BIOLOGICAL PROPERTIES OF SODIUM HYALURONATE PRODUCED BY *Streptococcus zooepidemicus immobilized*

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ABSTRACT

The aim of this study was to purify sodium hyaluronate produced by *Streptococcus zooepidemicus* cells immobilized in situ on flexible polyurethane (PU) and evaluate the biological properties (cytotoxicity, antioxidant and antimicrobial activity). Purification of sodium hyaluronate was carried out using ethanol and sodium chloride. Sodium hyaluronate showed 56,65% purification and/or recovery, did not exhibit cytotoxicity in HEK-293 cells at concentrations up to 1000 µg mL^{-1}, presented minimum inhibitory concentrations (MIC) for the bacteria *Escherichia coli* ATCC 8739, *Listeria monocytogenes* NCTC 11994, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923 at a concentration above 50 mg/mL. The antioxidant action of sodium hyaluronate presented a value of 77,82 mg/ml of sodium hyaluronate to eliminate 50% of the ABTS radical. These results are promising and indicate that the sodium hyaluronate produced can be used for possible applications, mainly in the food sector, as it presents antioxidant and antimicrobial activity.

Keywords: Hyaluronic acid. Bioproduction. Toxicity. Antioxidant. Antimicrobial.

1 INTRODUCTION

Hyaluronic acid (HA) is ca biomaterial with wide applications. Due to its physical and biological properties, such as non-toxicity, acting as an antimicrobial and antioxidant agent, sodium hyaluronate can be used in the food industry (films, food supplements, milk and dairy products industry).[1] With the commercialization of HA increasingly high, the industrial production of this biopolymer depends on microbial fermentation carried out mainly by strains of *Streptococcus zooepidemicus*, with the most used mode of operation being batch culture.[2]

The purification processes themselves generally include a combination of different techniques such as centrifugation, precipitation, filtration, chromatography or ultrafiltration. The use of solvents such as ethanol, methanol, acetone and propanol that act by promoting dehydration and reducing the dielectric constant of the medium, increasing electrostatic interactions, leading to intra and intermolecular aggregation. This is one of the most used operations for removing proteins. In these cases, the use of ethanol and propanol are preferable due to their non-toxicity. Furthermore, the cost of ethanol is cheaper when compared to other alcohols and ketones, which is why it is the solvent most used for HA precipitation.[3] The use of sodium chloride also serves to aid in the precipitation of the biopolymer resulting in more homogeneous particles and due to this stage of the purification process, hyaluronic acid is converted into sodium hyaluronate.

In this sense, the objective of this stage of the study was to recover sodium hyaluronate produced by submerged fermentation of *Streptococcus zooepidemicus* immobilized in polyurethane foam and evaluate its biological properties.

2 MATERIAL & METHODS

The *S. zooepidemicus* strain was reactivated in plates containing Brain Heart Infusion – BHI broth (Merck KGaA/Germany) as described by Silva et al (2021).[4] The immobilization process in polyurethane occurred following the methodology described by Souza et al.[5], with adaptations, using 7 g of polyol, 0,02 g of silicone, 8 g of *S. zooepidemicus* biomass and 3 g of isocyanate. The microbial production of hyaluronic acid, as well as its release and quantification followed the methodology described by Silva et al (2021)[4].

HA recovery/purification was carried out according to Amado et al.[6], with modifications and quantified according to Equation 1.

$$AH\ recovered\ (\%) = \frac{AH_{recovered}}{AH_{without\ recovering}} * 100 \quad (1)$$

Where: recovered HA is the amount of HA in g/L after the recovery process; Unrecovered AH is the amount of AH in g/L before the recovery process.

To obtain powdered sodium hyaluronate, the precipitate diluted in 20 mL of distilled water was frozen for 24 h and subsequently lyophilized in a freeze dryer (model: Modulyo; manufacturer: Edwards), coupled to a vacuum pump (model: RV8; manufacturer: Edwards); at a temperature of -40 °C to -60 °C for 48 h.

For sodium hyaluronate cytotoxicity assays, the Vero monkey kidney endothelial cell line was used, acquired from the Rio de Janeiro Cell Bank (BCRJ). For the tests, hyaluronic acid dissolved in ultrapure water was used and filtered with a sterilized syringe filter (0,22 µm; polyethersulfone). Cells were treated with increasing concentrations (10, 50, 100, 500 and 1000 µg/mL) of hyaluronic acid solution (m/v) for 24, 48 and 72 h. To assess cell viability, the MTT assay was performed according to Denizot and Lang.[7] Nitric oxide (NO) levels were determined using an indirect method based on the use of Greiss.[8] Quantification of extracellular Reactive Oxygen Species (ROS) using a 2,7 dichlorofluorescein diacetate fluorometric. To evaluate the release of double-stranded DNA (ds-DNA), the PicoGreen assay was used.[9] All results were expressed as a percentage.

To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (CBM), the evaluation of the antimicrobial activity of sodium hyaluronate was carried out using four different bacteria*: Escherichia coli* ATCC 8739; *Listeria monocytogenes* NCTC 11994; *Salmonella typhimurium* ATCC 14028; *Staphylococcus aureus* ATCC 25923. The MIC and MBC results were determined according to Equation 2.

$$MIC/MBC\ (mg/mL) = Abs\ (sample) - Abs\ (control) \quad (2)$$

In this work, the antioxidant activity was performed against the ABTS●+ radical according to the method described by Larrauri, Rupérez and Saura-Calixto[15] with adaptations.

3 RESULTS & DISCUSSION

The methodology used, which led to the formation of sodium hyaluronate, enabled the purification and/or recovery of 56,65% of the HA, that is, some impurities were still present in the product and/or in the conversion into sodium hyluronate.

Cytotoxicity assays are essential to determine the impact of the product on cell survival, as well as to identify the optimal concentrations at which there is no reduction in the rate of cellular metabolism greater than 20%.[8] Figure 1 (a) shows cell viability after 72 h of treatment of VERO cell lines (monkey) with different concentrations of sodium hyaluronate where it did not show cytotoxicity. The generation of free radical molecules, such as nitric oxide (NO), is an indicative marker of cellular inflammation. In this context, within the evaluated concentration range of sodium hyaluronate there was no change in NO levels in VERO cells (Figure b), which suggests that sodium hyaluronate has active anti-inflammatory activity.

ROS levels are important mediators in several pathways and are known toxic products in cellular metabolism. Through Figure 1 (c), it was possible to observe that in the concentrations of sodium hyaluronate studied, the generation of oxidative stress was not observed, maintaining the same amount of reactive oxygen species for up to 72 h in VERO cells, a value statistically equal to the cell control. Through Figure 1 (c), it was possible to observe that in the concentrations of sodium hyaluronate studied, the generation of oxidative stress was not observed, maintaining the same amount of reactive oxygen species for up to 72 h in VERO cells, a value statistically equal to the control of the cell. The PicoGreen assay is used to quantify ds-DNA released into the medium due to cell membrane damage, with the aim of investigating cytotoxicity. As shown in Figure 1 (d), it is possible to observe that ds-DNA levels in VERO cells did not show significant changes ($p>0.05$) in any of the sodium hyaluronate exposure conditions subjected to.

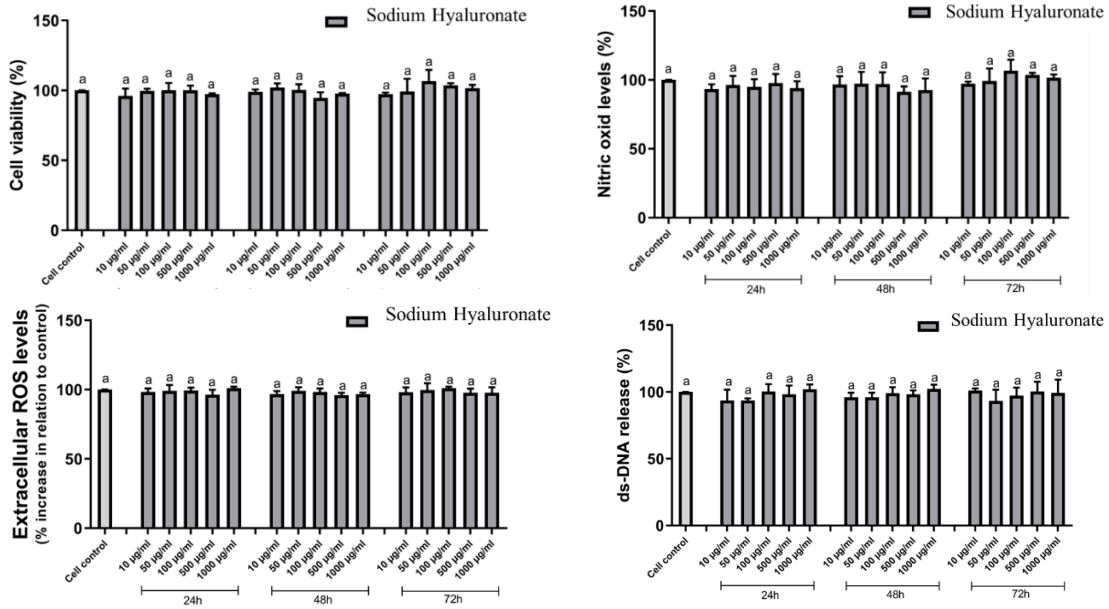

Figure 1 (a) Effect of sodium hyaluronate on VERO cell viability; (b) Effect of sodium hyaluronate on nitric oxide levels in VERO cells; **(c)** Effect of sodium hyaluronate on ROS levels in VERO cells; (d) Cytotoxicity of sodium hyaluronate according to Quant-iT™ PicoGreen® ds-DNA reagent in VERO cells.

Therefore, it can be concluded that the sodium hyaluronate produced and purified in this work does not represent a toxic role for the VERO cells studied, however, it does not present satisfactory responses as an antioxidant.

The antimicrobial activity of sodium hyaluronate was tested against four bacteria, namely: *Escherichia coli; Listeria monocytogenes; Salmonella typhimurium*; and *Staphylococcus aureus* and the results of the inhibitory and bactericidal concentration are presented in Table 1.

Table 1 Minimum inhibitory and bactericidal concentration of sodium hyaluronate.

Pathogenicity	Bacterium	MIC (mg/mL)	CBM (mg/mL)
Gram-positive	*Escherichia coli*	50	> 50
	Listeria monocytogenes	50	> 50
Gram-negative	*Salmonella typhimurium*	50	> 50
	Staphylococcus aureus	> 50	> 50

Sodium hyaluronate showed antimicrobial activity with a minimum inhibitory concentration (MIC) of 50 mg/mL against 3 of the 4 bacteria studied (*Escherichia coli, Listeria monocytogenes* and *Salmonella typhimurium*). However, studies on the minimum bactericidal concentration (MBC) potential of sodium hyaluronate did not produce favorable results for any of the microorganisms examined, as none of the bacteria demonstrated susceptibility to cell death.

The results obtained indicate that 1 g of sodium hyaluronate was capable of inhibiting the ABTS radical cation in a manner equivalent to 15,36 µmol trolox and that 77,82 mg/ml of sodium hyaluronate are needed to eliminate 50% of the ABTS radical.

4 CONCLUSION

Based on the results obtained, we can conclude that the hyaluronic acid (HA) produced, although not reaching the maximum level of purity reported in other studies, demonstrated to be a safe and promising material for a variety of industrial applications. The purification methodology used, using ethanol as a solvent together with NaCl, resulted in an efficient purification of sodium hyaluronate, with an impurity removal rate of 56,65%. Cytotoxicity analysis indicated the safety of HA produced at tested concentrations, suggesting its potential for use in biological applications.

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