0273-2289/05/121–124/753–762/$30.00

Production of Hyaluronic Acid by *Streptococcus*

The Effects of the Addition of Lysozyme and Aeration on the Formation and the Rheological Properties of the Product

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Abstract

The effects of the addition of lysozyme and forced aeration on the rheological properties and production of hyaluronic acid by *Streptococcus zooepidemicus* were investigated. Lysozyme was added to the culture broth in two pulses during the exponential and stationary phases of a fermentation carried out in a rotary shaker (150 rpm), using 200 mL Erlenmeyer flasks. The effect of aeration was evaluated by feeding air into a 2.5 L fermentor at a 2 vvm rate. The effects were analyzed in terms of concentration, viscosity, viscoelasticity, and molecular weight of the hyaluronic acid produced.

Index Entries: Hyaluronic acid; fermentation; *Streptococcus;* lysozyme and aeration.

Introduction

Hyaluronic acid (HA) is a linear high-molecular-weight glycosaminoglucan polysaccharide composed of repeating disaccharide units of alternating D-glucuronic acid (GlcUA) and *N*-acetylglucosamine (GlcNAc), as shown in Fig. 1. HA has a great potential for medical and cosmetic applications owing to its ability to retain large volumes of water and its rheological properties *(1)*.

It is well known that HA and its salts can be obtained from at least three sources: human umbilical cords, rooster combs, and bacterial streptococci cultures (A and C hemolytic groups). However, some disadvantages are associated with the former two sources, such as relatively low yields, contamination, and labor-intensive processing during the purification steps.

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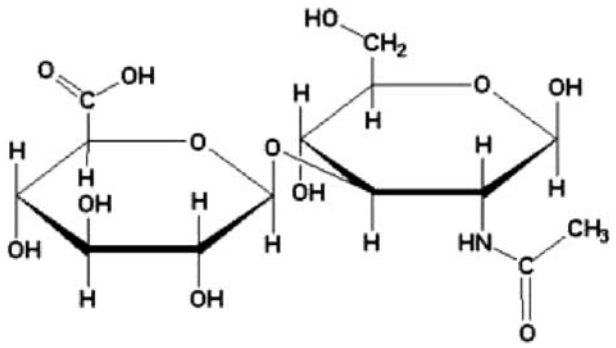

Fig. 1. Molecular structure of repeating disaccharide units of hyaluronic acid.

Production by *Streptococcus* ssp. has been advocated for several reasons, technical and economical as well as ethical. The bacterial polymer produced is identical to the eukaryotic HA *(2–6)*.

Kim et al. *(7)* studied the effects of addition of lysozyme to the broth on the production and molecular weight of HA produced by a *S. equi* mutant. The addition of lysozyme in batch fermentation when the optical density of the culture reached 1.0 and 3.0 increased the production and molecular weight of the HA. Johns et al. *(8)* studied the effect of pH, agitation, and aeration on the production of HA by *S. zooepidemicus*. They suggested the improvement caused by aeration on HA yield from glucose was probably due to improved energy yields.

In the present work the effects of addition of lysozyme and of forced aeration on HA production and HA rheological properties are investigated.

Materials and Methods

Microorganism and Inoculum

The strain ATCC 39920 of *S. zooepidemicus* Lancefield group C (ATCC, Manassas, VA, USA), which had previously been grown during 48 h at 37°C in Trypticase Soy Agar–BBL (TSA media), was maintained lyophilized or refrigerated at 5°C in 5 mL tubes. The inoculum was prepared in a 500 mL Erlenmeyer flask containing 200 mL of brain heart infusion (BHI medium) incubated at 37°C and 150 rpm during 48 h.

Culture medium

The synthetic culture medium was composed of 60 mg L^{-1} glucose, 60 mg L^{-1} yeast extract, and the salt composition proposed by Swann et al. *(9)*: 1.3 mg L^{-1} K_2SO_4, 1.0 mg L^{-1} $MgSO_4 \bullet 7H_2O$, 0.2 mg L^{-1} $Na_2SO_4 \bullet 12H_2O$, 5 g L^{-1} $CaCl_2 \bullet 2H_2O$, 5 g L^{-1} $FeSO_4 \bullet 7H_2O$, 1.0 g L^{-1} $MnSO_4 \bullet 4H_2O$, 1.0 g L^{-1} $ZnSO_4 \bullet 7H_2O$, 1.0 g L^{-1} $ZnSO_4 \bullet 7H_2O$, 0.1 g L^{-1} $CuSO_4 \bullet 5H_2O$, and 1.0 mL H_3PO_4 in 1 L deionized water.

Cultivation

Batch culture experiments were carried out in a rotary shaker (Logem) at 150 rpm using 500 mL Erlenmeyer flasks containing 200 mL medium or in a 3-L Bioflo III fermentor (New Brunswick Scientific Co Inc., Edison, NJ, USA), using 2.5 L medium, 10% v/v inoculum, and a 2 vvm (air volume/medium volume/min) aeration rate.

Addition of Lysozyme

The lysozyme was added twice to the broth during cultivation, at the exponential phase when the optical density at 600 nm reached 0.8 and at the stationary phase (1.2 OD).

Separation of Hyaluronic Acid

The HA was separated from the fermentation broth by precipitation with ethanol *(10)*. The culture broth was centrifuged at 3000 rpm for 20 min. After the cells were removed, 1.5 vol of ethanol were added to 1 vol of supernatant and the solution was refrigerated at 4°C for 1 h for HA precipitation. The precipitate was dissolved in 0.15 *M* NaCl and analyzed for HA concentration.

Concentration of Hyaluronic Acid

The concentration of HA was determined by high-performance liquid chromatography (HPLC) according to the protocol previously described by Armstrong and Jons *(11)*. The standard curve was prepared using commercial eukaryotic 1% v/v HA (Nikko Chemicals Co. Ltd, Tokyo, Japan).

Rheological Properties

The viscosity of the HA solutions was determined using a Haake rheometer CV20 with an oscillatory parallel plate modulus for viscoelasticity measurements. From the results of the oscillating strain tests at various frequencies, the storage modulus *G′* (elastic component), the loss modulus *G″* (viscous component), and the complex viscosity η^* were calculated. *G′*, *G″*, and η^* are functions of frequency and can be expressed in terms of the amplitude ratio and phase shift relative to the strain, as defined by Eq. (1) and (2) *(12)*:

$$G' = 5\ (\sigma_0/\gamma_0)\cos(\delta) \quad (1)$$

$$G'' = 5\ (\sigma_0/\gamma_0)\sin(\delta) \quad (2)$$

where (σ_0/γ_0) is the amplitude ratio, δ is the phase shift, σ_0 is the amplitude of shear stress, and γ_o is the amplitude of the strain equal to L/h, when the motion of the upper (oscillating) plate is $L \sin(\omega t)$, ω is the frequency expressed in rad/s, which is equivalent to $\omega/2\pi$ Hz, and *h* is the distance between the plates.

Table 1
Effect of Addition of Lysozyme on the Production and Viscous and Viscoelastic Properties of Hyaluronic Acid Produced by *S. zooepidemicus*

Lysozyme* ($U\ L^{-1}$)	HA ($g\ L^{-1}$)	η_∞ (mPas•s)	G' (Pa)
0	1.57	514	No
20,000	1.05	353	31.5

* Lysozyme addition at 0.8 and 1.2 OD_{600}.
HA, Hyaluronic acid; η_∞, Limit viscosity; G', Viscoelasticity storage modulus.

The complex viscosity η^*, defined by Eq. (3), describes the total resistance to a dynamic shear:

$$\eta^* = \sigma_0/(\gamma_0 \cdot \omega) \quad (3)$$

Results and Discussion

Effect of Addition of Lysozyme

Table 1 shows the influence of the addition of lysozyme when the optical density at 600 nm was 0.8 and 1.2 in comparison with the fermentation without lysozyme. The results show that the addition of lysozyme decreased the concentration of HA produced. This contradicts the tendency of the results from Kim et al. *(7)*, probably due to the differences in bacterial strains and fermentation conditions. Otherwise, the HA obtained when lysozyme was added had viscoelastic properties, indicating a higher molecular weight. This effect is probably due to the stress induced by the *Streptococcus* when lysozyme tends to destroy its cell wall and the microorganism reacts protecting itself with the HA produced.

Figure 2 shows the rheological curves for the standard HA and the HA produced by fermentation. The curves show a pseudoplastic behavior for each HA solution, and from its asymptotic behavior the limit viscosity η_∞ was determined. The limit viscosity of the HA produced by fermentation decreased from 514 to 354 mPa • s when lysozyme was added. In both cases the viscosities were higher than that of the standard HA (314 mPa • s).

Despite the decrease in viscosity of the solutions, the HA obtained with lysozyme was viscoelastic.

Figure 3 depicts the results of dynamic measurements of viscoelasticity and the calculated G' (elastic) and G'' (viscous) moduli. Undefined elastic behavior could be observed for the HA produced without lysozyme. It is evident that after a 1.5 Hz frequency, the elastic component G' is greater than the viscous component G'' for HA solutions produced by fermentation. Thus, the HA produced is highly elastic. This behavior is similar to

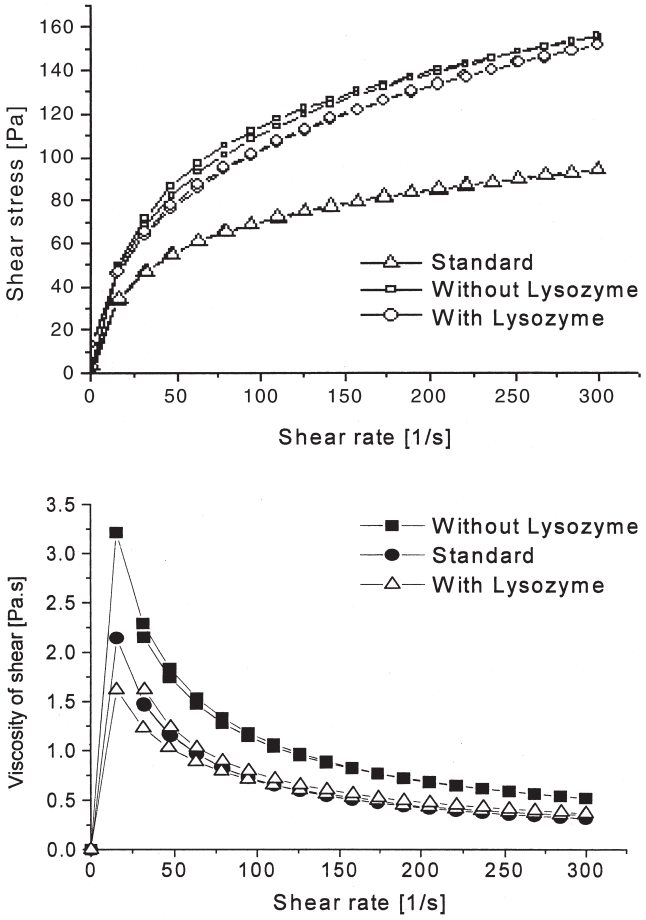

Fig. 2. Characterization through rheological curves of the hyaluronic acid (HA) produced by *S. zooepidemicus* with and without addition of lysozyme, and comparison with the standard HA.

that of the standard HA including the absolute values of the *G'* modulus. In fact, the HA produced with lysozyme can be thought of as a viscoelastic solid, because the deformed sample recoils to its original coordinates like a dampened spring after the strain is released, if the strain is not too large. When frequencies higher than 7 Hz are applied, the elastic structure breaks apart and thus the value of *G'* decreases. It is also evident that the HA is shear thinning, since the complex viscosity η^* decreases with increasing frequency. This is a common result for concentrated polymer solutions.

Effect of Forced Aeration

The forced 2 vvm aeration increased HA production to 3.75 g/L, while HA production in Erlenmeyer flasks was 1.57 g/L. The limit viscosity increased from 518 to 913 mPa•s and the HA produced was viscoelastic. For frequencies higher than 1.5 Hz, the elastic component *G'* is greater than

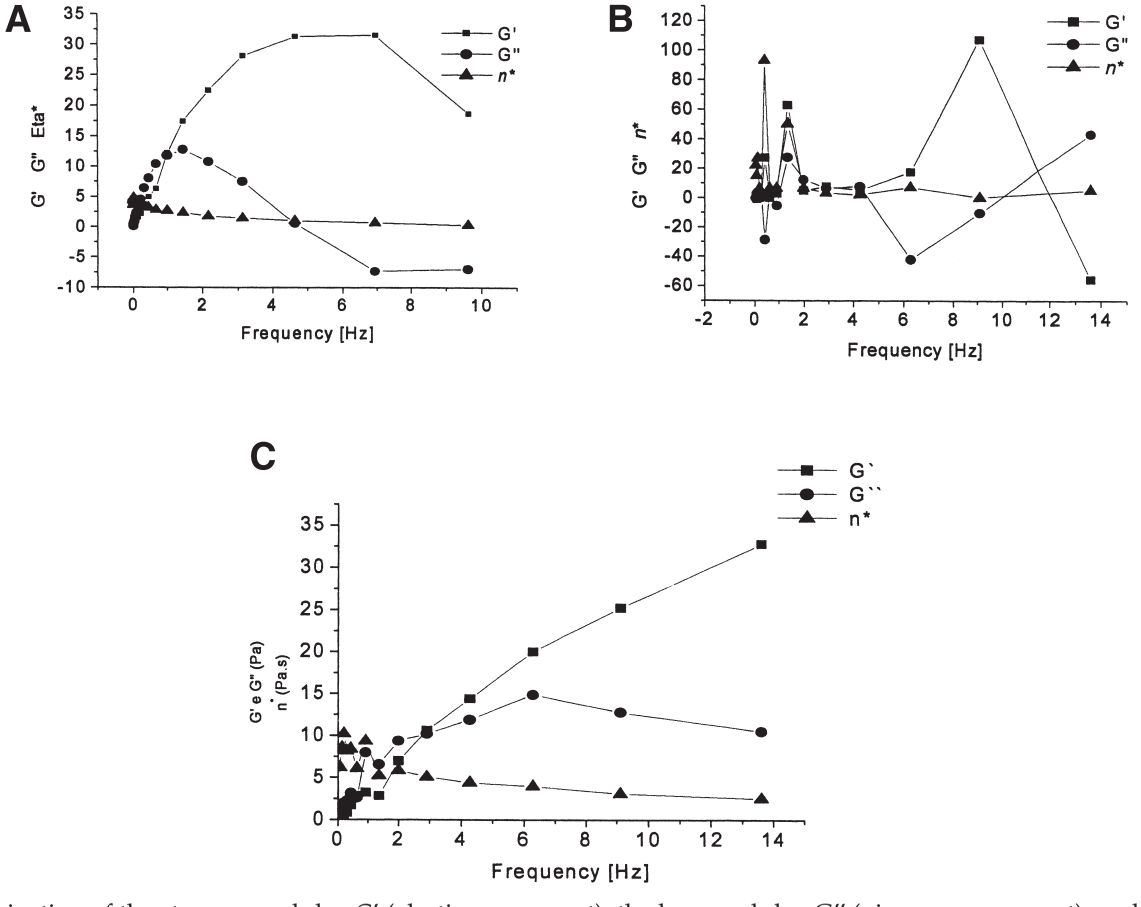

Fig. 3. Characterization of the storage modulus *G′* (elastic component), the loss modulus G″ (viscous component), and complex viscosity η^* for hyaluronic acid (HA) as a function of frequency sweep: **(A)** fermentation with lysozyme, **(B)** without lysozyme, **(C)** standard HA.

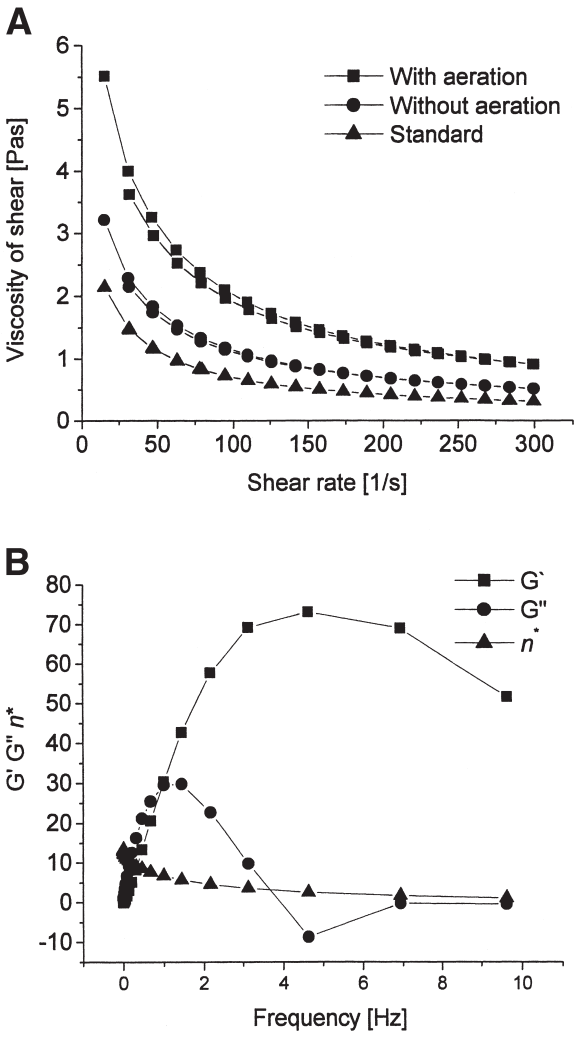

Fig. 4. Rheological **(A)** and viscoelastic **(B)** characterization of the hyaluronic acid produced by *S. zooepidemicus* fermentation using 2 vvm aeration.

the viscous component *G″*. Figure 4 shows these effects in viscosity and viscoelasticity curves.

The rheological properties are strongly influenced by the molecular weight of long chain polymers. Because the magnitude of the storage modulus *G′* corresponds to the molecular weight of the HA chains, a comparison of the values of this parameter should give us an indirect comparison of the molecular weights of the HA. Figure 5 clearly demonstrates that the elasticity and molecular weight are different for the three solutions obtained from fermentations and the HA commercial standard. The molecular weight of the HA is thus the highest in solutions obtained from fermentation with forced aeration (*G′* = 73.1 Pa), followed by the product obtained

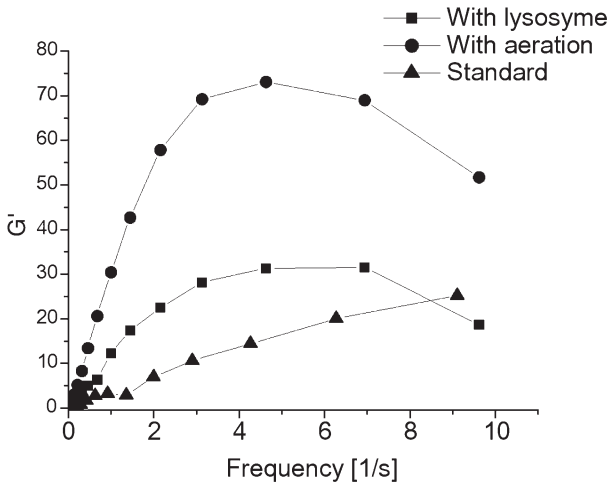

Fig. 5. Comparison of the viscoelastic modulus *G′* for the commercial and hyaluronic acid obtained from fermentations with lysozyme or using 2 vvm aeration.

with lysozyme (G' = 31.5 Pa). In both cases the viscoelastic properties were higher than those of commercial eukaryotic HA used as standard (G' = 25.3 Pa).

Conclusion

Production of hyaluronic acid (HA) was affected by the addition of lysozyme or aeration. The amount of HA produced with the addition of lysozyme was less than that obtained without the enzyme. The viscosity of the solutions also decreased, but the HA produced with lysozyme had viscoelastic properties. Forced aeration increased the production of HA and its viscous and viscoelastic characteristics. Only viscous properties were identified in the HA produced without lysozyme or forced aeration. An indirect comparison of molecular weight related to the magnitude of elastic modulus *G′* showed that HA produced with forced aeration had a higher molecular weight than that obtained with the addition of lysozyme or the 1% v/v commercial HA extracted from a eukaryotic source.

Acknowledgments

The authors acknowledge the financial support of the Brazilian financial agencies FAPESP and CNPq.

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