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Microbial production of hyaluronic acid from agro-industrial by-products: Molasses and corn steep liquor

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ABSTRACT

Agro-industrial by-products are being explored as alternative low-cost nutrients to produce hyaluronic acid (HA) by *Streptococcus zooepidemicus*. In this study, we formulated three culture media containing corn steep liquor (CSL) and sugarcane molasses (M), to produce microbial HA using batch bioreactor conditions (pH 6.7, 500 rpm and 1 vvm aeration). Final HA concentrations of 3.48 g L^{-1} were produced in culture medium containing corn steep liquor (10% v/v) and glucose, being comparable (3.60 g L^{-1}) to the control medium containing tryptone and glucose. The use of molasses (10% v/v) as carbon source produced a marked inhibition of *S. zooepidemicus* growth and HA production due to a low sugar consumption. The HA produced in CSL culture media had a high molecular weight of 3.8×10^3 kDa, greater than HA produced in tryptone-containing medium (3.0×10^3 kDa).

This is the first report achieving HA productions comparable to synthetic a medium in a batch bioreactor using CSL as the main nitrogen source. However, further optimization of culture conditions must be carried out towards using this agricultural by-product for the sustainable industrial production of HA.

1. Introduction

Hyaluronic acid (HA) is a glycosaminoglycan found in vertebrate tissues as an essential component of the extracellular matrix. This polysaccharide has a linear structure consisting of β-1,3-*N*-acetyl glucosamine-β-1,4-Glucuronic acid disaccharide repeating units [1]. Despite the simplicity of its structure, the polymer is semi-flexible and adopts an expanded wormlike random coil conformation in solution [2], exhibiting an unusual rheological behaviour. HA is an attractive molecule for specific applications in the cosmetic [3], pharmacological [4] and medical sectors [5] due to its viscoelasticity together with other advantages such as biocompatibility, angiogenic and immunostimulatory properties.

HA was traditionally recovered from rooster combs, synovial fluid, vitreous humour and umbilical cords [6] from terrestrial animals, but also from marine supplies [7]. In recent years, the microbial production by *Streptococci* was extensively investigated [8] due to improved HA yields, more efficient downstream processes, and reduced risk of cross-species viral infection [9]. In spite of these advantages, microbial cultivation must be cost competitive with HA recovery from animal sources. *Streptococci* have complex nutrient requirements on organic nitrogen [10], and nutritive media commonly used to grow these microorganisms contain high amounts of rich nutrients [11]. The continuous increment in the cost of these raw materials reduces the commercial competitiveness of microbial HA production [8] and therefore, the use of low-cost renewable resources and agro-industrial by-products as culture media contributes towards making HA production economically feasible.

Molasses is a by-product of the sugar cane industry containing valuable compounds for the fermentation process like sucrose, minerals, organic compounds and vitamins [12]. CSL is a by-product of the corn wet milling industry rich in vitamins, minerals, amino acids and proteins, and an important source of nitrogen [13]. The high nutritive value of both substrates suggests they could be useful for the formulation of culture medium to produce HA using a bacterium with complex nutrient requirements like *S. zooepidemicus*.

The production of HA using renewable resources as ingredients for the formulation of culture media is being explored nowadays [14]. The substitution of commercial peptones by marine

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by-products [15–17], and cheese-whey protein [18] yielded high concentrations of HA in batch cultures of *Streptococcus zooepidemicus*. Vegetable by-products are also extensively explored because HA for cosmetic and pharmacological applications must be produced from non-animal sources [19]. De Macedo and Santana [20] found juice-moisturized bagasse with cashew apple fruit was a promising (>6 mg/g) source for the production of low molecular weight HA (10^4–10^5 Da) in solid-state fermentation. In another study, HA was produced in culture medium containing corn steep liquor (CSL, 0.86 g L^{-1}), or soy protein hydrolysate alone and combined with CSL (0.17 g L^{-1}) as nitrogen sources [21]. Pan et al. [22] reported the replacement of yeast extract by soy protein resulted in a polymer production of 0.22 g L^{-1} while these authors did not find any HA production using CSL as the nitrogen source.

The objective of this work is the development of a low-cost alternative medium for the production of HA by *S. zooepidemicus* using molasses and corn steep liquor. The appropriate conditions (pH control, agitation, and aeration) for the production of HA were first defined in culture medium containing glucose and tryptone, and performances compared to alternative culture media.

2. Material and methods

2.1. Strain and culture conditions

The HA-producing strain *Streptococcus equi* subsp. zooepidemicus ATCC 35246 was stored at −80 °C in complex medium (CM) with 25% glycerol. The composition of CM medium was (g L^{-1}): glucose 50.0; tryptone 15.0; yeast extract 2.75; KH_2PO_4 2.00; K_2HPO_4 2.0; $MgSO_4$ 2.0; $(NH_4)_2SO_4$ 0.5; pH 6.7. The inoculum consisted of a 10% (v/v) as reported by Armstrong et al. [10], and detailed in Vázquez et al. [15].

Cultures were carried out in 0.75 L-bioreactor with a working volume of 0.5 L (Biostat Q, Braun Sartorius), at 37 °C. We tested different agitation (200, 500 and 800 rpm) and aeration (0, 1 vvm) conditions in CM medium, and the pH maintained at 6.7 using 5 M NaOH.

2.2. Streptococcus zooepidemicus *culture using alternative substrates*

Sugarcane molasses, kindly provided by RAR: Refinarias de Açúcar Reunidas, S.A. (Portugal), and CSL kindly provided by COPAM: Companhia Portuguesa de Amidos, S.A. (Portugal) were the alternative substrates for HA production by *Streptococcus zooepidemicus* ATCC 35246. Molasses contained 495 g L^{-1} of carbohydrates and 20 g L^{-1} of protein, while CSL contained 69 g L^{-1} of carbohydrates, and 57 g L^{-1} of protein. Molasses (M) were dissolved in distilled water (10% (v/v)), and tested as culture media containing either tryptone or 10% (v/v) corn steep liquor (CSLM). A third culture medium containing 50 g L^{-1} glucose, and 10% (v/v) corn steep liquor was prepared (CSL). All media were supplemented with yeast extract, KH_2PO_4, K_2HPO_4, $MgSO_4$, and $(NH_4)_2SO_4$ at the same levels as CM medium (Table 1). The initial pH was adjusted to pH 6.7 and the cultures carried out under previously defined conditions (37 °C, 500 rpm, 1 vvm).

Table 1
Composition of culture media (g L^{-1}) utilised for the production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 35246. M: culture medium containing sugarcane molasses, CSL: culture medium containing corn steep liquor, CSLM: culture medium containing sugarcane molasses and corn steep liquor, CM: complex medium.

	M	CSL	CSLM	CM
Glucose	–	50.00	–	50.00
Yeast extract	5.00	5.00	5.00	5.00
Tryptone	15.0	–	–	15.00
KH_2PO_4	2.00	2.00	2.00	2.00
K_2HPO_4	0.50	0.50	0.50	0.50
$MgSO_4$	0.50	0.50	0.50	0.50
$(NH_4)_2SO_4$	0.50	0.50	0.50	0.50
Molasess (%v/v)	10.0	–	10.0	–
CSL (%v/v)	–	10.0	10.0	–

2.3. Analytical methods

Samples were taken at different time points of fermentation and incubated with a 10% of 5% (w/v) SDS for 10 min. Biomass was removed by centrifugation at 15000 × *g* for 15 min and the optical density (OD) measured at 700 nm. We quantified the concentration of total sugars and soluble proteins in the supernatant using the methods phenol-sulphuric [23] and Lowry [24], respectively. Glucose and sucrose were quantified by HPLC using an ION-300 column (Transgenomic, USA) with 6 mM sulphuric acid as the mobile phase (flow = 0.4 mL/min) at 65 °C and a refractive index detector.

The production of HA was quantified after selective precipitation in the supernatant using ethanol (3:1) followed by centrifugation (10000 × *g*, 10 min). The sediment was dissolved in 1.5 M NaCl (1:1) and re-precipitated under the same conditions. Finally, the HA was suspended in distilled water and the concentration determined by the method of Blumenkrantz and Asboe-Hansen [25], following the modifications proposed by Murado et al. [26]. The molecular weight (MW) of HA was determined by size-exclusion chromatography with an Ultrahydrogel linear column (Waters, USA) with 0.1 M $NaNO_3$ as the mobile phase (flow = 0.8 mL/min) and a refractive-index detector. Standards of polystyrene sulphonate (Sigma) with different molecular weights (32, 77, 150, 330, 990 and 2600 kDa) were used for calibration.

2.4. Numerical and statistical analysis

S. zooepidemicus growth (X) kinetics were modelled using the following logistic equation [16]:

$$X = \frac{X_m}{\left(1 + exp\left[2 + \left(\frac{4\upsilon_X}{X_m}\right)(\lambda_X - t)\right]\right)} \quad (1)$$

where X is the biomass production (g L^{-1}), X_m is the maximum biomass (g L^{-1}), ν_x is the maximum growth rate (g L^{-1} h^{-1}) and λ_x is the growth lag phase (h).

Also a logistic equation was employed to model HA production data [16]:

$$H = \frac{H_m}{\left(1 + exp\left[2 + \left(\frac{4\upsilon_H}{H_m}\right)(\lambda_H - t)\right]\right)} \quad (2)$$

where H is the HA production (g L^{-1}), H_m is the maximum HA concentration (g L^{-1}), ν_H is the maximum HA production rate (g L^{-1} h^{-1}) and λ_H is the delay in HA production (h).

We calculated the yield of HA production on biomass by means of the following equation [27]:

$$H = Y_{H/x}\frac{X_m}{1 + exp\left[2 + \frac{4\upsilon_X}{X_m}(\lambda_X - t)\right]} - Y_{H/x}X_0 \quad (3)$$

where $Y_{H/x}$ is the yield of HA production per biomass (g HA g^{-1} biomass).

Plotting and data fitting were performed using the software GraphPad PrismTM 5 (GraphPad Software Inc., San Diego, CA, USA). The significance of the mathematical models (Fisher's F-test) was assessed using the "SolverAid" macro (Levie's Excellaneous website: http://www.bowdoin.edu/~rdelevie/excellaneous).

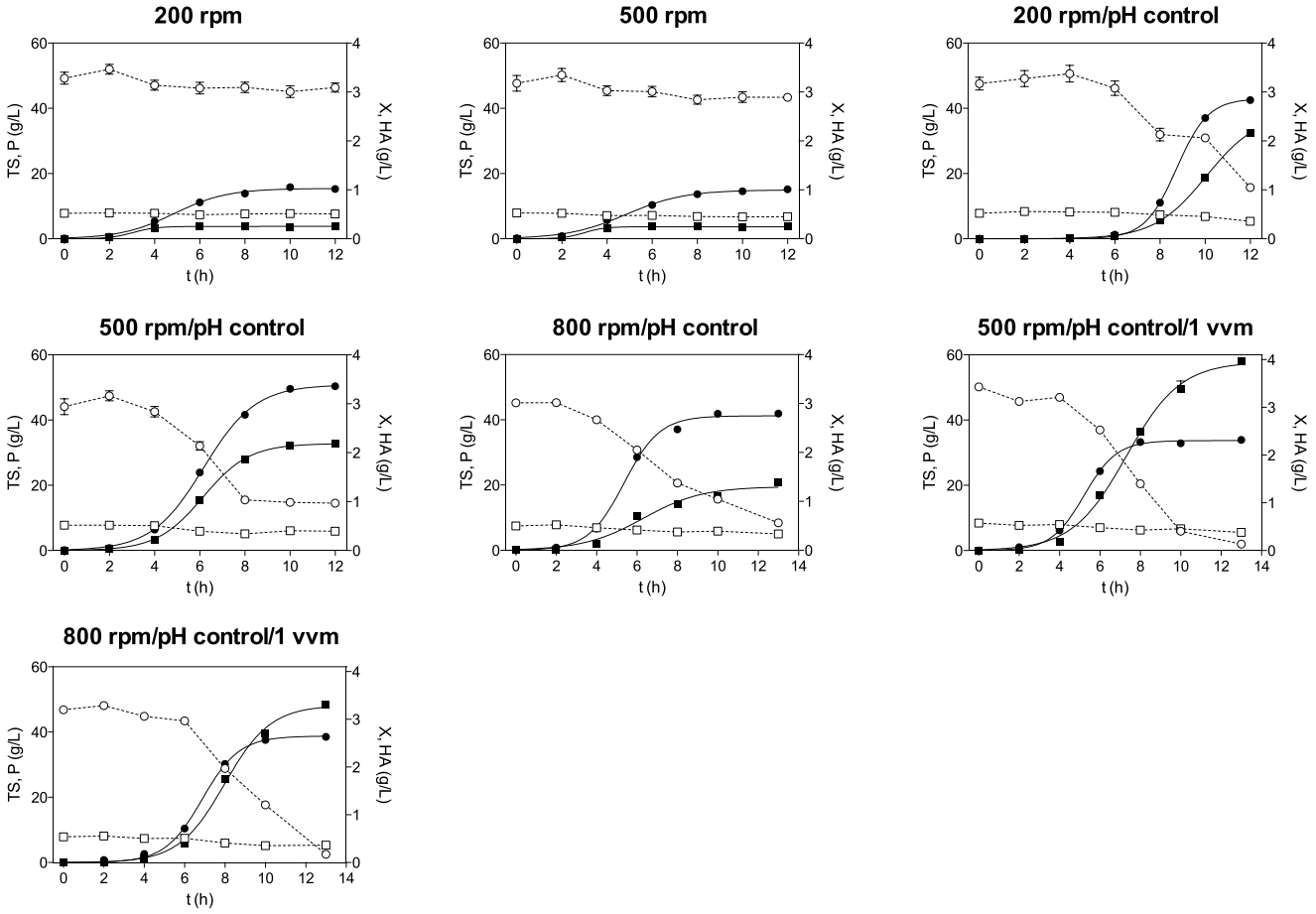

Fig. 1. Representation of the experimental data (points) and calculated (continuous lines) time course of biomass (X; ●), hyaluronic acid (HA; ■), total sugars (TS; ○) and protein (P; □) concentrations during *S. zooepidemicus* ATCC 35246 cultivations in complex medium at 37 °C, without pH control (200 and 500 rpm), with pH control (200, 500 and 800 rpm) and with 1 vvm aeration (500 and 800 rpm).

3. Results and discussion

3.1. Effect of pH control, agitation, and aeration on HA production

The production of HA by *S. zooepidemicus* ATCC 35246 was studied in complex medium (CM) at 37 °C under different agitation rates: 200, 500 and 800 rpm, with (1 vvm) and without aeration. Also, the effect of pH control (at 6.7) was initially studied. The experimental data (biomass: *X*, and hyaluronic acid: *H*) were quantified and modelled using the logistic Eqs. (1) and (2), respectively. The agreement between predicted and experimental data was excellent (Table 2), with R^2- values higher than 0.973. The *p*-values from Fisher's *F*-test also indicated the consistency of the models to describe the experimental data. In general, the kinetic parameters defined by Eqs. (1)–(3) were statistically significant for all the conditions assayed (Student's *t*-test, Table 2).

We studied the effect of pH control on HA production by carrying out four cultures at 200 and 500 rpm, with or without pH control (Fig. 1). Our results showed maximal HA concentrations achieved without pH control were almost negligible (0.25 g L^{-1}), regardless of the agitation rate applied (Table 2). After 6 h of cultivation, the pH dropped to average values below 5 (data not shown), sugar uptake stopped, and biomass and hyaluronic acid productions reached the stationary phase (Fig. 1). The pH control had a remarkable effect on the maximum HA concentration, yield and volumetric production rates in batch cultures of *S. zooepidemicus* (Table 2). Cultivation at controlled pH led to a 7 to 8-fold increment in maximal HA productions compared to non-controlled pH cultures at identical agitation rates (Table 2). The highest maximal HA productions were 2.56 g L^{-1} and 2.19 g L^{-1} at 200 rpm and 500 rpm, respectively. These results are in agreement with previous reports highlighting the importance of pH control on the parameters of HA production in batch cultures of *S. zooepidemicus* [28]. The maintenance of stable pH conditions such as those utilised in the present research (6.0–7.0) was reported to improve HA production [10,28], by counteracting the lactic and acetic product formation that causes a strong inhibition of cell growth and polysaccharide synthesis [29].

The production of HA typically increases broth viscosity and thus agitation and oxygen mass transfer rates significantly influence HA production [8]. However, there is no agreement on whether high agitation enhances HA production or not. In this regard, some reports have shown a decrease in the polysaccharide production rates with increased mixing speed [30], while others have found opposite results [28,31]. Although agitation is needed to facilitate the transport of nutrients and to remove lactate in the environment of the cell [28], high agitation rates can be detrimental to HA production [31,32], and to the structure of the polymer [28]. Indeed, we observed increasing the agitation rate from 200 rpm to 800 rpm markedly reduced the maximum production of HA and the productivity while increasing the maximum biomass compared to lower agitation conditions (Table 2). The negative relationship between growth and specific HA productivity at increased stirring rates is due to HA production competing for limited resources with biomass

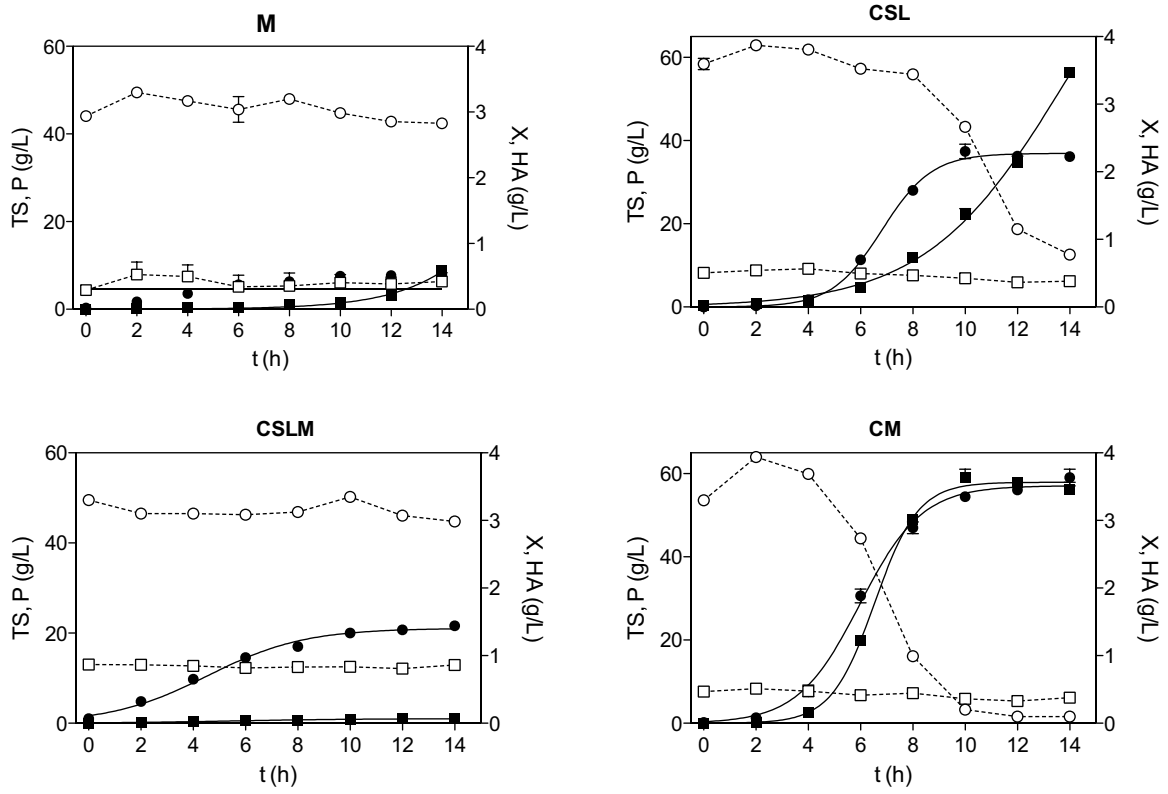

Fig. 2. Representation of the experimental data (points) and calculated (lines) time courses of biomass (X; ●), hyaluronic acid (HA; ■), total sugars (TS; ○) and protein (P; □) concentrations during *S. zooepidemicus* cultivations carried out in culture medium M (10% (v/v) molasses as carbon source), or medium CSL (10% (v/v) corn steep liquor as nitrogen source), or medium CSLM (10% (v/v) molasses + 10% (v/v) corn steep liquor), or complex medium.

synthesis, as previously reported [33]. We also found higher agitation rates reduced the yield of HA on biomass ($Y_{H/X}$; Table 2), which dropped from 0.66 g g^{-1} at 200 rpm to 0.51 g g^{-1} at 500 rpm (Table 2). On the other hand, increasing the stirring speed significantly shortened (>50%) the delay in HA production (λ_H), being this time reduced from more than 7 h at 200 rpm to only 3.37 h at 800 rpm. The inverse relationship between agitation rate and λ_H agrees with previous findings [31,32], while contradicts others that report a delayed rate of HA synthesis under high shear stress conditions [34]. In addition to maximizing the production, culture conditions should speed up the process and reduce the production costs, and so only 500 rpm and 800 rpm conditions were considered for further experiments.

Aeration of *S. zooepidemicus* cultures was reported to enhance ATP production and increase acetyl-CoA accumulation, leading to higher HA titres [29]. Therefore, in principle, it is possible to shift the carbon flux towards HA synthesis in microbial cultures under aerobic conditions and so, an experiment followed where we studied the effect of aeration (1 vvm). Indeed, our results showed a 1.8 and 2.5- fold increment in maximal HA concentrations in aerated cultures at 500 rpm and 800 rpm compared to their non-aerated counterparts (Table 2). The yields of HA per biomass ($Y_{H/X}$) were lower than 1.0 (Table 2), indicating glucose was utilised preferably for cell growth than HA synthesis, as previously reported [29]. However, cultivation conditions of 500 rpm and 1 vvm aeration provided a $Y_{H/X}$ value greater of 1.08 g g^{-1}, suggesting the carbon source goes mainly towards HA production rather than biomass synthesis (Table 2). Aeration at 1 vvm doubled the value of $Y_{H/X}$ compared to the culture at 500 rpm without aeration (Table 2) suggesting the carbon flux was diverted towards HA synthesis.

The average final molecular weight of HA in non-aerated cultures was 3.2×10^3 kDa and 3.0×10^3 kDa in aerated cultures. The slight reduced molecular weight observed might be related to the shear stress produced by high agitation rates and aeration of HA in accordance with previous results [28,34].

3.2. HA production in low-cost formulated media

The HA production in culture media formulated using agricultural by-products was tested using the conditions previously defined in CM medium providing the highest yields of HA per biomass: controlled pH (6.7), agitation of 500 rpm and aeration of 1 vvm. We tested two culture media containing sugarcane molasses (10% v/v) as the carbon source with either tryptone (M) or CSL (CSLM) as the nitrogen source, and a third culture medium containing 50 g L^{-1} glucose and 10% (v/v) CSL (CSL). We compared these results with a synthetic medium (CM) containing 50 g L^{-1} glucose and tryptone. All media contained yeast extract and total sugars at a concentration of 50 g L^{-1} (Table 1).

Both glucose-containing media either with CSL (3.48 g L^{-1}) or tryptone (3.60 g L^{-1}) as the main nitrogen source produced similar final HA concentrations (Fig. 2), despite the first showed a lower productivity and the production was delayed (Table 3). Contrarily, molasses-formulated media (M and CSLM) produced the lowest biomass and HA synthesis, achieving almost negligible HA concentrations (Fig. 2). According to HPLC analysis, sucrose was the primary sugar (98%) in sugarcane molasses. The uptake of this disaccharide was reported in *S. zooepidemicus* [22,29,33] up to an initial content of 60 g L^{-1}, although with a strong inhibition on cell growth at high concentrations (70 g L^{-1}) [29]. Jagganath and Ramachandran reported replacing glucose with sucrose increased

Table 2
Results of non-linear adjustment of biomass (Eq. (1)), and hyaluronic acid (Eq. (2)) time courses in the cultivation of *S. zooepidemicus* ATCC 35246 under different conditions. X_m, maximum biomass; ν_X, maximum growth rate; λ_X, growth lag phase; H_m, maximum hyaluronic acid production; ν_H, maximum hyaluronic acid production rate; λ_H lag phase of hyaluronic acid production. The table also shows yields of hyaluronic acid on biomass ($Y_{H/X}$) calculated using Eq. (3). The determination coefficients (R^2) and *p*-values from Fisher's F-test for the mathematical are shown. NS, not significant.

Cultivation conditions	Biomass					Hyaluronic acid production					Yield		
	X_m (g L^{-1})	ν_X ($g L^{-1} h^{-1}$)	λ_X (h)	R^2	*p*-value	H_m (g L^{-1})	ν_H ($g L^{-1} h^{-1}$)	λ_H (h)	R^2	*p*-value	$Y_{H/X}$ ($g g^{-1}$)	R^2	*p*-value
200 rpm	1.03 ± 0.05	0.22 ± 0.04	2.49 ± 0.51	0.991	<0.001	0.25 ± <0.01	0.12 ± 0.02	2.00 ± 0.23	0.996	<0.001	0.23 ± 0.17	0.949	<0.001
500 rpm	1.08 ± 0.06	0.19 ± 0.03	2.25 ± 0.50	0.992	<0.001	0.25 ± <0.01	0.13 ± <0.01	2.06 ± 0.09	0.999	<0.001	0.23 ± 0.13	0.778	0.002
200 rpm/pH control/0 vvm	2.87 ± 0.03	1.02 ± 0.03	7.33 ± 0.05	0.999	<0.001	2.56 ± 0.06	0.56 ± 0.01	7.76 ± 0.06	0.999	<0.001	0.66 ± 0.13	0.776	0.002
500 rpm/pH control/0 vvm	3.39 ± 0.06	0.74 ± 0.04	3.88 ± 0.15	0.999	<0.001	2.19 ± 0.03	0.53 ± 0.03	4.10 ± 0.14	0.999	<0.001	0.51 ± 0.36	0.658	0.005
800 rpm/pH control/0 vvm	2.748 ± 0.09	0.78 ± 0.12	3.59 ± 0.33	0.996	<0.001	1.30 ± 0.15	0.22 ± 0.07	3.37 ± 1.00	0.973	<0.001	0.36 (NS)	0.964	<0.001
500 rpm/pH control/1 vvm	2.30 ± 0.03	0.71 ± 0.05	3.59 ± 0.13	0.999	<0.001	3.95 ± 0.19	0.74 ± 0.09	4.63 ± 0.36	0.996	<0.001	1.08 ± 0.44	0.881	<0.001
800 rpm/pH control/1 vvm	3.65 ± 0.06	0.72 ± 0.06	5.03 ± 0.17	0.999	<0.001	3.29 ± 0.14	0.72 ± 0.09	5.71 ± 0.30	0.997	<0.001	0.84 ± 0.16	0.968	<0.001

Table 3
Results of non-linear adjustment of biomass (Eq. (1)), and hyaluronic acid (Eq. (2)) time courses in the cultivation of *S. zooepidemicus* ATCC 35246 in different by-product culture media. X_m, maximum biomass; ν_X, maximum growth rate; λ_X, growth lag phase; H_m, maximum hyaluronic acid production; ν_H, maximum hyaluronic acid production rate; λ_H lag phase of hyaluronic acid production. The table also shows yields of hyaluronic acid on biomass calculated using Eq. (3). The determination coefficients (R^2) and *p*-values from Fisher's F-test for the mathematical are shown. NS, not significant.

Culture medium	Biomass					Hyaluronic acid production					Yield		
	X_m (g L^{-1})	ν_X ($g L^{-1} h^{-1}$)	λ_X(h)	R^2	*p*-value	H_m (g L^{-1})	ν_H ($g L^{-1} h^{-1}$)	λ_H (h)	R^2	*p*-value	$Y_{H/X}$ ($g g^{-1}$)	R^2	*p*-value
M	0.52 ± 0.08	0.07 ± 0.04	0.68 (NS)	0.973	0.003	40.9 (NS))	4.64 (NS)	19.0 (NS)	0.974	0.002	0.80 ± 0.71	0.765	0.002
CSL	2.27 ± 0.06	0.59 ± 0.08	4.90 ± 0.29	0.996	<0.001	6.82 ± 2.88	0.63 ± 0.16	8.65 ± 1.25	0.995	<0.001	1.01 ± 0.60	0.884	0.002
CSLM	1.40 ± 0.08	0.19 ± 0.03	0.84(NS)	0.985	<0.001	0.07 ± 0.01	0.007 ± <0.01	0.62 (NS)	0.967	0.005	0.05 ± 0.01	0.915	<0.001
CM	3.51 ± 0.11	0.74 ± 0.10	3.59 ± 0.37	0.996	<0.001	3.56 ± 0.08	1.07 ± 0.14	4.88 ± 0.23	0.997	<0.001	1.02 ± 0.13	0.987	<0.001

the HA productivity and molecular weight, together with the specific HA synthesis rate [33]. Unlike these reports and in accordance with the results presented in this study, the use of sugarcane molasses has shown low productions of HA ($0.376 \pm 0.020\ g\ L^{-1}$), compared to commercial sucrose ($0.488 \pm 0.002\ g\ L^{-1}$) and glucose ($0.429 \pm 0.028\ g\ L^{-1}$) [22]. These findings suggest the inhibition observed in *S. zooepidemicus* growth in molasses-containing media must be due to other components of this by-product, such as lactic acid [29] and phenolic compounds [35] that were reported as inhibitory against *Streptococci*. A further pre-treatment of molasses, *i.e.* the use of acid hydrolysis and/or an activated carbon resin to reduce the content of inhibitory compounds and improve the HA titres.

The use of CSL as alternative nitrogen source for HA production has been scarcely studied to date, and has shown mixed results. For instance, Pires et al. [21] found nearly identical maximal HA production in culture media ($45\ g\ L^{-1}$ of glucose and $57\ g\ L^{-1}$ yeast extract) with ($0.84\ g\ L^{-1}$) and without ($0.86\ g\ L^{-1}$) CSL (10% v/v) supplementation. These authors reported however, a 2- fold increment in the yield of HA on glucose when using the by-product. On the other hand, Pan et al. [22] did not find any HA production in culture media containing CSL and yeast extract ($30\ g\ L^{-1}$) as the nitrogen sources, using either glucose or sucrose ($30\ g\ L^{-1}$) as carbon sources. The final HA concentration reported in the present study was higher ($3.60\ g\ L^{-1}$) than any other reported before in culture media containing CSL. These differences must be related to variations in experimental conditions, such as different *S. zooepidemicus* strains, ATCC 39920 [21,22] or ATCC 35246, changes in the composition of the culture media, and cultivation mode. The latter may be primarily responsible for the differences in polysaccharide productions, *i.e. S. zooepidemicus* cultivation in bioreactor instead of Erlenmeyer flasks [21,22]. This type of culture does not allow automatic control of pH and a high concentration of lactic acid accumulates in the cell environment, lowering the pH and inhibiting cell growth and HA synthesis [29].

The yields of HA per biomass in CSL and CM media were very similar (Table 3), suggesting CSL could be a low-cost alternative to expensive commercial peptones such as tryptone (~300 € kg^{-1}). Previous findings have shown the use of different substrates changes the glycolytic end products [36], and regulate the molecular weight of HA [33]. The average molecular weight of HA produced in CSL medium was 3.8×10^3 kDa, which is higher than in CM medium (3.0×10^3 kDa) using the same culture conditions. Cheese whey formulated medium [18] produced HA with the average molecular weight (3.8×10^3 kDa) to CSL medium. These differences might be related to the regulation of the molecular weight of HA caused by the use different nutritive sources [33].

The final production of HA in CSL medium was higher than the concentrations reported in culture media containing cheese whey [16] or marine peptones from fishing by-products ($1.51–2.41\ g\ L^{-1}$) as the nitrogen sources [15–17]. Both CSL and whey protein enhanced the conversion of the carbon source towards HA production instead of biomass synthesis, leading to yields of HA on biomass ($Y_{H/X}$) higher than 1 while marine peptones produced more biomass than polysaccharide ($Y_{H/X}$ = 0.4–0.7). Remarkably, the use of CSL as the nitrogen source in *S. zooepidemicus* cultures delayed HA synthesis (8.65 h) compared to fish and cheese by-product formulated media (3.30–7.66 h), which would be a limiting factor for the use of this alternative nitrogen source for the industrial production of HA. To overcome this issue, further optimization of culture medium composition and the use of alternative cultivation strategies must be approached. For instance, fed-batch cultures were reported to increase HA productions in different alternative culture media [15,17,29]. Also the use of a two-step culture model consisting of an initial exponential fed-batch culture phase maintaining low sugar concentrations (0–8 h) followed by batch fermentation (8–20 h), increased HA production by 36% compared to *S. zooepidemicus* batch cultivation [29,37].

4. Conclusions

In this study, low-cost culture media were formulated using two agro-industrial by-products (corn steep liquor and/or molasses) for HA production by *S. zooepidemicus*. Molasses-containing media with tryptone or CSL as nitrogen source produced a strong inhibition in *S. zooepidemicus* growth under optimized culture conditions. The amount of HA produced in glucose and CSL-containing medium was $3.48\ g\ L^{-1}$, comparable to that of complex medium ($3.60\ g\ L^{-1}$), and higher than those reported using animal-sourced agrifood wastes ($1.51–2.41\ g\ L^{-1}$). The HA production was delayed in CSL formulated medium and so, further optimization of the nutrient composition, and/or alternative culture strategies must be explored.

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