

Research Paper

Cashew apple juice as microbial cultivation medium for non-immunogenic hyaluronic acid production

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Abstract

In this work, natural cashew apple juice was used as cultivation medium as an alternative to substitute brain heart infusion medium. The effect of aeration and juice supplementation with yeast extract on the production of hyaluronic acid in batch fermentation was also investigated. Similar levels of cell mass were obtained in inoculum using cashew apple juice supplemented with yeast extract or the conventional brain heart infusion medium. Fermentation in Erlenmeyer flasks produced low biomass and hyaluronic acid concentrations. The hyaluronic acid concentration and viscosity increased from 0.15 g/L and 3.87 cP (no aeration or medium supplementation) to 1.76 g/L and 107 cP, when aeration (2 vvm) and 60 g/L of yeast extract were used. The results suggest the production of low-molecular weight hyaluronic acid oligomers instead of the high molecular weight polymer.

Key words: hyaluronic acid, cashew apple juice, submerged fermentation.

Introduction

Hyaluronic acid (HA), also commercially referred to as *hyaluronan*, is a linear high molecular weight glycosaminoglycan polysaccharide, composed by D-glucuronic acid and N-acetylglucosamine, which exists in many mammalian connective tissues such as joints, vitreous bodies, umbilical cords, cartilages, skins, and combs of fowls as a constituent (Yamada and Kawasaki, 2005). In rooster comb, for instance, HA is complexed with proteoglycans and often contaminated with HA degrading enzymes, making the isolation of high purity and high molecular weight HA very difficult and costly (Blank *et al.*, 2005). Furthermore, the risk of cross-species viral and other infection agent has been pointed out when using animal-derived biochemicals for human therapeutics (Blank *et al.*, 2005; Yamada and Kawasaki, 2005).

As an alternative, HA also can be produced by gram-positive bacterium *Streptococcus equi* subspecies *zooepidemicus*, which is generally the strain used in industry since it synthesizes HA as an extracellular capsule (Patil *et al.*, 2011). Microbial HA is non-immunogenic and chemi-

cally identical to mammalian polysaccharide. Consequently it is regarded as a viable substitute for the HA currently derived from other sources (Chong and Nielsen, 2003). The microbial HA production is becoming more and more interesting due to the possibility of process control and optimization, therefore, achieving higher product yields. In addition, the main advantage of the fermentative over traditional process, umbilical cords extraction, for instance, is the attainment of a product free of antigens. Other advantages include less laborious purification steps and achievement of a high molecular mass HA (Swann *et al.*, 1990; Ellwood *et al.*, 1996).

Streptococci strains used in HA production are fastidious with respect to their nutrient and organic nitrogen requirements (Hofvendahl and Hahn-Hägerdal, 2000; Fitzpatrick and Keeffe, 2001). Natural resources have been recently studied aiming to obtain low-cost cultivation media and a better purity grade of HA. Pires *et al.* (2010) evaluated different agricultural resource derivatives (ARDs), such as hydrolysate soy protein concentrate, whey protein concentrate, and cashew apple juice aiming at the production of HA by *Streptococcus zooepidemicus*. Corn steep li-

quor was also evaluated as a supplement. Among the studied ARDs, only cashew apple juice supplemented with yeast extract (CAJY) showed to be a promising medium due to the presence of complex nutrients, such as B-vitamins, particularly thiamine, riboflavin, niacin and folic acid (Sancho *et al.*, 2007). HA produced in CAJY presented an average molecular weight of 104 Da, which finds applications in biomedical and healthcare fields (Pires *et al.*, 2010). Likewise submerged fermentation of the cashew apple juice, the production of HA by solid-state fermentation of the juice-moisturized cashew apple bagasse was found to be significant (Macedo and Santana, 2012). The authors achieved a yield of 761.25 mg HA per kilo of total cashew apple fruit, which shows that HA production from the total cashew apple pseudo-fruit is promising and economically viable. Additionally, cashew is produced in 32 countries in the world and Brazil is one of the major cashew apple-producing countries. The production figures for the year of 2010, based on the Food and Agriculture Organization, are 104,342 tons (FAO, 2010). However, only 18% of the total peduncle is exploited for obtaining various products, from concentrated juice to desserts, and 80% of the pulp is wasted (Oliveira *et al.*, 2013).

Likewise the nutrient requirements, oxygen transfer also plays an important role on the HA production by submerged cultivation. In the presence of oxygen, *S. zooepidemicus* grows faster and has a higher biomass yield. Furthermore, HA yield and molecular weight are higher when compared to anaerobic cultivation (Chong and Nielsen, 2003). Under aerobic conditions, glucose uptake and growth rates are increased and, as expected; more acetate was formed with little or no ethanol and formate production. This altered metabolism enhances HA productivity and molecular weight (Johns *et al.*, 1994; Nielsen, 2005; Ogrodowski *et al.*, 2005).

Considering the above mentioned aspects, in this work, cashew apple juice, here named CAJ, was used as culture medium for HA production using *S. zooepidemicus*. Since the biopolymer can be produced anaerobically or aerobically, the effect of aeration on biomass production and HA concentration was investigated. Furthermore, the effect of medium supplementation with yeast extract as a substitute for the conventional medium Brain and Heart infusion (BHI) on the final HA concentration was also studied.

Material and Methods

Cashew apple juice (CAJ) preparation

Cashew apple juice (CAJ) was used as medium for inoculum preparation and batch fermentation for HA production due to its rich composition (Rocha *et al.*, 2007) that includes 10% of reducing sugars (glucose and fructose), phosphorous and amino acids. The juice was withdrawn by compressing the cashew apple (*Anacardium occidentale L.*). Afterwards, pH was adjusted to 7.0 and the medium

was submitted to ultraviolet exposition for 2 h for sterilization in order to avoid loss of labile components by heat.

Microorganism and inoculum preparation

Streptococcus zooepidemicus (ATCC 39920) was obtained from the American Type Culture Collection as a lyophilized culture in ampoules and it was maintained on Trypticase Soy Agar (TSA) at 5 °C. The inoculum was incubated at 37 °C on a rotary shaker at 150 rpm for 48 h in a 500 mL Erlenmeyer flask containing 200 mL of medium. Natural cashew apple juice (CAJ), CAJ supplemented with 37.5 g/L of BHI and CAJ supplemented with 60 g/L of yeast extract, here named CAJY, were used for inoculum preparation. Conventional BHI medium was used as control. The cells were harvested by centrifugation and were used latter to inoculate the fermentation broth.

Medium and fermentation conditions

Cashew apple juice and CAJ supplemented with 60 g/L of yeast extract were used in batch fermentations. The inoculum concentration was 10% v/v and the fermentation was conducted at 37 °C and 150 rpm. First, assays were carried out in 500 mL Erlenmeyers flasks containing 200 mL medium. After, to investigate the effect of aeration, a 3 L BioFLO III fermenter (New Brunswick Scientific Co. Inc., Edison, NJ, USA) with an operating volume of 2.0 L and 2 vvm aeration was used. The culture pH in the fermenter was controlled at 7.0 by automatic addition of sodium hydroxide solution.

Hyaluronic acid purification

The separation and purification of HA was performed by precipitation with ethanol according to Brown *et al.* (1988). Cells were removed from culture broth by centrifugation and the supernatant was treated with ethanol in a proportion 3:2 (v/v) ethanol:supernatant. The solution remained at rest for the precipitation of HA. The precipitate was recovered by centrifugation at 3000 rpm for 20 min and redissolved in NaCl 0.15 M. In order to increase the yield of HA, three steps of precipitation and redissolution were performed (Pires *et al.*, 2010).

Analytical methods

Biomass concentration was monitored by measuring the dry weigh (g/L). Samples of culture medium (approx. 100 mL) were centrifuged at 3000 rpm for 20 min. The harvested cells were then dried to constant weight at 60 °C to determine the dry weight content of the medium. Relatively higher values of dry weight measurements might be obtained due to interference from the presence of capsular HA, which might not be removed before analysis. This interference is more pronounced with aerated cultures, since cells under such conditions tend to possess very large capsules (Cooney *et al.*, 1999). The total reducing sugars concentration was determined according to Somogy-Nelson

method (Somogyi, 1952). Hyaluronic acid concentration in the culture broth, measured as sodium hyaluronate, was determined by high performance liquid chromatography (Johns *et al.*, 1994), using a refractive index detector (Waters 410). Filtered broth samples were injected into a HPLC equipped with OHPak SB-806 HQ column, working at 40 °C. The mobile phase consisted of 10 mM NaNO₃ solution pumped at a flow rate of 1 mL/min. The standard curve was prepared using sodium hyaluronate (Hylumed@ - Genzyme Corporation). The viscosity of the produced sodium hyaluronate was determined using a Haake rheometer CV20 with parallel plate modules. All measurements were performed in triplicate and results represent the means.

Yield and Kinetic parameters

The yields in product ($Y_{P/S}$) and cells ($Y_{X/S}$) related to substrate, and the specific rates for growth, μ_x , and product formation μ_p , were calculated from the measured concentrations of product, substrate and cells. HA productivity (P_p) was calculated as the ratio of maximum product concentration P_m to the fermentation time, see Eq.1, and biomass productivity (P_x) was calculated as the ratio of ($X_m - X_0$) to the fermentation time, see Eq. 2

$$P_p = \frac{P_m}{t_{fm}} \tag{1}$$

$$P_x = \frac{X_m - X_0}{t_f} \tag{2}$$

where X_0 is initial biomass concentration, X_m is the maximum biomass concentration after a fermentation time t_f . P_m is the maximum product concentration after a fermentation time t_{fm} .

Results and Discussion

Effect of culture medium on cell growth and inoculum preparation

Optimum culture conditions for HA production in batch mode is well described in the literature (Armstrong and Kim *et al.*, 1996; Johns, 1997; Stangohl, 2000; Chong *et al.*, 2005). Blood or serum is usually described as essential for colony growth and development in the strains of group C *Streptococcus* (Chong *et al.*, 2005). However, there is a potential risk for blood contamination (Zhang *et al.*, 2006). Therefore, in this work a different serum-free media for cell growth was studied and results were compared to a medium prepared using BHI (control).

Figure 1 shows the effect of culture medium on *S. zooepidemicus* growth during inoculum preparation. It can be observed that every medium induced colony growth and development, but low cell concentration (7.8 g/L) was obtained when pure CAJ was used. Enriching CAJ with 37 g/L BHI or using CAJY medium yielded similar bio-

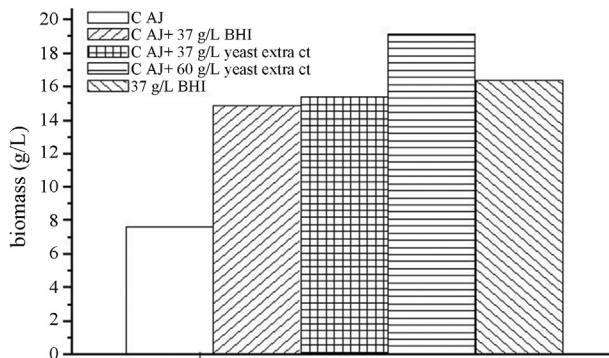


Figure 1 - Comparative performance of culture media for *S. zooepidemicus* growth: Crude cashew apple juice, CAJ, crude cashew apple juice, CAJ with 37 g/L or 60 g/L yeast extract, CAJ with 37 g/L Brain Heart Infusion, BHI and BHI medium 37 g/L.

mass concentrations. The highest cell concentration was produced in CAJY as compared to the other media, including the control.

Lancefield group A and C *streptococci* bacteria require complex nutrients due to their limited ability to synthesize specific amino acids and B-vitamins (Hofvendahl and Hahn-Hägerdal, 2000; Fitzpatrick and Keffe, 2001). Additionally to this fact, there is the nutritional requirement with respect to organic nitrogen, which also supplies a large proportion of carbon for the cellular biosynthesis (Armstrong and Johns, 1997). As reported in the literature, CAJ has low total nitrogen content (0.04% w/v) and requires an additional nitrogen supplementation to reach an ideal glucose/nitrogen ratio (Pires *et al.*, 2010). When yeast extract was used to enrich CAJ (CAJY), a favorable cultivation condition was established by the presence of purine and pyrimidine bases and the B-vitamins, which are essential to the *streptococci* cultivation. The CAJ also contains B-vitamins, mainly thiamin, riboflavin, niacin and folic acid, which are beneficial to the *streptococci* cultivation (Amrane and Prigent, 1994; Sancho *et al.*, 2010).

The yeast extract that was added to CAJ was able to create a suitable condition for *Streptococcus zooepidemicus* growth. These results demonstrate that the conventional BHI medium can be advantageously substituted by CAJY medium for inoculum preparation.

Influence of medium supplementation with yeast extract and aeration on hyaluronic acid production

The kinetic profiles in Figure 2 (A and B) describe the average behavior of fermentations carried out in Erlenmeyer flasks with CAJ and CAJY media. When CAJ medium was used, sugars were starved from medium in 16 h remaining at a low level (approximately 0.05 g/L), Figure 2A. Similar but faster (4 h) uptake of sugars was observed on CAJY medium. Analyzing the data, it can be observed that the presence of essential nutrients in CAJY increased cell growth and HA production, when compared to the CAJ. Biomass and HA production were 3-fold higher

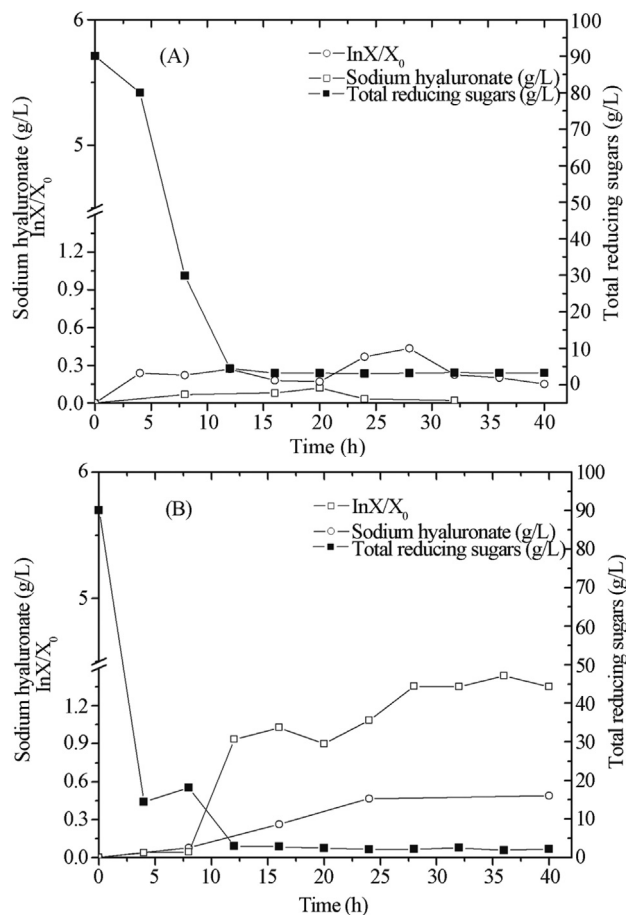


Figure 2 - Kinetic profiles of biomass ($\ln x/x_0$), reducing sugars and hyaluronic acid for fermentation using *S. zooepidemicus* and natural aeration (Erlenmeyer flasks) (A) crude cashew apple juice, CAJ (B) crude cashew apple juice enriched with 60 g/L yeast extract, CAJY.

on CAJY medium (Figure 2B). A diauxic behavior could also be observed, in CAJ medium, when a second exponential phase appeared after 20 h, with a concomitant decrease in HA concentration (Figure 2A).

The pH variation during *S. zooepidemicus* growth in CAJ and CAJY medium (Figure 3) was also compared. It can be observed that at the beginning of the assay, pH decreased in both CAJ and CAJY medium. From 5 to 15 h, while pH of CAJ continued to decrease, pH of CAJY remained almost constant. After 15 h, the pH of CAJ started to increase, while pH of CAJY decreased again. According to the literature (Armstrong and Johns, 1997), lactic acid is the main metabolite arising from glucose catabolism in *S. zooepidemicus*, which explains the decrease in pH values observed for both mediums. The fact that pH values decrease earlier in CAJ medium compared to CAJY may suggest that more lactic acid is produced in CAJ medium. This decreased synthesis of lactic acid in CAJY favored strain growth, as observed by other authors (Zhang *et al.*, 2006) when studying serum-free medium for colony growth and hyaluronic acid production by *Streptococcus zooepi-*

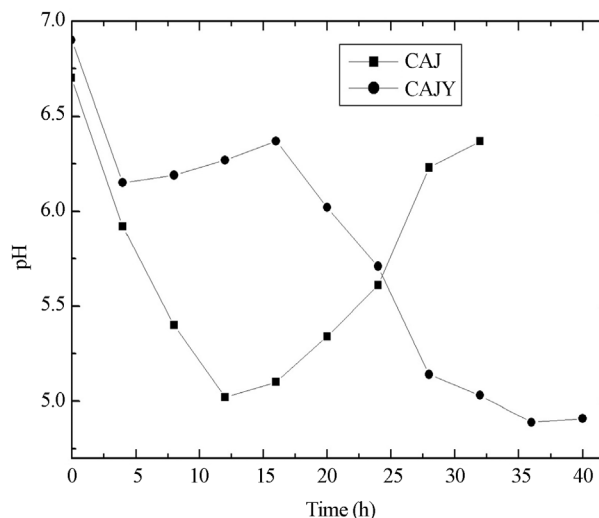


Figure 3 - Variation of pH during fermentation of CAJ and CAJY using *S. zooepidemicus* and natural aeration (Erlenmeyer flasks).

demicus NJUST01. It is known from the literature (Liu *et al.*, 2011) that lactic acid accumulation causes a strong inhibition of cell growth and HA.

Based on cell growth, HA production and pH values observed for both mediums, CAJ supplementation with yeast extract was favorable for HA synthesis under natural aeration. Nevertheless, it is well known that microbial production of HA is significantly influenced by culture conditions, such as aeration rate, dissolved oxygen concentration and bioreactor type (Swann *et al.*, 1990; Nielsen, 2005; Liu *et al.*, 2011). Therefore, the effect of aeration and medium supplementation on HA production were investigated in a 3 L BioFLO III fermenter with an operating volume of 2.0 L at 150 rpm, 37 °C and 2 vvm aeration. Figure 4 shows HA production, cell growth and substrate uptake under aerobic conditions. Table 1 compares the influence of aeration and juice supplementation on sodium hyaluronate production. It can be observed that the juice supplementation under aerobic conditions was not beneficial since HA concentration decreased from 1.88 g/L, when pure juice was used (Figure 4 A), to almost 1.76 g/L, when yeast extract was added to the juice (Figure 4 B). Aeration plays a more pronounced effect on sodium hyaluronate production, since the sodium hyaluronate concentration increased from 0.12 g/L to 1.88 g/L in CAJ. The higher HA concentrations obtained under aerobic conditions is probably caused by a large energetic yield, since, in the presence of oxygen, there is a deviation from pyruvate to acetate rather than to lactate (Swann *et al.*, 1990). In other words, in the presence of dissolved oxygen, the carbon flux towards lactic acid to acetic acid is redirected and more ATP is generated. This extra ATP is used to achieve higher HA titer (Liu *et al.*, 2011). Another explanation for the effects of aeration is that HA is produced in the microbial surroundings to protect themselves against the toxic effects provided by oxygen, espe-

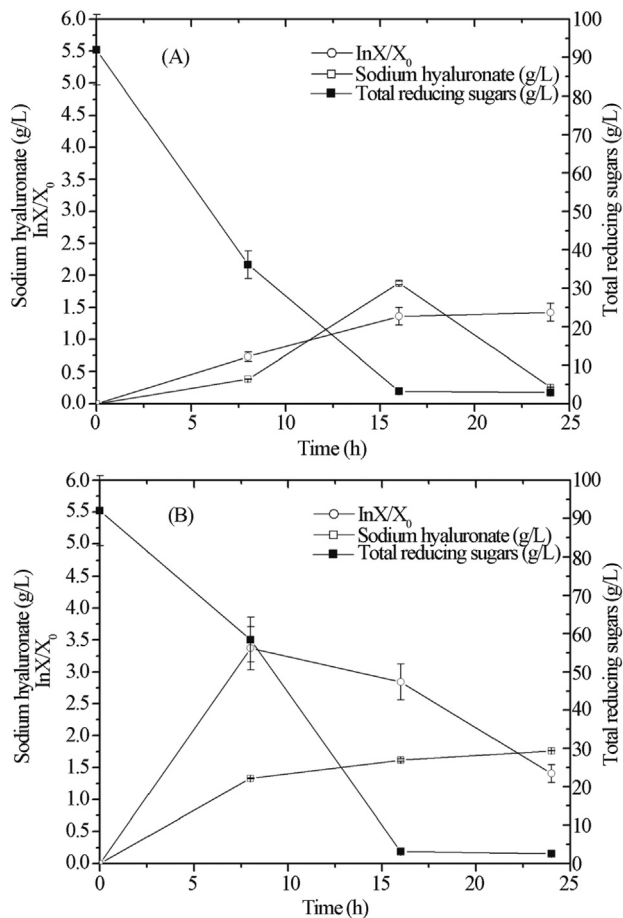


Figure 4 - Kinetic profiles of biomass ($\ln x/x_0$), reducing sugars and hyaluronic acid for fermentation using *S. zooepidemicus* with 2 vvm aeration using: (A) crude cashew apple juice, (B) CAJ crude cashew apple juice enriched with 60 g/L yeast extract, CAJY. All assays were performed in duplicate and results represent the means \pm standard deviation.

cially those from the univalent reduction of molecular oxygen and hydroxyl free radicals conditions (Swann *et al.*, 1990; Ellwood *et al.*, 1996; Nielsen, 2005).

In addition, the presence of glucose and fructose as main sugars in CAJ also may have an important role on the HA synthesis due to the metabolic route of glucose conversion to HA. Under limiting aeration (Erlenmeyer flasks), the biosynthesis needs were provided by the complex nitrogen source added to the medium (Pires and Santana, 2010), which explains the 4-fold enhancement in AH concentration when yeast extract was added to CAJ (Table 1). Nevertheless, under forced aeration (BioFLO III fermenter),

oxygen was available to complete sugar oxidative metabolic processes and, therefore, medium supplementation with yeast extract did not promote an increase in HA concentration.

Figures 5 and 6 show the effects of aeration and juice supplementation on the specific rates of cell growth and product formation along fermentation. In aerated conditions, the HA production rate is associated to the cell growth rate until 8 h fermentation. Afterwards, HA and cells compete for energy and metabolic intermediates, and in the supplemented medium, the HA production rate increases at the expenses of decline of cell growth rate. Indeed, it is reported in the literature that glycolysis and HA synthesis compete for the carbon flux (Liu *et al.*, 2011). In other words, a reduction in the rate of biomass formation is effective for the enhancement of HA production, as observed in this work.

Table 2 resumes the kinetic parameters estimated from the experimental data showed on Figures 2 and 3. Under anaerobic conditions, $Y_{P/S}$, P_P and μ_{Pmax} were higher on CAJ, while P_X and μ_{Xmax} were higher on CAJY. Under aerobic conditions, maximum specific growth rate (μ_{Xmax}) was much lower on CAJ (0.09 h^{-1}) as compared with CAJY (0.42 h^{-1}). The slower growth rate in CAJ might be explained by the mechanism of amino acid uptake. Certain amino acids compete for the same transport system. The presence of peptide in the yeast extract, used to compose CAJY, allows alternative routes for amino acid uptake (Chong *et al.*, 2005). Furthermore, peptides transport can be more efficient that the transport of individual amino acids (Guirard and Snell, 1962). Maximum specific HA production rate (μ_{Pmax}) was also lower on CAJ (0.007 h^{-1}) as compared to CAJY (0.009 h^{-1}), under aerobic conditions.

Maximum specific growth rate (μ_{Xmax}) CAJY was lower without aeration (0.06 h^{-1}) as compared to the assay when 2 vvm of aeration (0.42 h^{-1}) was used, keeping all other conditions constant. The same behavior is also observed for maximum specific HA production rate, see Table 2. It can also be observed that higher HA ($Y_{P/S}$) and growth yields ($Y_{X/S}$), HA (P_P) and biomass productivities (P_X) were obtained under aeration and using CAJY. Similar results were obtained by different authors (Kim *et al.*, 1996; Armstrong and Johns, 1997; Chong *et al.*, 2005) when using a chemically defined media. As mentioned before, higher HA production is achieved in an aerobic culture when compared with an anaerobic culture, which may be

Table 1 - Comparative performance of culture media for sodium hyaluronate production without aeration or with 2 vvm aeration: crude cashew apple juice, CAJ, crude cashew apple juice enriched with 60 g/L yeast extract, CAJY.

Fermentation media	Sodium hyaluronate concentration (g/L)	
	Without aeration	With 2 vvm aeration
Cashew Apple Juice (CAJ)	0.12	1.88 ± 0.03
Cashew Apple Juice supplemented with yeast extract (CAJY)	0.49	1.76 ± 0.01

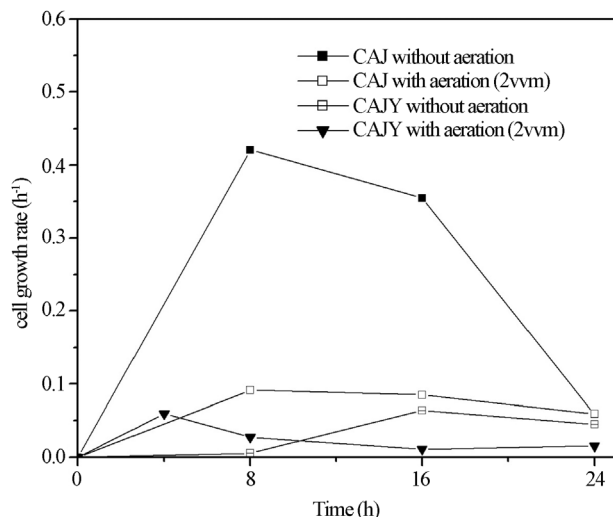


Figure 5 - Specific rate of cell growth of *S. zooepidemicus* without aeration or with 2 vvm aeration using: crude cashew apple juice (CAJ) or cashew apple juice enriched with 60 g/L yeast extract (CAJY).

explained by: (i) redirection of the carbon flux towards lactic acid to acetic acid in the presence of oxygen, which provides more ATP; (ii) oxygen may promote the synthesis of AH since cells aggregation may protect them from oxygen metabolites; (iii) acetyl-CoA accumulation may be enhanced by aeration, which can be deviated from the carbon metabolism in order to supply the synthesis of AH (Liu *et al.*, 2011).

Rheological characterization

The viscous behavior of the product obtained by fermentation using CAJY medium in Erlenmeyers flasks or in a 3 L BioFLO III fermenter (with aeration) was investigated. It was observed that culture aeration (viscosity varied from 3.87 cP without aeration to 107.00 cP with 2 vvm aeration), together with supplementation of medium with yeast extract (viscosity varied from 73.27 cP to 107.00 cP), caused a positive influence on the HA viscosity. The viscous behavior indicates a low molecular weight HA, less than 500 kDa, according to the rheological parameters obtained by other authors (Prieto *et al.*, 2005) for similar concentration of commercial HA. Although precursor compounds were produced, the HA was not polymerized. The production of HA by *S. zooepidemicus* is due to con-

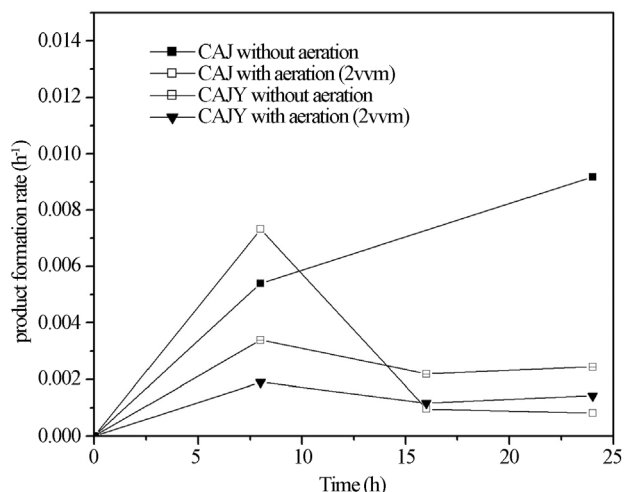


Figure 6 - Specific rate of product formation for fermentation using *S. zooepidemicus* without aeration or with 2 vvm aeration using: crude cashew apple juice (CAJ) or cashew apple juice enriched with 60 g/L yeast extract (CAJY).

version of glucose and fructose-6-phosphate in the precursors glucuronic acid and N-acetylglucosamine. The biopolymer is formed by reaction of the precursors mediated by a hyaluronidase synthase (HAS). An abortive translocation model proposed by Forsee *et al.* (2000) for polymerization of type 3 capsule in *Streptococcus pneumoniae* by a glycosyltransferase similar to HAS says that in the absence, or at low concentrations, of one of the substrates, while at normal levels of the other substrate, membrane-bound polymer chains were released into solution. Although this mechanism has not been observed directly for HA polymerization, it seems to explain the data obtained using CAJY as a fermentation medium. The absence of extended polymerization could be due to the presence of some inhibitions in CAJY broth, which unbalanced the HA precursors produced. The absence of the required balance produced low molecular weight HA.

A possible synergic redox-depolymerization mechanism provides by ascorbate presence in cashew apple juice possibly causes HA molecular weight reduction. oltés *et al.* (2006) and Liu *et al.* (2009), investigated the reduction of HA molecular weight due to depolymerization, by adding to a high molecular weight HA solution, or hydrogen peroxide and ascorbate along submerged fermentations with *S.*

Table 2 - Parameters obtained for fermentation using *S. zooepidemicus*, crude cashew apple juice (CAJ) or crude cashew apple juice enriched with 60 g/L yeast extract (CAJY), without aeration or under 2 vvm aeration.

Medium/parameter	Y_{PS}	Y_{XS}	P_P (g.L ⁻¹ .h ⁻¹)	P_x (g.L ⁻¹ .h ⁻¹)	μ_{Xmax} (h ⁻¹)	μ_{Pmax} (h ⁻¹)
CAJ (*)	0.001	0.01	0.008	0.03	0.01	0.002
CAJY (*)	0.006	0.01	0.019	0.24	0.06	0.002
CAJ (**)	0.021	0.07	0.118	0.57	0.09	0.06
CAJY (**)	0.039	0.38	0.176	3.72	0.42	0.06

* Without aeration. ** with 2 vvm aeration.

zooepidemicus in synthetic medium. The authors described the basic mechanism for redox depolymerization, involving the appearance of reactive oxygen species (ROS) that are capable of breaking the HA into smaller molecules.

Conclusion

The results obtained at this work show that cashew apple juice is a suitable substrate for the growth of *Streptococcus zooepidemicus* and the production of oligomers of HA. Furthermore, cashew apple juice supplemented with yeast extract is a proper substitute for the conventional medium BHI used for inoculum propagation, which is expensive and has a risk for contamination. It was observed that the composition of the culture medium as well as oxygen supply influenced the microbial production of HA (concentration and viscosity), highest concentrations were obtained when the fermentation was carried out under aeration. The hyaluronic acid viscosity increased when cashew apple juice was supplemented with 60 g/L of yeast extract. The potentialities of CAJY medium are evident, with the advantages that BHI did not need to be added, resulting in a fermentation medium without risk of cross-species viral and other infection agent. These results point out to a straight and safe process to produce HA oligomers suitable to important medical applications as a high value-added product. Nowadays, most of methods for production of HA oligomers involve enzymatic digestion or sonication of polymeric HA followed by purification of fractions.

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