in 24 h. At 300 rpm, the specific growth rate was 0.255 h$^{-1}$, and the hyaluronic acid production level reached 2.43 g·L$^{-1}$ in 24 h. (Figure 2)

Figure 2 - Production of hyaluronic and organic acids, biomass and sugar consumption by S. zooepidemicus in bioreactor at 100 (•) and 300 rpm (△)
Amado et al. [2] observed that in controlled pH and aeration conditions, HA production was dependent on agitation speed. However, this effect was not observed in the present work. In previous studies, our group found that pH 8.0 was the optimum level for fermentation in Erlenmeyer flasks [33]; pH control in bioreactors improved hyaluronic acid production by a factor of 2.86 [32].

Lactate and acetate production levels were higher in the bioreactor (p<0.002). Lactate production reached 24.60 g·L⁻¹ in 24 h at 100 rpm and 16.80 g·L⁻¹ at 300 rpm and were 3 and 2 times greater, respectively, than the production levels in Erlenmeyer flasks (Fig. 2). Acetate production levels at 100 and 300 rpm were 5.72 and 5.27 g·L⁻¹, respectively, i.e., 5 times higher than the smaller scale flasks. Lactate production and sugar consumption at 100 rpm was 3 times higher than in the flasks, reaching 27.24 g·L⁻¹ in 24 h. At 300 rpm, sugar consumption in 24 h was 28.19 g·L⁻¹. While the larger mass transfer at the highest agitation favored microbial growth instead of lactate production, the agitation speed had no effect on hyaluronic acid production.

3.4 HYALURONIC ACID CHARACTERIZATION

The UV-Vis spectrum showed similarity of hyaluronic produced by S. zooepidemicus with standard in absorbance profile in both conditions of speed agitation. FT-IR analysis confirmed the presence of functional groups characteristic of hyaluronic acid, with produced equal to standard polymer, like the elevated peak in ≈ 3440 cm⁻¹, related with hydrogen bonded H-O stretching and N-H stretching vibrations of the N-acetyl side chain [22]; in ≈ 1650-1617 cm⁻¹, amide I of C=O carboxyl; in 2929 cm⁻¹, according with stretching bound of C-H of glucuronic acid; and in 1029 cm⁻¹, the stretching of C-O bond of primary alcohol [8].

The NRM spectrum of produced and standard hyaluronic acid showed the same peaks. The peak around 175 ppm is correlated with carbonyl carbons from carboxylic and acetyl. The 102-106 ppm region was assigned to C1 linked with two oxygen atoms. Peak at 75.4 ppm was attributed to C-OH, at 54.4 ppm to C2 linked to N and 23.5 ppm to methyl from N-acetyl group [15].

3.5 ANTIOXIDANT ACTIVITY

Free radical production can result in tissue damage, resulting in the loss of function. Oxidative damage protection provided by antioxidant and repair systems are typically insufficient. Because of that, too much attention has been focused on the role of hyaluronic acid as an antioxidant [6,19,23,25]. As shown in Fig. 3, the antioxidant activity levels of hyaluronic acid produced at 100 and 300 rpm were like hyaluronic acid standard and were lower than that of ascorbic acid. The higher antioxidant
activity for produced polymer was 38.40 % (1 g·L\(^{-1}\), 100 rpm) and 41.00 % for standard polymer, as observed by Pan et al. [32].

Figure 3 - Antioxidant activity (%) by the reduction of the DPPH radical of standard and produced hyaluronic acid by S. zooepidemicus in a reactor with agitation of 100 and 300 rpm. The ascorbic acid was used as a positive control.

3.6 ANTIMICROBIAL ACTIVITY

At the tested concentrations, the minimum inhibitory concentration of the produced and hyaluronic acid standard could not be determined. The use of hyaluronic acid at higher concentrations becomes a problem due to economic costs and increased cytotoxicity. As described by Cermelli et al. [7], 4 g·L\(^{-1}\) of hyaluronic acid showed 20 % of cytotoxicity on epithelial cell lines; hyaluronic acid levels at 10 times that concentration would certainly result in higher toxicity.

The agar diffusion test also showed a low antimicrobial effect of hyaluronic acid. No inhibition zones in any of the tested concentrations and higher concentrations (i.e., more viscous) were observed on the agar. These results corroborated with those of Di Cerbo et al. [16], who did not observe any antimicrobial activity of hyaluronic acid when tested on lactic bacteria.

In contrast, Zhang et al. [43] observed improved S. pyogenes biomasses in a medium supplemented with hyaluronic acid. Costagliola et al. [13] tested hyaluronic acid against Streptococcus and Staphylococcus strains and observed microbial growth improved by 50 % when the media were supplemented with 7.0 g·L\(^{-1}\) of the polymer. This group assumed that the probiotic growth was due to the Gram-positive bacteria produced hyaluronidases, enabling the release of small...
sugars as nutrients during polymer degradation. Drago et al. [17] showed that the antimicrobial effect of hyaluronic acid presented the growth of Gram-positive and Gram-negative bacteria due to disrupted biofilm formation.

However, Ardizzoni et. al [4] showed that some Staphylococcus aureus and Streptococcus epidermidis and S. mutans strains showed significantly decreased of microbial growth with increased hyaluronic acid concentration. However, these authors measured activity against positive controls using an inoculum of $5 \times 10^3$ UFC and exposition time of 72 h, parameters not advocated by CLSI [12].

4 CONCLUSION

The production of hyaluronic acid by S. zooepidemicus was bigger in sugarcane molasses than in glucose. Only glutamine present positive effect on production. Bioreactor fermentation (molasses 30.0 g.L$^{-1}$ of total sugars and glutamine 8 g.L$^{-1}$) improved polymer product about 3 times (2.55 g.L$^{-1}$) and agitation speed did not show influence. Hyaluronic acid produced in sugarcane molasses present 38.40 % of antioxidant activity in 1.0 g.L$^{-1}$, demonstrating its application potential to be used in biomedical products, but did not present antimicrobial activity.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.
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