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Metabolic Effects of the Initial Glucose Concentration on Microbial Production of Hyaluronic Acid

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Abstract The objective of the present work was to evaluate the metabolic effects induced by the initial glucose concentration (IGC) on the cultivation of *Streptococcus zooepidemicus* for the production of hyaluronic acid (HA). These effects were monitored along non-controlled pH cultivations, carried out in 250-mL Erlenmeyer flasks (natural aeration) and in a 3-L bioreactor (forced aeration) as well. Effects of the IGC were observed with focus on the main metabolites, cell growth, production, and average molecular weight of HA. The absence of glucose resulted in a mixed acid metabolism independent of the oxygen supply, while, for IGCs ranging from 5 to 90 gL^{-1}, the homolactic metabolism was prevalent. The IGC had no influence on the amounts of either biomass or HA produced in the cultivations carried out in flasks; however, cultivations in 3-L bioreactor were found to be strongly dependent on it. The highest concentration of HA (1.21 gL^{-1}) was obtained from 25 gL^{-1} IGC, the only cultivation where the conversion of glucose to HA was higher than the one of glucose to biomass. Average molecular weight of HA increased concomitant with the IGC, independently of aeration; nevertheless, it decreased along cultivation under forced aeration, due to the shear imparted by stirring.

Keywords Hyaluronic acid · Initial glucose concentration · Metabolites · *Streptococcus zooepidemicus*

Introduction

Hyaluronic acid (HA) is an unbranched polysaccharide with high molecular weight, composed of D-glucuronic acid and *N*-acetylglucosamine disaccharide units, linked alternately by β-1-3 and β-1-4 glycoside bonds [1]. Owing to its unique hydrodynamic properties, HA has been widely applied in the cosmetic and medical fields [2]. Final

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products containing HA present high aggregated values ranging from US$ 2,000 to 60,000 kg^{-1} when considering the above-mentioned applications.

Prokaryotes as group A and C streptococci synthesize HA as an extracellular capsule [3]. Streptococci are Gram-positive bacteria whose main fermentation product from carbohydrates is lactate [lactic acid bacteria (LAB)]. The homolactic metabolism accounts for the conversion, more than 90%, of sugars into lactate, while in the mixed acid metabolism, high amounts of formate (in an anaerobic environment), acetate, and ethanol are produced [4]. Both metabolisms are depicted in Fig. 1.

Common to all microbial syntheses of oligosaccharides and polysaccharides, the production of HA is a carbon-intensive and energy-intensive process [5]. Precursors for the HA synthesis (Uridine diphosphate–glucuronic acid and Uridine diphosphate–*N*-acetylglucosamine) are also precursors for the cell wall biosynthesis, specifically peptidoglycan, teichoic acids, and antigenic wall polysaccharides. Therefore, the HA synthesis competes with the cell growth for carbon source and energy [6, 7].

Bacteria may alter cell metabolism and consequently, the direction of carbon flux in response to environmental fluctuations. Thence, the analysis of the metabolic changes represents a valuable tool, which potentially contributes to the understanding of how to manipulate the culture conditions of an organism, in order to improve the production of interesting substances [8]. Since the 1990s, a myriad of metabolic studies have focused on the effects which nutrition and culture conditions exert upon the production of HA by *Streptococcus zooepidemicus*, such as carbon source, carbon-to-nitrogen ratio, initial glucose concentration, pH, agitation, aeration, and temperature [7, 9–19].

The only studies which address the effect of initial glucose concentration (IGC) on the production of HA were performed under controlled pH cultivations. Armstrong and Johns [14]

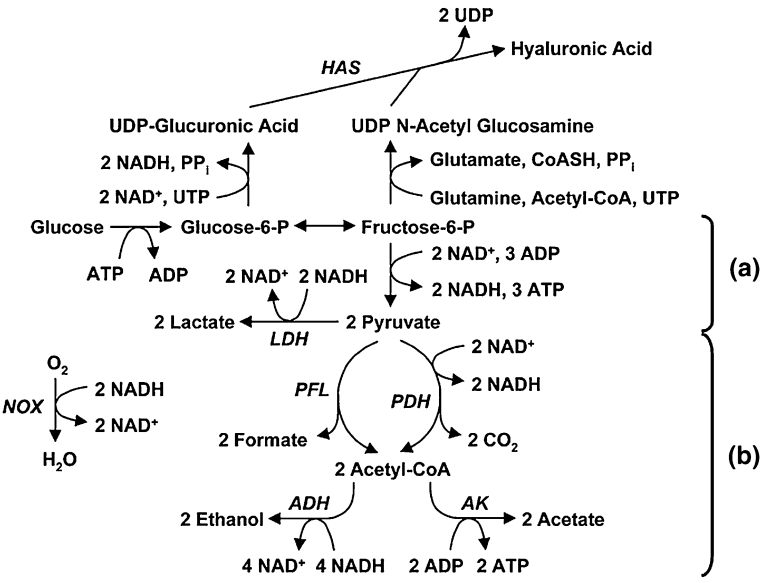


Fig. 1 Central metabolic pathways in *S. zooepidemicus* involved in the conversion of glucose to hyaluronic acid and fermentation products through the **a** homolactic and **b** mixed acid metabolisms. *HAS* hyaluronate synthase, *NOX* NADH oxidase, *LDH* lactate dehydrogenase, *PFL* pyruvate formate lyase, *PDH* pyruvate dehydrogenase, *ADH* alcohol dehydrogenase, *AK* acetate kinase (adapted from Chong and Nielsen, 2003 [13])

investigated this effect on the molecular weight of HA produced by *S. zooepidemicus* ATCC 35246; Chen et al. [15] explored the specific productivity of HA by *S. zooepidemicus* ATCC 39920. However, studies on this topic are scarce; besides, none of them examined the effects of IGC either on the metabolite quantification or metabolism shifting.

Concerning the significant role of metabolic studies on the improvement of HA production, we have extended the previous findings by systematically investigating the effects of IGC on the cultivation of *S. zooepidemicus* ATCC 39920 under forced or natural aeration. In both cases, the experiments were carried out at non-controlled pH, in order to understand the natural behavior of the cultivation. The metabolic effects were associated with the main metabolites involved in cell growth, production, and average molecular weight of HA.

Materials and Methods

Microorganism

Streptococcus equi subsp. *zooepidemicus* ATCC 39920 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) as a lyophilized culture kept in ampules.

Culture Medium

The synthetic medium contained 60 gL^{-1} yeast extract and salts according to the composition reported by Swann et al. [16]. The pH was adjusted to 7.5 prior to sterilization. A 20% (*w*/*v*) glucose solution was autoclaved separately and added to the medium to obtain IGCs in the range of 0 and 90 gL^{-1}.

Culture Maintenance and Inoculum Preparation

The stock culture was kept frozen in brain heart infusion (BHI) broth containing 10% glycerol and glass beads at −20 °C. The pre-inoculum was prepared by streaking the glass beads onto BHI agar plates supplemented with 5% sheep blood (Biotério Boa Vista, São Paulo, Brazil) and incubated at 37 °C during 24 h. Colonies were then transferred into the culture medium. For the cultivation in shake flasks, 5 mL of inoculum were prepared at 37 °C during 12 h. For the cultivation in bioreactor, the inoculum was prepared with a series of transfers into the volumes of 25 and 250 mL of the same culture medium, both incubated at 37 °C in a reciprocal shaker under 150 rpm during 12 and 6 h, respectively.

Cultivations

Cultivation Under Natural Aeration

All batch cultures were performed in 125-mL Erlenmeyer flasks with a working volume of 50 mL. The media were inoculated with 10% *v*/*v*. The flasks were incubated at 37 °C, under 150 rpm for 24 h. The evaluated IGCs were 0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 gL^{-1}. The cell mass, HA, glucose, lactate, acetate, formate, and ethanol concentrations were determined for each flask at the initial and final times. The average molecular weight of HA produced in the end of the cultivation was also determined.

Cultivation Under Forced Aeration

All batch cultures were performed using a 3 L BioFlo III fermentation system (New Brunswick Scientific Co. Inc., Edison, NJ, USA) with an operating volume of 2.5 L, during 24 h. The media were inoculated with 10% *v*/*v*. Agitation rate and temperature adopted were 250 rpm and 37 °C, respectively. The bioreactor was sparged with a continuous air supply (2 vvm), and the culture pH was not controlled during cultivation. The evaluated IGCs were 0, 5, 25, 45, and 90 gL^{-1}. Samples were withdrawn at intervals not longer than 2 h and their cell mass, HA, glucose, lactate, acetate, formate, and ethanol concentrations were determined. The kinetic of average molecular weight of HA was also determined.

Analytical Methods

Cell Growth

Cell growth was determined by the cell dry weight according to the gravimetric method.

Concentrations of Glucose, Lactate, Acetate, Formate, and Ethanol

Culture samples were filtered through membranes with a pore size of 0.2 μm (Sartorius, Goettingen, Germany), and 20 μL of filtered sample was injected into an ion exchange High Performance Liquid Chromatography (Shimadzu Corporation, Kyoto, Japan) equipped with a 7.8 mm×300 mm HPX-87H fast acid column Aminex (Bio-Rad, Hercules, CA, USA). The mobile phase was composed of 0.004 mol L^{-1} H_2SO_4 solution pumped at a flow rate of 0.6 mL min^{-1} as described by Chong and Nielsen [13]. The column was maintained at 65 °C. The peak elution profile was monitored with a Shimadzu RID-6A refractive index detector (Shimadzu Corporation, Kyoto, Japan).

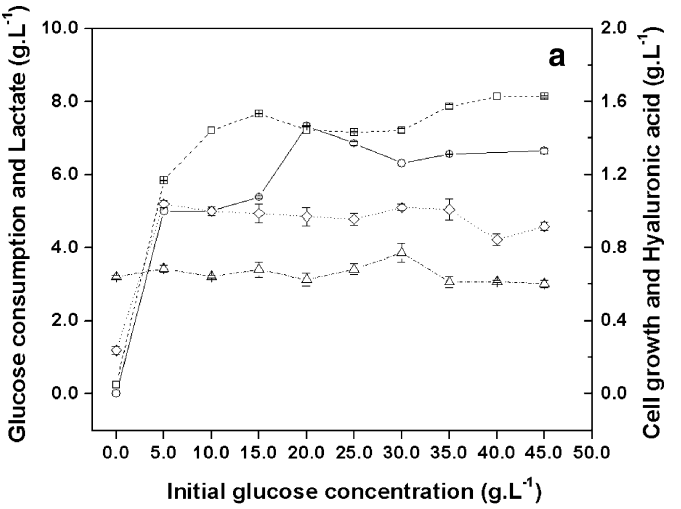
HA Concentration

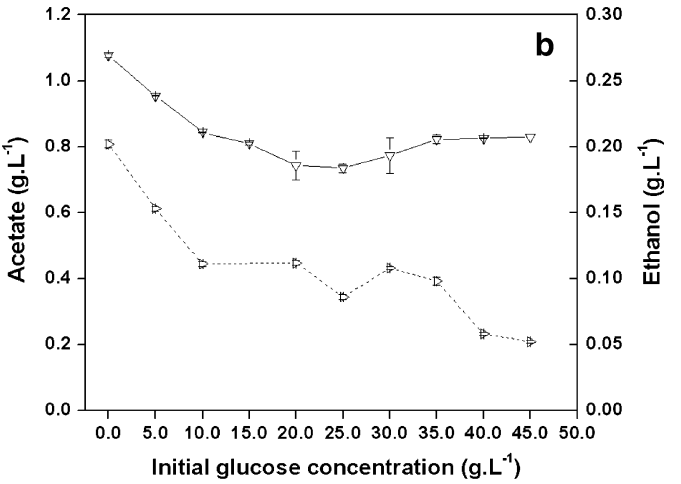
The culture broth was centrifuged at 3,200 rpm during 20 min. The cell-free broth was then treated with ethanol in a proportion 1.5:1 *v*/*v* ethanol/supernatant. The solution was cooled down, remaining at 4 °C during 1 h for the precipitation of HA. Finally, the HA precipitated was redissolved in a 0.15-mol L^{-1} NaCl solution. Three precipitation and redissolution steps were performed to increase the yield of HA precipitated. Its concentration was measured by the carbazole method [20].

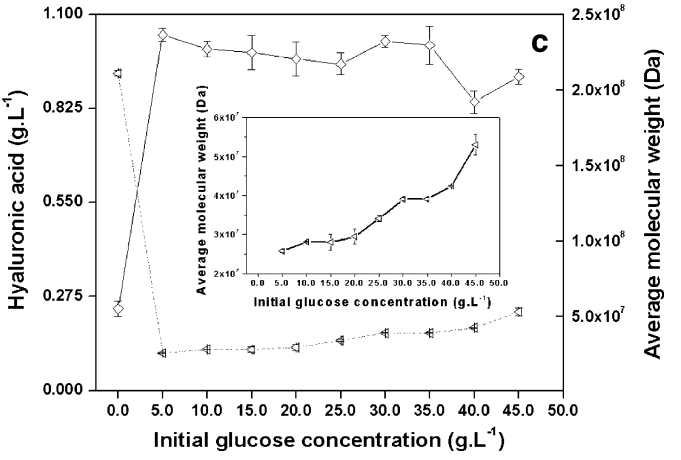
Molecular Weight of HA

The average molecular weight of HA was determined by size exclusion chromatography, using a Shimadzu chromatography system (Shimadzu Corporation, Kyoto, Japan), containing a 7.8 mm×35 mm Polysep–GFC-P column guard (Phenomenex, Torrance, CA., USA) mounted in series with a 7.8 mm×300 mm Polysep–GFC-P6000 column (Phenomenex, Torrance, CA.,

Fig. 2 Effects of the initial glucose concentration on the cultivation of *S. zooepidemicus* in shake flasks: **a** (*open circle*) glucose consumption; (*open square*) lactate; (*open diamond*) hyaluronic acid; (*open triangle*) cell growth; **b** (*open inverted triangle*) acetate; (*open right-pointing triangle*) ethanol; **c** (*open diamond*) hyaluronic acid; (*open left-pointing triangle*) average molecular weight of hyaluronic acid ▶


a
Glucose consumption and Lactate (g.L-1)
Cell growth and Hyaluronic acid (g.L-1)
Initial glucose concentration (g.L-1)
10.0
8.0
6.0
4.0
2.0
0.0
2.0
1.6
1.2
0.8
0.4
0.0
0.0 5.0 10.0 15.0 20.0 25.0 30.0 35.0 40.0 45.0 50.0


b
Acetate (g.L-1)
Ethanol (g.L-1)
Initial glucose concentration (g.L-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.0 5.0 10.0 15.0 20.0 25.0 30.0 35.0 40.0 45.0 50.0


c
Hyaluronic acid (g.L-1)
Average molecular weight (Da)
Initial glucose concentration (g.L-1)
Average molecular weight (Da)
Initial glucose concentration (g.L-1)
1.100
0.825
0.550
0.275
0.000
2.5x10^8
2.0x10^8
1.5x10^8
1.0x10^8
5.0x10^7
0.0 5.0 10.0 15.0 20.0 25.0 30.0 35.0 40.0 45.0 50.0

USA) and a refraction index detector. The analysis conditions were: injected a sample of 20 μL 0.1 mol L^{-1} $NaNO_3$ solution as the mobile phase, 1.0 mL min^{-1} flow rate, and 25°C temperature, as suggested by the column manufacturer. Dextran (American Polymer Standards, Mentor, OH, USA) with molecular weight ranging from 10^3 to 10^6Da was used as a standard for the calibration curve as described by Balke et al. [21].

Results and Discussion

Effects of the IGC on the Cultivation Under Natural Aeration

The IGC effects on the metabolism of *S. zooepidemicus* cultured under natural aeration in shake flasks are presented in Fig. 2. It can be observed that the cell growth was independent of the IGC, even in the absence of glucose (0 gL^{-1} glucose). These results are in accordance with the typical behavior for LAB, whose biosynthesis needs are provided by the complex nitrogen source [6, 17]. Furthermore, the vast majority of the glucose is recovered in the fermentation products, accounting for the low concentration of the lactate (0.25 gL^{-1}) obtained in the glucose-free cultivation (Fig. 2a). As a consequence, a higher production of the acetate (1.08 gL^{-1}) and ethanol (0.20 gL^{-1}) was observed in the absence of glucose as illustrated in the Fig. 2b.

Under glucose limitations, results indicated redirection of the carbon flux. LAB shifted from a homolactic to a mixed acid metabolism in order to make up for the decline in the production of cell energy (ATP) [4]. Whenever this occurs, one additional mole of ATP is generated per mole of glucose, when the pyruvate is converted into acetate instead of lactate as shown in the Fig. 1.

Concomitant with the increasing IGC in the medium, there is a substantial increment of lactate and a reduction in the concentrations of acetate and ethanol. This behavior demonstrates the change to the homofermentative metabolism, which is the most ordinary route for the glucose catabolism in non-limiting conditions [22]. No formate production was observed within the range of IGCs used in the cultivations.

Low production of HA (0.24 gL^{-1}) in the absence of glucose is due to the insufficient concentration of the activated monomers for the hyaluronate synthase [13]. These monomers are produced from the intermediates glucose-6-P e fructose-6-P (Fig. 1). Concentration of HA increased four times compared with its concentration in the absence of

Table 1 Effects of the initial glucose concentration (IGC) on the specific growth rate, the yield coefficients related to glucose and volumetric and specific productivities obtained in the cultivation of *S. zooepidemicus* under forced aeration.

IGC (g L^{-1})	μ_X	R^2	$Y_{HA/S}$	R^2	$Y_{X/S}$	R^2	P_V	P_S
0	0.32	0.9894	NC	NC	NC	NC	0.01	0.00
5	0.41	0.9985	0.06	0.9757	0.14	0.9916	0.02	0.02
25	0.34	0.9912	0.18	0.8441	0.11	0.9012	0.06	0.07
45	0.31	0.9967	0.09	0.7129	0.20	0.9655	0.04	0.01
90	0.28	0.8470	0.02	0.9543	0.10	0.9514	0.02	0.01

μ_X specific growth rate (h^{-1}), $Y_{HA/S}$ yield of HA from glucose (g g^{-1}), $Y_{X/S}$ yield of cells from glucose (g g^{-1}), P_V volumetric productivity of HA (g L^{-1} h^{-1}), P_S specific productivity of HA (g g^{-1} h^{-1}), *NC* not calculated.

glucose, when IGC reached 5 gL^{-1}. However, under limiting aeration of the shake flasks, the concentration of HA remained constant in other studied IGCs (Fig. 2a).

The IGC also affected the average molecular weight of HA as presented in Fig. 2c. The highest average molecular weight (2.11×10^8 Da) was obtained in the absence of glucose, nevertheless the lowest concentration of HA (0.28 gL^{-1}). The presence of glucose caused a slight increase in the average molecular weight of HA when the IGC ranged from 5 to 45 gL^{-1}.

Effects of the IGC on the Cultivation Under Forced Aeration

Table 1 shows the effects of IGC on the yield of cells ($Y_{X/S}$) and HA ($Y_{HA/S}$) from glucose, as well as the specific growth rate (μ_X) and volumetric and specific productivities of HA. The μ_X was somehow constant (0.28 to 0.34 h^{-1}), except for the 5 gL^{-1} IGC (0.41 h^{-1}). This was consequent of the faster exponential phase, 4 h, compared with the other IGCs, in which this phase remained between 8 and 12 h.

Among the considered IGCs, only the cultivation with 25 gL^{-1} of glucose presented a higher conversion of glucose into HA than into cells, as observed in the yield coefficient values ($Y_{HA/S}$, $Y_{X/S}$) in Table 1. This result, which has no precedent in the literature, indicates an inversion of the direction of the carbon source to the HA synthesis, at the expenses of the cell growth in acidifying cultivation. A similar inversion was observed when *S. zooepidemicus* WSH-24 was grown under the intermittent alkaline-stress (pH 8.5) strategy at carbon-to-nitrogen ratio (C/N) of 2.8 [7]. By correlating these effects, it may be suggested that there is a relationship between the C/N ratio and pH extremes related to neutral pH 7.0, which deserves to be approached in depth. This behavior may be in consequence of the exposition of the microorganism to stress conditions, in which the cells produce a capsule of HA as a way to shield from the acid or alkaline medium pH. This protection strategy has been described for group A streptococci as a protective shield from oxygen [23]. In the other IGCs, the carbon source was preferentially converted into cell mass instead of HA.

The effect of medium C/N ratios at constant pH7.0 influenced the specific productivity of HA. However, the changes were attributed more to the cell yield than to the HA yield [15]. In the present study, it was noted, in non-controlled pH cultivations, that the changes occurred in both yields. For this reason, the pH control could also be related to the inversion of the carbon source direction, previously observed.

Effects of the IGC on the volumetric and specific productivities of HA are presented in Table 1. The highest volumetric and specific productivities of HA happened for the 25 gL^{-1} IGC (0.06 $gL^{-1}h^{-1}$ and 0.07 $gg^{-1}h^{-1}$) which is in accordance with the observed behavior of the yield coefficients (Table 1).

Figure 3 shows the relationship between production and average molecular weight of HA, as well as the kinetics of average molecular weight of HA (b), both obtained at various IGCs in cultivations under forced aeration. The inverse behavior (Fig. 3a) agrees with hypothesis inferred by Weigel and De Angelis [24], regarding the hyaluronan synthase (HAS) controls the size of HA: the more time a chain is held and acted on by HAS, the larger the HA; conversely, if the chains are readily released by HAS, then the HA will be smaller. From our data, the velocity of HA release could be associated with the production of HA which is a function of IGC: the higher the intracellular accumulation of HA, the faster the release of chains, consequently, the smaller the molecular weight of HA. The kinetics of average molecular weight of HA (Fig. 3b) show the degradation of the polymer chains, imparted by the shear from the agitation. If the interaction between HA and glucose molecules can help the strengthening of the HA network [25], then the absence of glucose can explain the sharp decay of HA molecular weight in this cultivation.

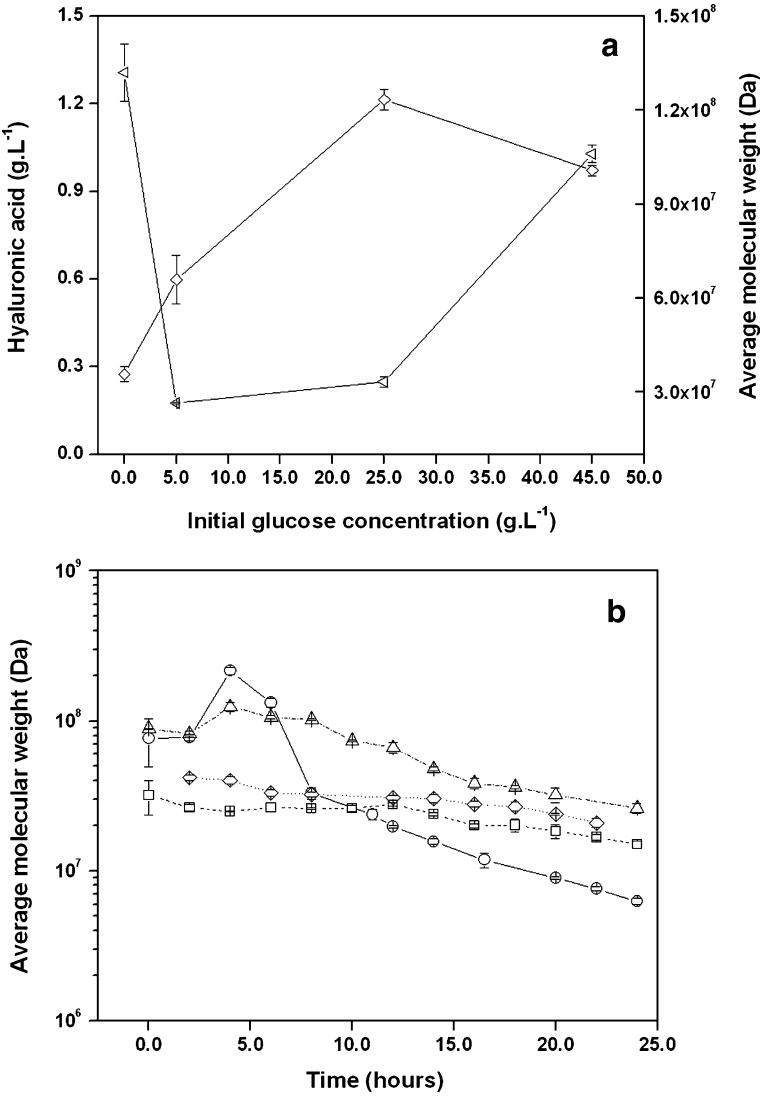


Fig. 3 Effects of the initial glucose concentration on the **a** final production of hyaluronic acid (*open diamond*) and average molecular weight (*open left-pointing triangle*) of HA after 6 h of cultivation and **b** kinetics of average molecular weight of HA at the initial glucose concentrations: (*open circle*) 0 gL^{-1}; (*open square*) 5 gL^{-1}; (*open diamond*) 25 gL^{-1}; (*open triangle*) 45 gL^{-1}. Both behaviors were observed in the cultivation of *S. zooepidemicus* under forced aeration

Effects of the IGCs on the concentrations of organic acids as a consequence of the metabolic changes, as well as the pH changes, are presented in Fig. 4. Lactate, which is the main product of the homofermentative metabolism, was produced in all cultivations. Nevertheless, the mixed acid metabolism observed in the absence of glucose yielded the lowest level of lactate, consumed by the cells as a carbon source after 12 h of cultivation (Fig. 4a), as well as the lowest decay of the pH along cultivation (Fig. 4e). The kinetic of acetate production (Fig. 4b) followed the one of cell growth, ranging from 5 to 45 gL^{-1}

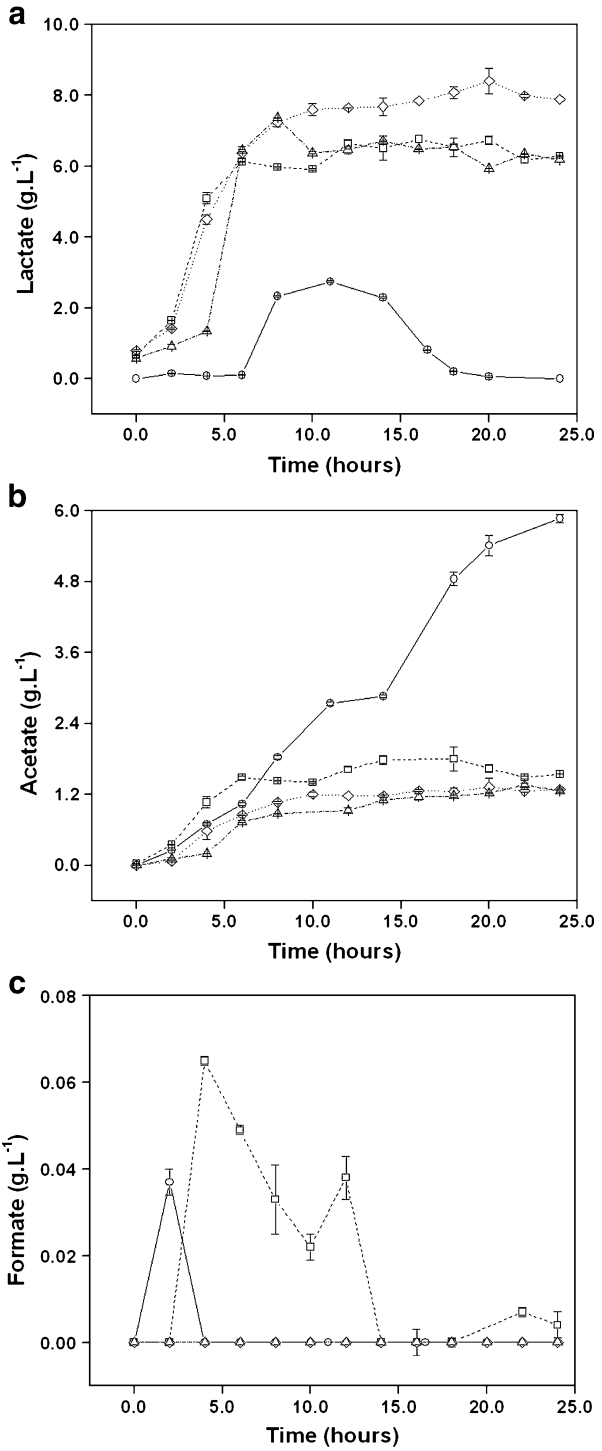
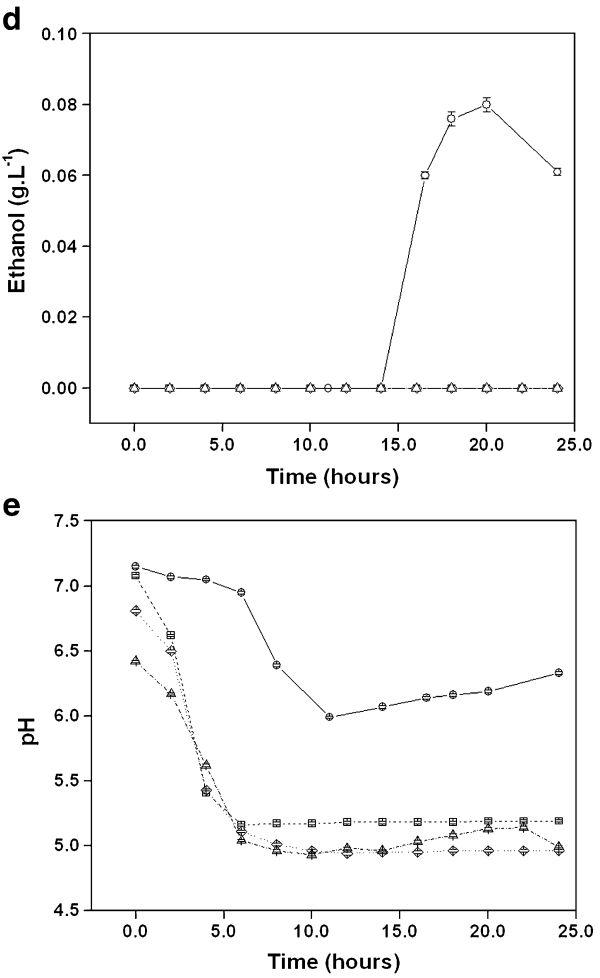


Fig. 4 Production of **a** lactate, **b** acetate, **c** formate, **d** ethanol, and **e** pH changes, in the cultivation of *S. zooepidemicus* under forced aeration at the initial glucose concentrations: (*open circle*) 0 gL^{-1}; (*open square*) 5 gL^{-1}; (*open diamond*) 25 gL^{-1}; (*open triangle*) 45 gL^{-1}

Fig. 4 (continued)



IGC, reaching concentrations between 1.3 and 1.5 gL^{-1}. In the absence of glucose, the metabolism of pyruvate shifted to acetate to compensate the reduction in the production of ATP as mentioned above (Fig. 1).

Formate was produced in low concentrations at IGCs of 0 and 5 gL^{-1} glucose; however, in both cases it was consumed by the cells due to either the absence or the total consumption of glucose at 5 gL^{-1} IGC (Fig. 4c). Even under forced aeration, formate production can be associated with anoxic conditions encountered for some cells given the greater cell aggregation in the presence of HA [13]. This aggregation avoids the pyruvate formate lyase inhibition under the excess of oxygen [26]. Formate was not produced in the other IGCs. By contrast, the production of ethanol was noted in the absence of glucose only. It was produced after 14 h, at the same time lactate was consumed by the cells (Fig. 4d).

Considering the above-mentioned findings, a metabolic flux analysis will certainly contribute to a better understanding of the metabolic changes and their influences on the production of HA.

Conclusions

In the present study, we demonstrated three main effects of IGC on the *S. zooepidemicus* metabolism: in the absence of glucose, the metabolism was mixed acid, independent of the oxygen supply, while the presence of glucose induced the homofermentative metabolism in which the production of HA and cell growth were dependent on the IGC under non-limiting oxygen supplying conditions only. A higher production and productivity of HA were observed in the cultivation for the 25 gL^{-1} IGC, which was the only condition where the inversion of the carbon source direction to HA occurred. And last but not least, the molecular weight of HA increased concomitant with the IGC within the studied range.

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