Agroindustrial Byproducts For The Production Of Hyaluronic Acid By Streptococcus Zooepidemicus ATCC 39920

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Abstract: Agroindustrial derivatives are alternative nutritional sources employed in bioprocesses that reduce costs and corroborate with social sustainability. In this study, alternative carbon (sugarcane juice, sugarcane molasses and soy molasses) and nitrogen sources (corn steep liquor, soy protein and whey protein) were evaluated for hyaluronic acid production by Streptococcus zooepidemicus ATCC 39920. The medium containing sugarcane molasses archived high yield of hyaluronic acid (0.066 $g.g^{-1}$), when compared to the medium composed of glucose or sucrose. The replacement of yeast extract by soy protein was also effective for the production of the polymer resulting in 0.219 $g.L^{-1}$. In general, the organic acids production was also evaluated and the results showed that the main metabolic products were lactate. In contrast, the acetate synthesis was detected only in the medium containing yeast extract. This study showed that sugarcane molasses is a promising carbon source for the hyaluronic acid production. This is the first study in which a culture media containing sugarcane molasses, a cheap substrate extensively produced in Brazil, has been successfully used for the microbial hyaluronic acid production.

Index Terms: hyaluronic acid, organic acid, soy protein, Streptococcus zooepidemicus, sugarcane molasses.

1 Introduction

According to FAO (2010), the Brazilian economy is strongly dependent on agricultural products, being the largest world producer of sugarcane and oranges, the second largest for soybean and the third largest for corn [1]. However, this great production is responsible for the generation of excessive amounts of residues which may cause serious environmental problems. Thus, the utilization of renewable resources such as agricultural residues and byproducts as alternative substrates in bioprocesses may result into bioenergy and valuable biomolecules [2], [3]. Although carbohydrates in general hold many other important functions, bulk carbohydrates have been used as a nutrient source of carbon for the largest scale cultivation of microorganisms. Cheap carbohydrates such as beet and sugarcane molasses, sucrose, glucose syrup, starch or its hydrolysates are almost universally used as renewable carbon sources in large scale fermentation process.

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The worldwide total usage of carbohydrate-nature feedstock for industrial fermentation process has been estimated at 4.10^7 tons per year [4]. The same occurs with renewable nitrogen source, where vegetable peptones replace animal derived substance [5], [6]. The sugarcane molasses and juice, soy molasses, soy protein, corn steep liquor and whey protein are some examples of agroindustrial substrates extensively produced in Brazil. These byproducts, relatively of low cost, can be employed at the cultivation medium, reducing the cost of production and generating polymers of high value aggregated, such as hyaluronic acid. Hyaluronic acid is a linear polysaccharide with high molecular weight formed of disaccharide units of D-glucuronic acid and N-acetylglucosamine linked alternately by β (1→3) and β (1→4) glycosidic bonds [7]. Due to its hydrophilic and viscoelastic properties, hyaluronic acid constitutes an essential component in a wide variety of cosmetics. Likewise, it has been extensively used in viscosupplementation of synovial fluids of patients with arthritis, tissue repair, ophthalmological surgery, and drug delivery [8]. The hyaluronic acid exhibits high aggregated value ranging from US$ 2,000 to 60,000 kg, depending on its applications [9]. The production of hyaluronic acid by Streptococcus zooepidemicus demands an enrichment culture medium comprising amino acids, nucleotide bases and vitamins, necessary to the microbial growth and polymer production [10]. The sources of organic nitrogen are considered essential for the growth of the microorganism, once there are evidences that these components supply high quantity of carbon required for the cellular biosynthesis [11]. Consequently, the high nutritional demand required by S. zooepidemicus to produce hyaluronic acid and the gradually rise in the cost of synthetic materials, weakens the commercial competiveness of microbial hyaluronic acid production when compared to the one obtained through extraction of animal tissues [12]. Additionally, more than 80% of these costs are due to sugars and proteins and commercial formulations are not an economical resource for industrial production of hyaluronic acid [13]. In this sense, the aim of this study was to evaluate the production of hyaluronic acid using agroindustrial byproducts as alternative source of carbon and nitrogen. Our results pointed out the high yield of hyaluronic acid using

sugarcane molasses and the possibility of the substitution of yeast extract by soy protein for hyaluronic acid production by S. zooepidemicus. The novelty of this study was the utilization of sugarcane molasses, a cheap and abundant substrate found in Brazil, for the efficient production of hyaluronic acid by S. zooepidemicus ATCC 39920.

2 MATERIALS AND METHODS

2.1 Microorganisms and agricultural byproducts

Streptococcus equi subsp. zooepidemicus ATCC 39920 was obtained from the Brazilian Collection of Environmental and Industrial Microorganisms - CBMAI. The sugarcane juice and molasses were acquired from COROL - Cooperativa Agroindustrial (Rolândia, Paraná, Brazil), soy molasses from IMCOPA - Importação, Exportação e Indústria de Óleos S. A. (Cambé, Paraná, Brazil), soy protein from Solabia Biotecnológica (Maringá, Paraná, Brazil), whey protein from Confepar Agro-Industrial Cooperativa Central (Londrina, Paraná, Brazil) and corn steep liquor from Corn Products Brasil -Ingredientes Industriais Ltda. (Mogi Guaçu, São Paulo, Brazil).

2.2 Culture maintenance and inoculum preparation

S. zooepidemicus ATCC 39920 was maintained in saline solution containing 50% glycerol at -80°C. The inoculum was prepared transferring 1 mL of the stock culture to 125 mL Erlenmeyer flasks, containing 25 mL of the medium Brain Heart Infusion (BHI). These flasks were incubated under orbital shaking at 150 rpm, 37°C for 48 h.

2.3 Carbon and nitrogen sources to hyaluronic acid production

The basal medium was composed of ($g.L^{-1}$): glucose, 30; yeast extract, 30; K_2HPO_4, 2.5; NaCl, 2.0 and $MgSO_4.7H_2O$, 1.5. To study the effect of carbon sources on hyaluronic acid production, glucose in the basal medium was replaced with seven other carbon sources, such as sucrose, fructose, lactose, maltose, soy molasses, sugarcane molasses and sugarcane juice. The final concentration was standardized at 30 g L^{-1} of total sugars. To study the effect of nitrogen sources, yeast extract was replaced by corn steep liquor, soy protein and whey protein, in the concentration of 30 $g.L^{-1}$, in medium containing glucose or sucrose at 30 $g.L^{-1}$. The pH was adjusted to 8.0 according to the previously optimization condition (data not shown). The concentration of total nitrogen and protein nitrogen of the nitrogen sources studied were determined according to AOAC [14] and Lowry et al. [15], respectively. The results were showed on Table 1.

Table 1. *Concentration of total nitrogen and protein nitrogen at the carbon sources studied*

Nitrogen Source	Total Nitrogen $g.Kg^{-1}$	Protein Nitrogen $g.L^{-1}$
Yeast extract	117.792±9.809	345.970±13.939
Corn steep liquor	45.808±7.198*	299.319±2.414
Soy Protein	128.848±0.118	719.170±4.639
Whey Protein	17.344±0.152	95.086±1.534

*$g.L^{-1}$

2.4 Fermentation

Fermentations were carried out in triplicate batch culture, using 125 mL Erlenmeyer flasks containing 25 mL of the evaluated medium (item 2.3), which were maintained under orbital shaking at 100 rpm, 37°C for 24 h. The media were inoculated with 10% (v/v). Fermentations were interrupted by centrifugation at 9956 × g for 15 min, at 4°C. Hyaluronic acid, lactate, formate, acetate, and total sugar concentrations were determined from each flask at the initial and final times. The analyses were performed in triplicate.

2.5 Analytical methods

2.5.1 Hyaluronic acid concentration

The cell free supernatant was treated with ethanol in a proportion 3:1 (v/v) ethanol:supernatant at 4°C for 24 h. The precipitated hyaluronic acid was collected by centrifugation (8784 × g, 4°C, 20 min) and its concentration was estimated by Carbazol reagent, using sulfuric acid with 0.025 M sodium tetraborate according to Filisetti and Carpita [16] modified. The sodium hyaluronate (Sigma-Aldrich Brasil Ltda) was used as a standard.

2.5.2 Concentrations of lactate, formate and acetate

For the quantification of lactate, formate, and acetate, samples of the culture supernatant were filtered through membranes with a pore size 0.45 µm (Millipore) and 20 µL of filtered sample was injected into a High Performance Liquid Chromatography (HPLC) (Shimadzu Corporation, Kyoto, Japan), equipped with a 7.8 × 300 mm HPX-87H organic acid column Aminex (Bio-rad, CA, USA). The mobile phase was composed of 0.005 $mol.L^{-1}$ H_2SO_4 solution pumped at a rate of 0.7 $mL.min^{-1}$. The column was maintained at 60°C. The peak elution profile was monitored with a Shimadzu RID – 10A refractive index detector (Shimadzu Corporation, Kyoto, Japan).

2.5.3 Total sugar concentrations

Total sugar concentration was determined according to methodology described by Dubois and co-workers [17].

2.6 Statistical Analysis

The variance was analyzed by ANOVA method and the media compared by Tukey test at 5% probability level ($p < 0.05$), using the Software Statistica 9.0.

3 RESULTS AND DISCUSSION

3.1 Alternatives carbon sources for hyaluronic acid production

The table 2 shows the production of hyaluronic acid using different carbon sources. Interestingly, one of the highest productions was observed when the sugarcane molasses were used resulted in 0.376 $g.L^{-1}$ of hyaluronic acid. This result was very close to that observed to the media containing glucose (0.429 $g.L^{-1}$) and sucrose (0.488 $g.L^{-1}$). The microorganism also produced a great amount of hyaluronic acid of 0.266 $g.L^{-1}$ in medium containing sugarcane juice. This value was not significantly different of that of maltose (0.295 $g.L^{-1}$). The production was low in fructose (0.032 $g.L^{-1}$) and lactose (0.006 $g.L^{-1}$), whereas in soy molasses the production of hyaluronic acid was not detected. It is interesting to point out that the greatest yield of the hyaluronic acid in relation to

the consumption of total sugar (0.066 $g.g^{-1}$) was in medium composed of sugarcane molasses. This byproduct is a complex substrate with high concentration of sucrose, glucose and fructose, and a great variety of salts and some nitrogen sources, which are essential for the microbial metabolism [18], [19]. Few studies have reported the hyaluronic acid production using alternative sources. Pires and co-workers [9] evaluated the production of hyaluronic acid by S. zooepidemicus ATCC 39920 using agricultural resource derivatives such as hydrolysate soy protein concentrate, whey protein concentrate, and cashew apple juice. The authors showed that the cashew apple juice induced the highest amount of hyaluronic acid (0.89 $g.L^{-1}$). In another study, Macedo and Santana [20], used the cashew apple bagasse in solid state fermentation, obtaining productivity of 0.28 $mg.g^{-1}.h^{-1}$ of hyaluronic acid. The organic acids produced by S. zooepidemicus on different carbon sources were also studied (Table 2). The results indicated that the higher lactate production was obtained in media contained sugarcane molasses, fructose, sugarcane juice, sucrose or glucose. Additionally, lactate was the main product in media with lactose and maltose. The formate production was highest just in soy molasses medium. However, only in media with fructose and maltose the formate synthesis was not detected. Acetate production was observer in all conditions, ranged from 0.040 $g.L^{-1}$ in medium with sugarcane molasses at 1.546 $g.L^{-1}$ in lactose. Chong and Nielsen [21] developed a metabolic flux model for S. zooepidemicus ATCC 35246 to compare the metabolism of glucose and maltose during the aerobic batch cultivation. The authors verified that the main production of lactate was in medium with glucose. In the same way, the major production of acetate was observed when maltose was used. Nevertheless, the hyaluronic acid production had little impact over the carbon sources utilized. These same authors also observed the formate synthesis, even under aerated cultivation, and suggested that this behavior could be related to the anoxic conditions caused by the aggregation of cells at the presence of hyaluronic acid. This aggregation prevents the inhibition of the enzyme pyruvate formate lyase under forced aeration [22].

Table 2. *Performance of the fermentation with the alternatives carbon sources by S. zooepidemicus ATCC 39920.*

Carbon Source	Hyaluronic acid ($g.L^{-1}$)	Yp/s ($g.g^{-1}$)	Productivity ($g\ AH.L^{-1}.h^{-1}$)	Lactate ($g.L^{-1}$)	Formate ($g.L^{-1}$)	Acetate ($g.L^{-1}$)
Glucose	0.429 ± 0.028^{b}	0.052 ± 0.008^{a}	0.018 ± 0.001^{b}	4.766 ± 0.299^{a}	1.013 ± 0.142^{b}	1.079 ± 0.062^{c}
Sucrose	0.488 ± 0.002^{a}	0.064 ± 0.014^{a}	0.020 ± 0.000^{a}	4.974 ± 0.623^{a}	0.956 ± 0.133^{b}	$1.129\pm0.196^{b,c}$
Fructose	0.032 ± 0.021^{e}	$0.004\pm0.003^{b,c}$	0.001 ± 0.001^{e}	5.262 ± 0.178^{a}	0.000 ± 0.000^{d}	0.547 ± 0.012^{d}
Lactose	0.006 ± 0.004^{e}	0.000 ± 0.000^{c}	0.000 ± 0.000^{e}	2.997 ± 0.158^{c}	2.171 ± 0.184^{a}	1.546 ± 0.027^{a}
Maltose	0.295 ± 0.020^{d}	0.056 ± 0.005^{a}	0.012 ± 0.001^{d}	3.897 ± 0.329^{b}	0.000 ± 0.000^{d}	$1.386\pm0.177^{a,b}$
Soy Molasses	0.000 ± 0.000^{e}	0.000 ± 0.000^{c}	0.000 ± 0.000^{e}	0.000 ± 0.000^{d}	2.001 ± 0.160^{a}	0.360 ± 0.016^{d}
Sugarcane Molasses	0.376 ± 0.020^{c}	0.066 ± 0.006^{a}	0.016 ± 0.001^{c}	5.544 ± 0.247^{a}	$0.334\pm0.051^{c,d}$	0.040 ± 0.003^{e}
Sugarcane Juice	0.266 ± 0.017^{d}	0.022 ± 0.004^{b}	0.011 ± 0.001^{d}	5.062 ± 0.070^{a}	0.591 ± 0.112^{c}	1.008 ± 0.067^{c}

$Y_{p/s}$ – Hyaluronic acid production per sugar consumption. Different lower-case letters indicate statistical difference at α=0.05 level in each column. Values with the same letters in the same parameters indicate that the values did not differ by Tukey test at 0.95 confidence interval (a>b>c>d).

Thus, the formate production observed in the present work could be explained by the low agitation and the formation of anoxic regions on the cultivations flasks. Shah, Badle and Ramachandran [23] also evaluated the metabolic flux of S. zooepidemicus ATCC 39920 and showed that the hyaluronic acid concentration and molecular weight increased by decreasing of carbon flux towards glycolysis and pentose phosphate pathway and raising carbon flux towards hyaluronic acid precursor formation.

3.2 Alternatives nitrogen sources for hyaluronic acid production

The production of hyaluronic acid was also evaluated using alternatives nitrogen sources, in medium containing glucose or sucrose. The results were compared to the yeast extract (Table 3). In general, for the fermentations using sucrose as a carbon source, the hyaluronic acid production as well as the yield and the productivity, were higher than in medium containing glucose. As expected, the highest hyaluronic acid production was observed in the medium composed of yeast extract. But, in soy protein medium was detected a good production of the polymer for both carbon sources studied (0.192 g. L^{-1} for glucose and 0.219 g. L^{-1} for sucrose). On the other hand, in the medium composed of corn steep liquor the production was not detected (Table 3). It is well-known that the main contributions of yeast extract for hyaluronic acid production are the purine, pyrimidine bases and vitamin B [24]. Interestingly, among the nitrogen source investigated, the soy protein contains the highest concentration of protein nitrogen (Table 1), explaining its ability to induce the great amounts of the studied polymer. Although the fermentations with soy protein have resulted in lower amounts of hyaluronic acid in comparison to the yeast extract, its use may result in reduction in production costs of the polymer. In addition, the use of non-animal nutrients for the production of hyaluronic acid is a requirement for its utilization on the pharmaceutical and cosmetics applications, for safety reasons [5]. According to Pires et al. [9], the replacement of the yeast extract by whey protein and hydrolyzed soy protein concentrate, resulted in a production of hyaluronic acid of 0.1 $g.L^{-1}$ and 0.17 $g.L^{-1}$, respectively. Lai et al. [25] evaluated the polymer production by S. zooepidemicus ATCC 39920 in a medium containing yeast extract, tryptone, $(NH_4)_2S_2O_8$ and $(NH_4)_2PO_4$ and obtained the highest production when yeast extract and tryptone were used

in association. In addition, Vázquez and co-workers [13] studied the hyaluronic acid production by S. zooepidemicus ATCC 35246 in medium containing mussel processing wastewater and tuna peptone viscera and the results showing that the manufacturing costs were reduced by more than 50%. Taken together, these studies and the results presented here clearly showed the possibility of utilization of renewable sources for the production of hyaluronic acid. Considering the organic acids production, the nitrogen source was a factor that influences in the microbial metabolism. According to Table 3, the main metabolic products in the evaluated conditions were lactate. The acetate synthesis was detected only in the medium containing yeast extract. In medium containing glucose and yeast extract, 58.0% of the carbon from glucose was recovered as metabolites, being 45.0% as lactate, 2.0% as formate and 11.0% as acetate. Farther, in medium with sucrose and yeast extract, 64.0% of the carbon from sucrose was recovered as metabolites, being 52.0% as lactate and 12.0% as acetate. The production of acetate contributed for the increasing at the hyaluronic acid production by the synthesis of ATP necessary for the polymer production [21]. The formate synthesis was also detected in whey protein medium.

Table 3. *Performance of the fermentation with the alternatives nitrogen sources by S. zooepidemicus ATCC 39920.*

Nitrogen Source	Hyaluronic acid ($g.L^{-1}$)	$Y_{p/s}$ ($g.g^{-1}$)	Productivity ($g.L^{-1}.h^{-1}$)	Lactate ($g.L^{-1}$)	Formate ($g.L^{-1}$)	Acetate ($g.L^{-1}$)
Carbon Source: Glucose						
Yeast Extract	0.534 ± 0.012^{a}	0.053 ± 0.005^{a}	0.022 ± 0.000^{a}	4.580 ± 0.165^{a}	0.369 ± 0.063^{a}	1.074 ± 0.061^{a}
Corn Steep Liquor	0.000 ± 0.000^{c}	0.000 ± 0.000^{c}	0.000 ± 0.000^{c}	0.011 ± 0.012^{c}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}
Soy Protein	0.192 ± 0.047^{b}	0.030 ± 0.007^{b}	0.008 ± 0.002^{b}	4.099 ± 1.212^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}
Whey Protein	0.062 ± 0.003^{c}	0.005 ± 0.001^{c}	0.003 ± 0.000^{c}	1.764 ± 0.271^{b}	0.369 ± 0.044^{a}	0.000 ± 0.000^{b}
Carbon Source: Sucrose						
Yeast Extract	0.592 ± 0.034^{a}	0.061 ± 0.012^{a}	0.025 ± 0.001^{a}	4.533 ± 0.357^{a}	0.000 ± 0.000^{b}	1.085 ± 0.071^{a}
Corn Steep Liquor	0.000 ± 0.000^{d}	0.000 ± 0.000^{b}	0.000 ± 0.000^{d}	0.000 ± 0.000^{c}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}
Soy Protein	0.219 ± 0.049^{b}	0.042 ± 0.010^{a}	0.009 ± 0.002^{b}	3.834 ± 0.615^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}
Whey Protein	0.120 ± 0.011^{c}	0.015 ± 0.001^{b}	0.005 ± 0.000^{c}	1.594 ± 0.205^{b}	0.212 ± 0.008^{a}	0.000 ± 0.000^{b}

$Y_{p/S}$ – Hyaluronic acid production per sugar consumption. Different lower-case letters indicate statistical difference at α=0.05 level in each column. Values with the same letters in the same parameters indicate that the values did not differ by Tukey test at 0.95 confidence interval (a>b>c>d).

4 Conclusion

Nowadays, there has been an increasing trend towards efficient use of agroindustrial byproducts for biomolecules production. However, the searching for alternatives carbon and protein sources for the production of hyaluronic acid is still little explored. This study brought the real possibility of the use of sugarcane molasses and soy protein as substrates for the microbial hyaluronic acid production. According to the compositions of the medium, the bacteria may lead carbon flux to the synthesis of organic acids, which would reduce the polymer synthesis. Thus, studies of carbon flux contribute for bacterial metabolism understanding, aiming at increasing hyaluronic acid production.

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