Appl Biochem Biotechnol (2017) 182:276–293
DOI 10.1007/s12010-016-2326-y

Improvement Production of Hyaluronic Acid by *Streptococcus zooepidemicus* in Sugarcane Molasses

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Received: 8 September 2016 / Accepted: 3 November 2016 /
Published online: 30 November 2016

Abstract Microbial hyaluronic acid (HA) production has been preferred rather than extraction from animal tissue for medical and cosmetic applications. In this context, to obtain an economically competitive HA production by *Streptococcus zooepidemicus*, culture conditions were studied to improve the polymer production in sugarcane molasses. The highest HA production by *S. zooepidemicus* ATCC 39920 achieved was 2.825 g. L^{-1} in a 4.5 L bioreactor with controlled pH (8.0) and medium containing molasses (85.35 g.L^{-1} total sugar) pretreated with activated charcoal and yeast extract (50 g.L^{-1}). The HA produced exhibited a high molecular weight of 1.35×10^3 kDa and the DPPH radical scavenging activity of the polymer at 1 g.L^{-1} was 41 %. The FTIR and UV-Vis spectra showed no substantial differences in the spectral pattern between

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produced and standard HA. This study is a promising strategy for sugarcane molasses application by producing high value-added products such as hyaluronic acid.

Keywords Hyaluronic acid · Sugarcane molasses · *Streptococcus* · Fermentation · Bioreactor

Introduction

Hyaluronic acid (HA) is a linear polysaccharide composed of disaccharide units of (1,4)-β-linked glucuronic acid and (1,3)-β-linked N- acetylglucosamine [1]. Due to its unique physico-chemical properties, such as high intrinsic viscosity, antioxidant properties, chain stiffness and water-holding capacity [2], HA has been used for a wide variety of medical applications, including osteoarthritis [3, 4], ophthalmic surgery [5, 6] and cutaneous wound healing [7, 8]. In dermatology and cosmetic practices, HA has been employed to help the skin regain elasticity, turgor and moisture [9, 10]. Depending on the applications, the values of HA products and derivatives range from US $2000 to $60,000 Kg^{-1} [11].

Traditionally, hyaluronic acid is chemically extracted from animal waste such as rooster combs or umbilical cords. However, these resources are limited and HA from these tissues is generally associated with proteoglycans and often contaminated with HA degrading enzymes that make isolation of high purity and high molecular weight HA very difficult and costly [12]. Furthermore, the risk of cross-species viral and infection agent has been pointed out when using animal-derived biochemicals for human therapeutics. Therefore, microbial production is gradually replacing extraction as the preferred HA source with lower production cost, more efficient purification, higher yield compared to animal sources and less environmental pollution [1]. Although great progress has been achieved in HA microbial production, the price of culture media reduces the commercial competiveness of this alternative, and thus it is necessary to find a low-cost substrate replacement to reduce production cost [13].

More than 80 % of the production costs of microbial HA produced by *Streptococcus* (particularly *S. equi* subsp. *zooepidemicus*) are due to the fermentation medium [14]. Thus, one important goal of the fermentation process is to study a new cost-effective culture medium that can increase HA production. In most studies, glucose is used as the primary carbon source for HA production [15, 16]. The possibility of using byproducts rich in carbon and other essential nutrients for microorganism growth in industry is underexplored. Vázquez et al. [14, 17] reported the successful use of marine peptones from fishing byproducts for hyaluronic acid production. Agricultural resource derivatives such as cashew apple juice, cheese whey, and soy protein were also studied [11, 18, 19]. For instance, Amado et al. [20] saved 70 % of the nutrient cost by replacing commercial peptone with cheese whey during polymer production.

Brazil is the largest sugar producer and exporter in the world, with 2015/2016 harvests of 33,928 thousand tons [21]. Approximately 0.3 tons are discharged when one ton of sugar is processed [22]. It is estimated that around 10 million tons of sugarcane molasses are discharged annually, which makes this product an available raw material for fermentation processes. In the current market, glucose prices are approximately $0.39 Kg^{-1}, whereas sugarcane molasses is $0.1 kg^{-1} [23]. This raw material has previously been applied to polymer production, such as cellulose [24] welan gum [25], succinoglycan [26] and levan [27]. In our study, we confirmed that crude sugarcane molasses could also be used as a promising carbon source for HA production [18].

Sugarcane molasses contains approximately 50 % (*w*/w) total sugar (primarily sucrose, fructose and glucose), suspended colloids, metal ions, vitamins and nitrogen compounds [23]. The suspended particles and complex structures cause heterogeneity in the medium and may affect the cell growth rate. Therefore, many molasses pretreatments have been proposed to prepare a unique molasses medium and improve microbial production [24, 25]. Another important aspect of the use of agro-industrial byproducts for high value aggregated molecule production is the optimization of the concentrations of these substrates [28].

The aims of the present study were to improve HA production by *S. zooepidemicus* ATCC 39920 in sugarcane molasses medium, characterize its structure and estimate the antioxidant activity. The main highlight of this study is the originality of the pretreated sugarcane molasses application for hyaluronic acid production by *S. zooepidemicus*.

Material and Methods

Sugarcane Molasses

Sugarcane molasses was obtained from the Alltech group (São Pedro do Ivaí, PR, Brazil) and contained (*w*/w): 53 % sucrose, 4 % glucose, 5 % fructose, 6.6 % ash, 1.2 % amino acids such as glutamic acid, aspartic acid and alanine, and 0.17 % metal ions including sodium, aluminum, iron, nitride, phosphide and potassium. Sugars were analyzed by high-performance liquid chromatograph (HPLC) (Shimadzu RID-10A, Japan) coupled to a refractive index detector, with an Aminex Carbohydrate HPX-87C (300 × 7.8 mm, Biorad) column at 80 °C. The mobile phase was Milli-Q water at a 0.6 $mL.min^{-1}$ flow rate. Ash content was determined in an oven at 550 °C during 24 h. Amino acids and metal ions concentrations were provided by Alltech group. The sugarcane molasses (SM) was diluted to 15 % (*w*/*v*) of the total sugar concentration [29] with distilled water and centrifuged at 9956 x g at 4 °C for 15 min. To obtain pretreated sugarcane molasses (PSM), the molasses solution was treated with 12 % (*w*/*v*) activated charcoal at 70 °C with constant stirring for 1 h, centrifuged at 9956 g at 4 °C for 15 min and filtered (Whatman, n° 1) [28].

Microorganism and Medium

Streptococcus equi subsp. *zooepidemicus* ATCC 39920 was obtained from the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI). The strain was maintained in glycerol stock at −80 °C and cultured on brain heart infusion (BHI) agar plates at 37 °C for 24 h. For the inoculum, colonies from the agar plates were transferred to 125 mL Erlenmeyer flasks containing 25 mL BHI medium for 24 h. These cultures were used to inoculate 1 L Erlenmeyer flasks containing 200 mL BHI medium for 6 h. The flasks were incubated at 37 °C in a reciprocal shaker under 150 rpm. The cell concentration was determined by measuring turbidimetry at $\lambda = 600$ nm; for each fermentation, the inoculum was standardized to 0.2 $g.L^{-1}$.

The fermentation media contained ($g.L^{-1}$): yeast extract (YE) ranging from 14.65 to 85.35; SM or PSM ranging from 14.65 to 85.35 of the total sugar content [29]; 2.5 K_2HPO_4; 2.0 NaCl and 1.5 $MgSO_4$. SM, PSM and $MgSO_4$ were autoclaved separately.

Hyaluronic Acid Production

Effect of Sugarcane Molasses and Yeast Extract

To investigate the effect of the molasses pretreatment, the first experiment was performed in a 125 mL Erlenmeyer flask containing 25 mL fermentation media with SM or PSM at a 30 $g.L^{-1}$ concentration total sugar and 30 $g.L^{-1}$ YE. The experiment was performed in quadruplicate, and the samples were collected at the initial and final fermentation. Because the molasses pretreatment increased polymer production, we investigated the effect of the PSM (14.65–85.35 $g.L^{-1}$ of the total sugar) and YE (14.65–85.35 $g.L^{-1}$) concentrations using a central composite design (CCD) (Table 2). The CCD required 13 experiments with four factorial points, four axial points and five central points. The factor level choice was based on Pan et al. [30]. Samples were collected at the initial and final fermentation. Based on the CCD results, fermentation in triplicate was performed in a 1 L Erlenmeyer flask containing 200 mL fermentation medium with 85.35 $g.L^{-1}$ PSM and 50 $g.L^{-1}$ YE. Samples were collected every 2 h to study the HA production profile. All experiments were incubated at 37 °C with an initial pH of 8.0 and rotation at 100 rpm for 24 h.

Effect of pH on the Bioreactor

The pH control effect was evaluated under the HA production conditions optimized by CCD. Batch fermentation in a 4.5 L bioreactor (Tecnal, Brazil) containing 2 L fermentation medium with 85.35 $g.L^{-1}$ PSM and 50 $g.L^{-1}$ YE was performed at 8.0 pH that was controlled by an 8 $mol.L^{-1}$ NaOH solution. This fermentation was compared to a batch reactor without pH control (initial pH 8.0). The temperature was maintained at 37 °C, the rotation at 100 rpm and the aeration rate at 0.5 vvm. Samples were collected every 2 h for 24 h.

Biomass, Hyaluronic Acid, Organic Acids, Ethanol and Total Sugar Determination

Fermentation samples were centrifuged at 9956 g for 15 min. The biomass was determined by measuring the turbidimetry at $\lambda = 600$ nm and correlated to the biomass curve in $g.L^{-1}$. Total sugar concentration was determined from the supernatant sample according to methodology described by Dubois and co-workers [29]. For the HA, lactate, formate, acetate and ethanol quantifications, the culture supernatant samples were filtered (0.45 μm pore size, Millipore) and 20 μL was injected into a HPLC instrument (Shimadzu Corporation, Kyoto, Japan). The HA evaluation was performed in an OHpak SB-806 M HQ 80 × 300 mm column (Shodex, Japan) at 40 °C with a 0.1 M $NaNO_3$ mobile phase and a 1 $mL.min^{-1}$ flow rate. Lactate, acetate, formate and ethanol were evaluated using an Aminex 7.8 × 300 mm HPX-87H organic acid column (Bio-Rad, CA, USA) at 60 °C; the mobile phase was composed of a 0.005 $mol.L^{-1}$ H_2SO_4 solution with a 0.7 $mL.min^{-1}$ flow rate. The peak elution profile was monitored with a Shimadzu RID – 10A refractive index detector (Shimadzu Corporation, Kyoto, Japan).

Hyaluronic Acid Characterization

To characterize HA, the 24 h samples from the bioreactor with the controlled pH were centrifuged (9956 g, 15 min, 4 °C), and the cell-free supernatant was precipitated with

ethanol at a 1.5:1 (*v*/v) ratio of ethanol:supernatant at 4 °C for 1 h. The HA was re-dissolved in a 0.15 mol.L^{-1} NaCl solution. Three precipitations were performed to increase the HA purity. Then, trichloroacetic acid (1 %) was added until the HA solution reached pH 2.0 and was maintained for 1 h at 4 °C. The solution was centrifuged at 7744 g at 4 °C for 30 min. The supernatant was dialyzed for 48 h with six distilled water changes. The frozen dialysis product was lyophilized to characterize the structural and antioxidant properties of the produced HA. Sodium hyaluronate with a molecular weight of 1.5–1.8 × 10^3 kDa (Sigma-Aldrich, Brazil Ltd.) was used as the standard.

The HA homogeneity was determined by high performance steric exclusion chromatography (HPSEC) coupled to a refractive index (RI) detector model RID 10A. The chromatography system consisted of an HPLC pump (Model Shimadzu-10 AD), a manual injection valve (Shimadzu) fitted with a 200-μL loop and Ultrahydrogel columns (7.8 × 300 mm) arranged in series with different exclusion limit connected in order of decreasing pore size 7 × 10^6, 4 × 10^5, 8 × 10^4 and 5 × 10^3 Da corresponding to Ultrahydrogel (Waters) 2000, 500, 250 and 120, respectively. The mobile phase was 0.1 M $NaNO_3$ with sodium azide (0.03 %), and a 0.6 mL.min^{-1} flow rate. Data analysis was performed using LC solution software (Shimadzu Corporation). A standard curve of dextran with MW of 2000, 1400, 670, 500, 410, and 266 kDa was made to determine the HA apparent molecular weight.

Fourier transform infrared spectroscopy (FTIR) was recorded at wavelengths between 4000 and 400 cm^{-1} on an IR PRESTIGE-21 (Shimadzu, Kyoto, Japan) spectrophotometer. Thirty-two scans at a 4 cm^{-1} resolution were averaged and referenced against air. The powdered samples were compressed into KBr disks to measure the FTIR.

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 HA samples.

Antioxidant activity was estimated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich Brazil Ltd.). Briefly, 300 μL 0.1 mM DPPH in ethanol was added to 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 $(\%) = [1 - (A_1 - A_2)/A_0] \times 100$, where A_0 is the control absorbance (water instead of sample solution), A_1 is the sample absorbance and A_2 is the sample absorbance under identical conditions as A_1 with ethanol instead of DPPH solution. Ascorbic acid (Sigma-Aldrich Brazil Ltd.) was used as a standard for the assay.

Statistical Analysis

The data analysis was performed using the Statistica 9.0 software (StatSoft Inc., USA). The averages were compared using the Tukey test at a 5 % probability level ($p < 0.05$). For optimal point prediction, a second order polynomial function was fitted to the CCD experimental results:

$$Y_i = b_0 + \sum_i b_i x_i + \sum_i b_{ii} x_i^2 + \sum_{ij} b_{ij} x_i x_j \quad (1)$$

in which Y_i is the response, $x_i x_j$ are independent variables, b_0 is the offset term, is the i th linear coefficient, b_{ii} is the i th quadratic coefficient, and b_{ij} is the ij th interaction

coefficient. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The fit quality of the polynomial model equation was expressed by the determination coefficient R^2, and its statistical significance was assessed by the F-test.

Results and Discussion

Hyaluronic Acid Production

Effect of Sugarcane Molasses Pretreatment

Molasses and molasses pretreated with activated charcoal were evaluated as carbon sources for HA production. PSM increased HA production by 19 % compared to SM (Table 1). This result suggested that activated charcoal pretreatment decreased the level of inhibition by factors such as excessive metal ions and raised the fermentation quality. Although sugarcane molasses generally contains essential nutrients beneficial to microorganism growth, it also has metal ions and suspended colloids, which may be detrimental to microorganisms because they influence the medium pH and inactivate enzymes associated with product biosynthesis [25]. The individual presence of Na^+ and Fe^{2+} in medium containing glucose and yeast extract inhibited HA production by *S. zooepidemicus* ATCC 39920 [31]. Moreover, Tlapak-Simmons et al. [32] reported that hyaluronan synthase was sensitive to the concentrations of monovalent cations such as Na^+ and K^+. This result complied with research on the production of bacterial cellulose [24], welan gum [25] and polyhydroxyalkanoates [33] that reported an increase in polymer production when crude molasses was replaced by pretreated molasses. The total sugar consumption was 8.839 ± 0.297 $g.L^{-1}$ in SM and 8.433 ± 0.343 $g.L^{-1}$ in PSM and these results did not show significant difference ($p > 0.05$). Acetate synthesis (Table 1) was also increased in PSM compared to SM, which confirmed the change in cellular metabolism in favor of polymer production. This finding was explained by Chong and Nielsen [34], who observed that extra ATP concurrently generated during acetate formation by acetate kinase facilitated HA production. Biomass and lactate synthesis were not significantly different in PSM and SM media. Thus, the pretreatment of molasses with activated charcoal was advantageous because the cost of this pretreatment was low and the product has a high aggregated value. Therefore, we used PSM for the subsequent experiments.

Table 1 Hyaluronic acid, biomass, lactate and acetate production by *S. zooepidemicus* ATCC 39920 in sugarcane molasses and pretreated sugarcane molasses

Molasses	Hyaluronic acid $g.L^{-1}$	Biomass $g.L^{-1}$	Lactate $g.L^{-1}$	Acetate $g.L^{-1}$
SM	0.557 ± 0.038^b	2.178 ± 0.133^a	4.021 ± 0.229^a	0.582 ± 0.031^b
PSM	0.662 ± 0.003^a	2.123 ± 0.286^a	4.315 ± 0.161^a	0.678 ± 0.018^a

SM – sugarcane molasses; PSM – pretreated sugarcane molasses. Different lower-case letters indicate significant differences at the $\alpha = 0.05$ level in each column

Effect of Pretreated Sugarcane Molasses and Yeast Extract

The PSM and YE concentrations were assessed by CCD to evaluate their effects on HA production, biomass, lactate and acetate synthesis (Table 2). Because molasses has a low total nitrogen content, supplementation with yeast extract is necessary to achieve an ideal carbon/nitrogen ratio. Lancefield group A and C streptococci bacteria require complex nutrients due to their limited ability to synthesize specific amino acids and vitamins [19, 35]. Previous studies suggested that certain essential amino acids cannot be synthesized from an inorganic nitrogen source, because hyaluronic acid production in this medium is very low [36, 37]. Other studies that evaluated different organic nitrogen sources demonstrated that the highest HA production was obtained using yeast extract [11, 18, 37]. The main contributions of yeast extract to hyaluronic acid production are purine, pyrimidine bases and vitamin B [38]. Therefore, yeast extract was the nitrogen source used in this research. The maximum observed HA production was 0.860 $g.L^{-1}$ and was achieved in medium containing PSM with 85.35 $g.L^{-1}$ total sugar content and 50 $g.L^{-1}$ YE. HA production was 30 % higher than the production level obtained in the previous experiment using PSM (0.662 $g.L^{-1}$) (Table 1). The effect estimated for each variable was also reported (Table 3). An increase in the PSM and YE concentrations from 25 to 75 $g.L^{-1}$ led to an increase in the HA production of 0.106 and 0.108 $g.L^{-1}$, respectively. In contrast, the interaction between PSM and YE had a negative influence. This result supports the finding that agro-industrial byproducts have complex compositions in which a substrate can show an individual positive effect but have a negative effect in the overall system due to an

Table 2 Hyaluronic acid, biomass, lactate and acetate production by *S. zooepidemicus* ATCC 39920 at different pretreated sugarcane molasses and yeast extract concentrations

Run*	Factor level		Response				
	$\times_1$	$\times_2$	Hyaluronic acid ($g.L^{-1}$)	Biomass ($g.L^{-1}$)	Lactate ($g.L^{-1}$)		Acetate ($g.L^{-1}$)
1	-1	-1	0.555	2.304	4.256		0.682
2	-1	1	0.759	2.891	9.096		1.549
3	1	-1	0.749	2.529	4.796		0.659
4	1	1	0.758	2.634	8.692		1.245
5	-1.41	0	0.695	2.749	5.991		0.937
6	1.41	0	0.860	2.720	7.166		0.944
7	0	-1.41	0.623	2.261	4.378		0.698
8	0	1.41	0.776	2.933	9.172		1.357
9	0	0	0.809	2.760	7.329		1.131
10	0	0	0.796	2.566	7.549		1.167
11	0	0	0.766	2.677	6.898		1.060
12	0	0	0.775	2.607	6.968		1.068
13	0	0	0.767	2.772	7.094		1.204
Code	Variables		Coded variables levels				
			-1.414	-1	0	+1	+1.414
(X_1)	PSM (total sugar $g.L^{-1}$)		14.65	25	50	75	85.35
(X_2)	YE ($g.L^{-1}$)		14.65	25	50	75	85.35

(*)Assays were randomized; PSM – pretreated sugarcane molasses; YE – yeast extract

Table 3 Effect estimates for hyaluronic acid production by *S. zooepidemicus* ATCC 3992 from the CCD

Factor	Effect	Std. Err.	*t* – value	*p*- value
Average	0.783	0.011	74.171	0.00000*
PSM (L)	0.106	0.017	6.366	0.00037*
PSM (Q)	-0.022	0.018	-1.221	0.26175
YE (L)	0.108	0.017	6.448	0.00035*
YE (Q)	-0.100	0.018	-5.574	0.00084*
PSM YE	-0.098	0.024	-4.138	0.00436*

PSM – pretreated sugarcane molasses; YE – yeast extract. * Significant factors ($p < 0.01$)

increment in the concentration of other constituents [28]. Only the PSM quadratic term was not significant ($p < 0.01$) for hyaluronic acid production at a 99 % confidence level. These results showed the importance of the amounts of the PSM and YE variables for hyaluronic acid production. The effects of these variables in biomass, lactate and acetate synthesis were also evaluated to observe the carbon flux at the studied conditions. The total sugar consumption in the assays (Table 2) was 18.238 ± 3.698 % initial sugar concentration, except for assays 2 (42.469 %) and 5 (49.630 %). Of these metabolized carbons, 60–80 % was utilized for lactate production and 8–14 % for acetate. According to Chong and Nielsen [34] the HA and biomass synthesis account for 5–10 % of the carbon metabolized, whereas lactate and acetate are held responsible for the majority of the carbon. YE was the most significant variable for the biomass, lactate and acetate synthesis, with the YE linear term significant at 1 % level of significance. The increase from 25 $g.L^{-1}$ to 75 $g.L^{-1}$ YE caused an increase of 15 %, 30 % and 55 % in the biomass, lactate and acetate concentrations, respectively. The effect of PSM was not significant for these responses and the interaction between YE and PSM was significant only for biomass, and its was effect negative. Equations 2–5 represent the coded models used for HA production, biomass, lactate and acetate synthesis.

$$HA = 0.775 + 0.053 \times PSM + 0.054 \times YE - 0.048 \times YE^2 - 0.049 \times PSM \times YE \quad (2)$$

$$\text{Biomass} = 2.683 + 0.205 \times YE - 0.060 \times YE^2 - 0.120 \times PSM \times YE \quad (3)$$

$$\text{Lactate} = 7.037 - 0.262 \times PSM^2 + 1.940 \times YE \quad (4)$$

$$\text{Acetate} = 1.100 - 0.076 \times PSM^2 + 0.298 \times YE - 0.070 \times PSM \times YE \quad (5)$$

ANOVA was used to evaluate the adequacy of the fitted model (Table 4). The determination coefficient (R^2) was 93.83 % for HA production, 88.56 % for biomass, 94.05 % for lactate and 89.49 % for acetate synthesis. According to Haaland [39], R^2 values above 90 % are considered very good for the experimental design of biotechnological processes. Based on the *F* test, the models were predictive because *F*-calc was higher than *F*-tab. Furthermore, the pure error was very low and the lack of fit was not significant, indicating that the models were reproductive and adequately represented by the data in the experimental region.

Table 4 Analysis of the variance (ANOVA) of hyaluronic acid, biomass, lactate and acetate production by *S. zooepidemicus* ATCC 39920 from the CCD

Variance source	Level of freedom	Sum of Square	Mean Square	f-calc	f-tab	R^2	R^2_{adj}	*p* - value
Hyaluronic acid								
Regression	0.07185	4	0.01796	30.40	3.84	0.93828	0.90742	
Residue	0.00473	8	0.00059					
Lack of fit	0.00327	4	0.00082					0.22559
Pure error	0.00145	4	0.00036	2.25	6.39			
Total	0.07657	12	-					
Biomass								
Regression	0.42052	3	0.14017	23.21	3.86	0.88555	0.84740	
Residue	0.05435	9	0.00604					
Lack of fit	0.02132	5	0.00426	0.52	6.26			0.75731
Pure error	0.03303	4	0.00826					
Total	0.47487	12	-					
Lactate								
Regression	30.57855	2	15.28928	79.03	4.10	0.94050	0.92860	
Residue	1.93463	10	0.19346					
Lack of fit	1.64519	6	0.27420	3.79	6.16			0.10901
Pure error	0.28943	4	0.07236					
Total	32.51318	12	-					
Acetate								
Regression	0.77148	3	0.25716	25.56	3.86	0.89494	0.85993	
Residue	0.09056	9	0.01006					
Lack of fit	0.07499	5	0.01500	3.85	6.26			0.10781
Pure error	0.01557	4	0.00389					
Total	0.86204	12	-					

The response surfaces were obtained using eqs. 2–5 (Fig. 1). The biomass concentration was enhanced by the increase in YE and decrease in PSM (Fig. 1b). This result is in accordance with the typical behavior of lactic acid bacteria, whose biosynthetic need is met by the complex nitrogen source. Organic nitrogen sources are considered essential for good Streptococci growth because there is evidence that these components also supply a large proportion of the carbon for cellular biosynthesis [40]. Several *Streptococcus* strains can only grow in media containing vitamins, purines and amino acids which are mainly used as carbon sources for the cell skeleton due to a lack of tricarboxylic acid cycle (TCA) and precursors for the synthesis of most amino acids and nucleotides [41]. The lactate and acetate concentrations were raised by the increase in YE (Fig. 1c,d), and 50 $g.L^{-1}$ and 32 $g.L^{-1}$ of PSM were the best concentrations for the synthesis of these organic acids, respectively. Under the best condition observed for HA production (85.35 $g.L^{-1}$ PSM and 50 $g.L^{-1}$ YE), the predicted value was 0.850 $g.L^{-1}$ (Fig. 1a). To confirm the predicted results, experiments were performed in quintuplicate using these conditions, and a value of 0.842 ± 0.025 $g.L^{-1}$ was obtained. The good correlation between these results (p = 0.50095) verifies the model validation for HA production. In these experiments, the total sugar consumption, biomass, lactate and acetate

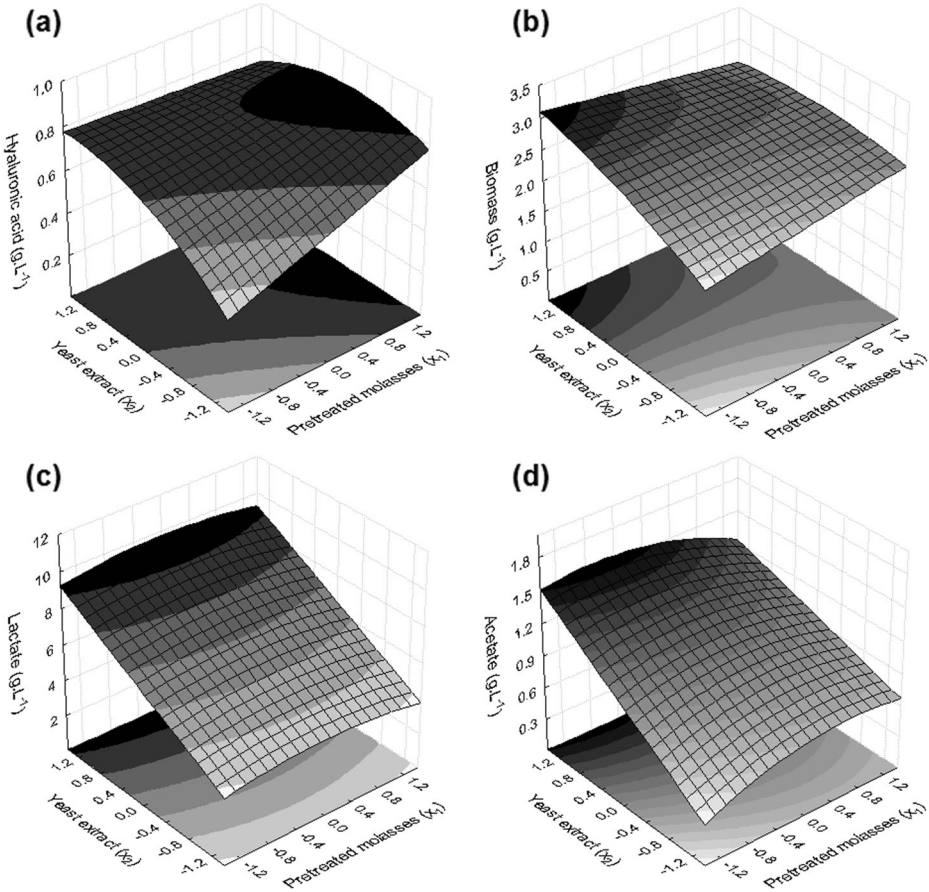

Fig. 1 Response surfaces of (a) hyaluronic acid, (b) biomass, (c) lactate and (d) acetate production by *S. zooepidemicus* ATCC 39920 in pretreated sugarcane molasses ($\times_1$) and yeast extract ($\times_2$)

results were 9.375 ± 0.598 g.L^{-1}, 2.398 ± 0.054 g.L^{-1}, 5.682 ± 0.639 g.L^{-1} and 0.706 ± 0.053 g.L^{-1}, respectively.

In the conditions optimized by CCD, medium containing PSM with 85.35 g.L^{-1} total sugar content and 50 g.L^{-1} YE, the assays were performed in 1 L Erlenmeyer flasks containing 200 mL medium to study the HA production profile. As shown in Fig. 2a, after 2 h of lag phase, cells began exponential growth until 6 h with a specific growth rate at 0.30 ± 0.10 h^{-1} and biomass concentration reached 2.075 ± 0.023 g.L^{-1}. The HA concentration followed the biomass synthesis trend and achieved a value of 0.825 ± 0.075 g.L^{-1} at 24 h. This value was not significantly different compared to the result predicted in the model by CCD (p = 0.622210). The maximum lactate and acetate synthesis were 7.840 ± 0.445 g.L^{-1} at 24 h and 0.531 ± 0.072 g.L^{-1} at 14 h, respectively, and the total sugar consumption at 24 h was 10.622 ± 0.895 g.L^{-1}.

Due to the lactate and acetate production, the final pH in the assays performed in Erlenmeyers ranged from 4.7 to 5.0. The decrease in pH which cannot be controlled in shake flasks may have caused inhibition of microbial growth and limited hyaluronic acid production.

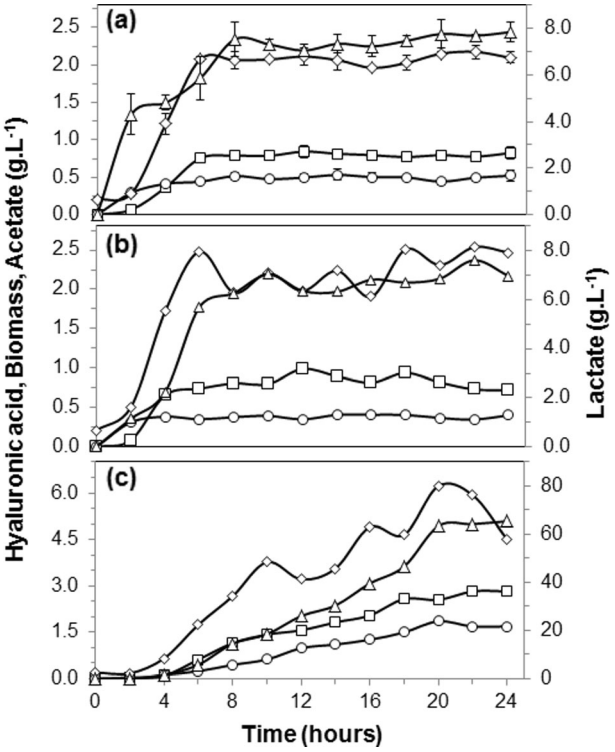

Fig. 2 Production of hyaluronic acid (□), biomass (◊), lactate (Δ) and acetate (○) by *S. zooepidemicus* ATCC 39920 in (a) Erlenmeyer flask uncontrolled pH, (b) bioreactor uncontrolled pH and (c) bioreactor with controlled pH

Then, fermentations in bioreactor were performed to evaluate the effect of pH control in optimized medium.

Effect of pH on the Bioreactor

Pan et al. [30] investigated the effect of the initial pH on *S. zooepidemicus* fermentation in a shake flask and concluded an initial pH of 8.0 was best for HA production. Shake flasks have been widely used in both studies and biotechnology process optimization because they allow experiments to be conducted with minimal costs and materials. However, shake flasks have several limitations. For instance, some are unable to control the pH and the dependency of the oxygen transfer rate on the agitation speed. Therefore, scaling from shake flasks to bioreactors is essential to obtain large quantities of the final product [42]. The influence of controlled pH on HA production was evaluated in a bioreactor using medium optimized by CCD and 0.5 vvm aeration (Fig. 2c). A control experiment was run at initial pH 8.0 that was not controlled (Fig. 2b). Under alkaline conditions, the maximum specific cell growth rate was 0.14 h^{-1} and the biomass concentration reached 6.22 $g.L^{-1}$, compared with 0.27 h^{-1} and 2.539 $g.L^{-1}$ in the control experiment, respectively. During fermentation without pH control, the pH decreased to 4.9 during the first 8 h, which may have caused the inhibition of microbial growth. The pH reduction was caused mainly by lactate production. According to other studies, batch culture at pH less than 6.0 slowed cell growth and resulted in very low HA production [35, 43]. Diauxic growth was observed when the pH was controlled, suggesting the preferential utilization of the carbon sources present in PSM (predominantly sucrose and lower concentrations of fructose

and glucose). The total sugar consumption in pH controlled medium was 75.754 $g.L^{-1}$ at 24 h, which was 6.12-fold greater than in the control experiment. HA, lactate and acetate were produced in parallel with the biomass. Under controlled pH conditions, the maximum HA production was 2.825 $g.L^{-1}$ at 24 h. This value was 2.86-fold greater than the maximum HA production in uncontrolled pH conditions (0.988 $g.L^{-1}$ at 12 h). The positive effect of intermittent alkaline-stress on HA production by *S. zooepidemicus* WSH-24 was reported by Liu et al. [44]. The authors suggested that the carbon flux was redirected, thereby increasing HA production by up to 30 % and decreasing the biomass by 24 %. However, in our results the relationship between biomass and HA production was positive. This effect was also observed by Shah and co-workers [16]. Pires and Santana [15] explained that the high HA concentration in an alkaline medium might be due to the exposition of the microorganisms to the stress condition in which the cells produce the capsule as a way to shield themselves. Lactate and acetate synthesis increased to 65.368 and 1.855 $g.L^{-1}$ compared with 7.623 and 0.408 $g.L^{-1}$ in the control, respectively.

Formate and ethanol were not observed in any experiments in this research. The lack of formate synthesis suggested good aeration conditions in the experiments because pyruvate formate lyase is extremely sensitive to oxygen [34].

HA productions by *S. zooepidemicus* ATCC 39920 in bioreactor reported in previous studies ranges from 1 to 5 $g.L^{-1}$. The highest HA concentration observed by Pires and Santana [15] was 1.21 $g.L^{-1}$ in a medium containing glucose, yeast extract and salts. Lai et al. [36] reported that about 2.442 $g.L^{-1}$ HA was synthesized at an optimal C/N of 1.5:1 using glucose and a mixture of yeast extract and tryptone. The highest polymer productions were observed in media containing casein, 3.32 $g.L^{-1}$ [45] and 5.0 $g.L^{-1}$ [46], and media supplemented with amino acid, oxygen vector, and other additives that aimed to redirect the carbon flux to HA production. Adding 5 $g.L^{-1}$ glutamine and 25 μM sodium iodoacetate increased the HA concentration to 5.0 $g.L^{-1}$ from 2.0 $g.L^{-1}$ in a control run [16]. Lai et al. [47] studied the potential of oxygen vectors for enhancing HA biosynthesis and obtained a high production of 4.25 $g.L^{-1}$ when 0.5 % (*v*/v) n-hexadecane was added. These supplementations to the fermentation medium increase the production cost, and are not an advantage when the aim of study is to reduce the polymer price. Considering the studies that evaluated alternative fermentation media for hyaluronic acid production (Table 5), the concentration of 2.825 $g.L^{-1}$ polymer obtained in our study was high. These results point out the possibility of obtaining a high HA production by replacing the commercial sources with an inexpensive alternative, emphasizing the use of sugarcane molasses as a promising carbon source.

Hyaluronic Acid Characterization

The homogeneity of the hyaluronic acid produced by *Streptococcus zooepidemicus* in a bioreactor with the pH controlled at 8.0 was verified by HPSEC/RID. The chromatography profile showed a single symmetrical peak (Fig. 3b) with an enlarged base that most likely indicated a higher polydispersity (Mw/Mn 1.32) than the hyaluronic acid standard (Fig. 3a), which eluted in 36.7 min and had 1.5–1.8 × 10^3 kDa molecular weight. The hyaluronic acid molecular weight from *S. zooepidemicus* was 1.35 × 10^3 kDa, using dextrans of known molecular weight as standards. HA applications depend on its molecular weight, a high molecular weight (>1 MDa) is used for clinical and cosmetic applications [48, 49].

The FTIR spectra of the produced HA (Fig. 4b) was similar to the HA standard (Fig. 4a). The strong band at approximately 3449 cm^{-1} can be attributed to hydrogen-bonded O-H and

Table 5 Hyaluronic acid production in different fermentation media containing alternative sources

Bacterial strain	Cultivation conditions	Carbon and nitrogen source ($g.L^{-1}$)	Hyaluronic acid ($g.L^{-1}$)	Reference
S. zooepidemicus ATCC 39920	250 mL Erlenmeyer; 37 °C; 150 rpm; pH initial 7.5	cashew apple juice (45 of glucose) and yeast extract (54)	0.89	[11]
S. thermophilus YIT 2084	2 L bioreactor, 40 °C; no aeration; 100 rpm; pH 6.8	10 % of skimmed milk and soybean peptides (10)	0.21	[43]
S. zooepidemicus ATCC 35246	2 L bioreactor; 37 °C; no aeration; 500 rpm; pH 6.7	glycogen of mussel processing wastewater (50), tuna peptone from viscera residue (protein 8) and yeast extract (5)	2.46	[14]
S. zooepidemicus ATCC 39920	3 L bioreactor; 37 °C; 150 rpm; 2 vvm, pH 7.0	2 L cashew apple juice and yeast extract (60)	1.76	[19]
S. zooepidemicus ATCC 39920	125 mL Erlenmeyer flasks; 37 °C; 100 rpm pH initial 8.0	sugarcane molasses (30) and yeast extract (30)	0.38	[18]
S. zooepidemicus ATCC 39920	125 mL Erlenmeyer flasks; 37 °C; 100 rpm pH initial 8.0	sucrose (30) and soy protein (30)	0.22	[18]
S. zooepidemicus ATCC 35246	2 L bioreactor; 37 °C; no aeration; 500 rpm; pH 6.7	glucose (50), peptones from alcalase hydrolyzed viscera (protein 5) and yeast extract (5)	2.32	[17]
S. zooepidemicus ATCC 35246	5 L bioreactor; 37 °C; 1vvm; 500 rpm; pH 6.7	glucose (50), lactose (50) cheese whey (protein 5) and yeast extract (5)	4.0	[20]
***S. zooepidemicus* ATCC 39920**	**4.5 L bioreactor, 37 °C; 100 rpm; 0.5 vvm; pH 8.0**	**sugarcane molasses pretreated (85.35) and yeast extract (50)**	**2.83**	**Present study**

N-H stretching vibrations of the N-acetyl side chain. A group of overlapping bands of moderate intensity is observed at approximately 2922 cm^{-1} due to the C-H stretching vibrations. The bands at 1622 and 1418 cm^{-1} can be attributed to the asymmetric (C = O) and symmetric (C-O) stretching modes of the planar carboxyl groups in the hyaluronate. According to Gilli et al. [50], after protonation, these peaks are shifted to 1735 and 1255 cm^{-1}, respectively. This explained the band at 1738 cm^{-1} observed in the produced HA. The protonation of the carboxyl group leading to carboxylic acid occurred during the purification process due to the use of trichloroacetic acid. The absorption bands at approximately 1653, 1564 and 1325 cm^{-1} are characteristic of the amide I, II and III bands, respectively. The C-O-C group at 1155 cm^{-1} (O-bridge), C-O (exocyclic) and C-C groups at 1082 cm^{-1} and the C-OH group at 1043 cm^{-1} are also present [2]. The band at 945 cm^{-1} can be assigned to an asymmetrical out-of-phase ring vibration [51].

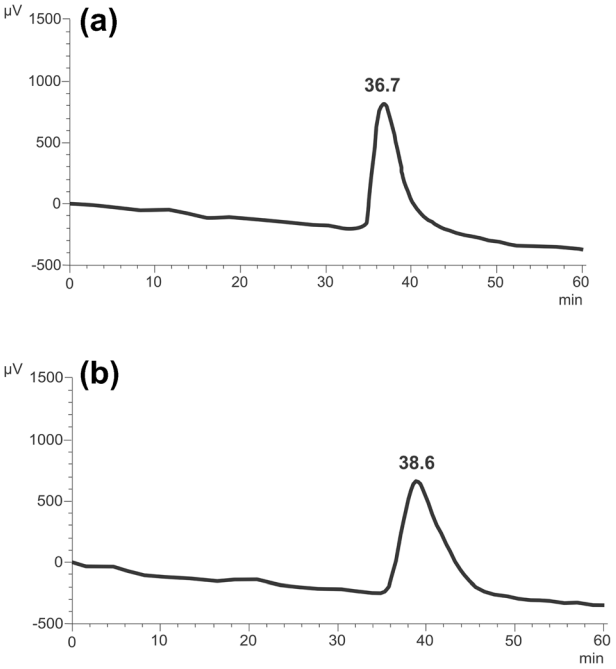

Fig. 3 Elution profile of (a) hyaluronic acid standard and (b) hyaluronic acid produced by *S. zooepidemicus* ATCC 39920 in a bioreactor with controlled pH analyzed by HPSEC/RID

The UV-vis spectra of the standard HA and the HA produced by *S. zooepidemicus* (Fig. 5) showed that both had the same absorption profile. The maximum absorbance wavelength was 205 nm. Absorbance at ~210 nm was attributed to carboxyl groups [2].

DPPH radical scavenging activity of the produced HA was evident et al.l of the tested concentrations but was lower than that of ascorbic acid (Fig. 6). The highest scavenging effects were 41 % for the produced and standard HA and 84 % for ascorbic acid at 1 $g.L^{-1}$. Produced HA showed higher scavenging activity than standard HA at the range of 0.2–0.8 $g.L^{-1}$. This result might be correlated with the lowest molecular weight of the

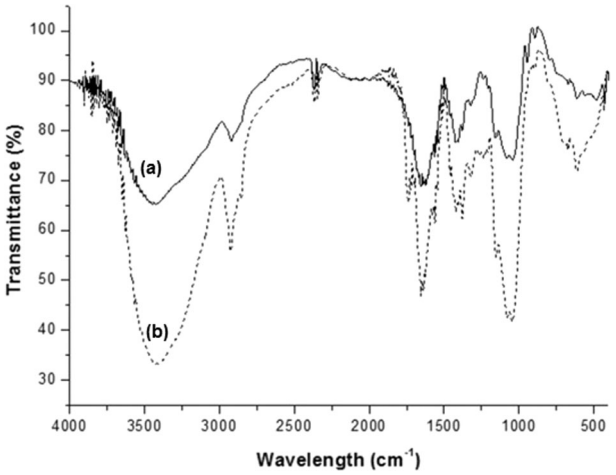

Fig. 4 FTIR spectra of the (a) hyaluronic acid standard and (b) hyaluronic acid produced by *S. zooepidemicus* ATCC 39920 in a bioreactor with controlled pH

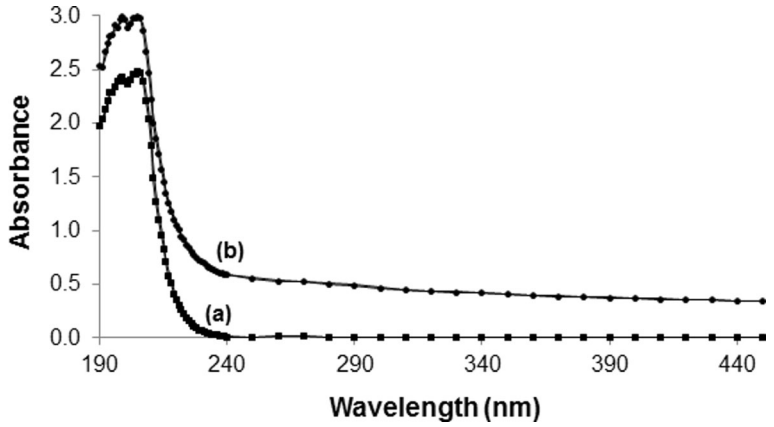

Fig. 5 UV-vis spectrum of the (a) hyaluronic acid standard and (b) hyaluronic acid produced by *S. zooepidemicus* ATTC 39920 in a bioreactor with controlled pH

produced HA. Several studies showed that polymer antioxidant activity is related to molecular weight [52–54]. Kim et al. [53] showed a gradual increase in DPPH radical scavenging ability of HA by the decreased polymer molecular weight. El-Safory and Lee [52] also observed a stronger radical scavenging activity of HA oligomers than native HA. The mechanism by which HA reduces damage from free radicals is based on its structure, which has cross-linked carboxylic groups. Thus, these carboxylic groups can interact with metal ions such as Cu^{2+} and Fe^{2+}, allowing these molecules to act as metal chelators [55]. These encouraging results reveal that the HA produced in sugarcane molasses can be employed as a natural antioxidant.

Conclusion

The present study demonstrated that it is possible to obtain high hyaluronic acid production in an alternative medium using pretreated sugarcane molasses such as carbon source. The

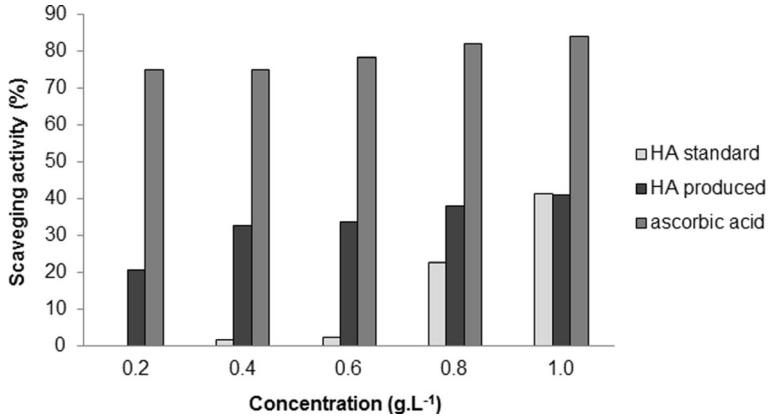

Fig. 6 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the hyaluronic acid standard, hyaluronic acid produced by *S. zooepidemicus* ATCC 39920 in a bioreactor with controlled pH and ascorbic acid

maximum HA production was 2.825 $g.L^{-1}$ at 24 h in pH 8.0 medium containing pretreated molasses with 85.35 $g.L^{-1}$ total sugar content and 50 $g.L^{-1}$ YE in a bioreactor by *S. zooepidemicus* ATCC 39920. The controlled pH of 8.0 increased the production 2.86-fold. Polymers characterization showed that sugarcane molasses fermentation medium provided an HA appropriate for medical and cosmetic application with 1.35 $\times 10^3$ KDa molecular weight and potential antioxidant activity. Thus, pretreated molasses may be an excellent substrate for cost-effective HA production.

Acknowledgements The authors thank Coordination for the Improvement of Higher Education Personnel (CAPES – Brazil) for financial support, Dr. Dionisio Borsato from Londrina State University for support with the statistical analysis, and the Laboratory of Spectroscopy (SPEC) – State University of Londrina for the analyses.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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

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