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Effect of fermentation conditions on the production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 39920

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ABSTRACT. The production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 39920 with varying rates of pH (6.0, 7.0, 8.0), temperature (34; 37; 40°C), agitation (100, 150, 200 rpm), glucose (10, 20, 30 g L^{-1}) and yeast extract concentration (10, 20, 30 g L^{-1}) was evaluated by statistical approaches. The best conditions for the production of hyaluronic acid was pH 8.0, 37°C and 100 rpm in a medium containing 30 g L^{-1} glucose and yeast extract, for a production of 0.787 g L^{-1}. Temperature, pH and yeast extract were significant variables ($p < 0.05$). Yeast extract and pH had a positive effect on the production of the polymer. Lactate, formate and acetate synthesis were also analyzed. Current assay showed the feasibility of statistical tools to optimize the physical and nutritional parameters for the production of hyaluronic acid and the improvement of the fermentation process.

Keywords: physical factors, nutritional factors, glycosaminoglycans, factorial design, microbial production.

Efeito das condições de fermentação na produção de ácido hialurônico por *Streptococcus zooepidemicus* ATCC 39920

RESUMO. A produção de ácido hialurônico por *Streptococcus zooepidemicus* ATCC 39920 foi avaliada variando pH (6,0; 7,0, 8,0), temperatura (34; 37; 40°C), agitação (100, 150, 200 rpm) e concentração de glicose (10, 20, 30 g L^{-1}) e extrato de levedura (10, 20, 30 g L^{-1}) por metodologias estatísticas. A condição otimizada foi pH 8,0, 37°C e 100 rpm, em meio contendo 30 g L^{-1} de glicose e extrato de levedura atingindo a produção de 0,787 g L^{-1}. O pH, temperatura e extrato de levedura foram as variáveis significativas ($p < 0,05$). Extrato de levedura e pH apresentaram efeito positivo para a produção do polímero. A síntese de ácido lático, fórmico e acético também foi analisada. Este estudo demonstra a viabilidade de utilização de ferramentas estatísticas para otimizar os parâmetros físicos e nutricionais para a produção de ácido hialurônico, permitindo a melhoria do processo fermentativo.

Palavras-chave: fatores físicos, fatores nutricionais, glicosaminoglicanos, delineamento fatorial, produção microbiana.

Introduction

Hyaluronic acid is a linear, high molecular weight, non-sulfated glycosaminoglycan polysaccharide composed of repeating disaccharide units of alternating D-glucuronic acid and N-acetylglucosamine by β-1,3 and β-1,4 bonds (KOGAN et al., 2007). Owing to its unique physical, chemical and biology properties, such as high water-holding capacity, viscoelasticity, and biocompatibility, hyaluronic acid is widely used in drug manufacture, ophthalmology, rheumatology, healthcare and cosmetic fields (KONG et al., 2011; KRETZ et al., 2014; SU et al., 2014; TOLG et al., 2014; YU et al., 2014; YANG et al., 2015). Native hyaluronic acid was traditionally extracted from animal tissues such as rooster combs but this technique is increasingly being replaced by microbial fermentation mainly due to the limited tissues sources and viral infection risks. *Streptococcus equi* subspecies *zooepidemicus* is one of the bacterial producers of the polymer (CHONG et al., 2005).

Streptococcus are lactic acid bacteria (KANDLER, 1983). Although homolactic metabolism converts more than 90% of sugars into lactate, under certain conditions the homolactic metabolism is lost, and high amounts of formate (in an anaerobic environment), acetate and ethanol are produced in what is called mixed acid metabolism (GARRIGUES et al., 2001). Shift control from homolactic to mixed acid may be attributed to several factors, such as pH (LIU et al., 2008), glucose concentration, aeration (PIRES; SANTANA, 2010) and the concentration of yeast extract (CHEN et al., 2009). Typical to lactic acid

bacteria, whose biosynthesis needs a large and complex nitrogen source, most glucose carbon is recovered in the fermentation products although only low levels are recovered in biomass (CHONG et al., 2005). Organic nitrogen sources are considered essential for the good growth of *Streptococcus*, since there is evidence that these components also supply a large proportion of the carbon for cellular biosynthesis (ARMSTRONG; JOHNS, 1997). Further, organic nitrogen source contains amino acids, nucleotide bases and vitamins are required by *Streptococcus* (MARCELLIN et al., 2009). For instance, purine, pyrimidine bases and B-vitamins are the main contribution of yeast extract in the production of hyaluronic acid (AMRANE; PRIGENT, 1994).

Growing conditions are crucial for cell development and synthesis of products. However, the diversity of nutritional components and their combinatory interaction with cellular metabolism makes difficult the development of an accurate mathematical model to describe the whole process. Thus, the optimization of growing conditions of a biological system is essential to maximize the product´s concentration and yield and to reduce the cost of raw material. Due to the many variables involved in the process and the complexity of microbial metabolism, statistical designs are used to optimize the composition for fermentation media (WEUSTER-BOTZ, 2000). Current study evaluates the effect of agitation, pH, temperature and glucose and yeast extract concentration in the production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 39920, from a statistical approach. Results show that the study may be useful for future researchers to find new applications for hyaluronic acid in various industrial sectors.

Material and methods

Microorganisms, culture maintenance and preparation of the inoculum

Streptococcus equi subsp. *zooepidemicus* ATCC 39920 was obtained from the Brazilian Collection of Environmental and Industrial Microorganisms - CBMAI. The strain was maintained in a saline solution containing 50% glycerol, at -80°C. The inoculum was prepared by transferring 1 mL of the stock culture to 125 mL Erlenmeyer flasks, containing 25 mL of Brain Heart Infusion medium (BHI). The flasks were incubated on an orbital shaker at 150 rpm, at 37°C, during 48h.

Culture media and fermentations

The culture media contained (g L^{-1}): glucose, 10 - 30; yeast extract, 10 - 30; K_2HPO_4, 2.5; NaCl, 2.0 and $MgSO_4$, 1.5, and pH was adjusted following Box-Behnken design (Table 1). The glucose solution was autoclaved separately and fermentations were carried out with 125 mL Erlenmeyer flasks with a working volume of 25 mL, maintained on an orbital shaker during 24h. Temperature, pH and agitation were evaluated in a factorial design (Table 1). Media were inoculated with 10% (v v^{-1}). Fermentations were interrupted by centrifugation at 9956 × g for 15 min., at 4°C. Hyaluronic acid, lactate, formate and acetate concentrations were determined for each flask at the initial and final periods.

Hyaluronic acid concentration

The cell-free supernatant was treated with ethanol at the ratio 1:3 (v v^{-1}) supernatant:ethanol, at 4°C, for 24h. The precipitation of hyaluronic acid was collected by centrifugation and its concentration was estimated by Carbazol reagent, using sulfuric acid with 0.025 M sodium tetraborate, following Filisetti-Cozzi and Carpita (1991), with modifications. Sodium hyaluronate (Sigma-Aldrich Brasil Ltda) was used as standard.

Concentrations of lactate, formate and acetate

For the quantification of lactate, formate and acetate, samples of the supernatant culture were filtered through membranes with 0.45 μm pores (Millipore) and 20 μL of the filtered sample was injected into a High Performance Liquid Chromatography (HPLC) (Shimadzu Corporation, Kyoto, Japan), equipped with 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 the 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). Lactate, formate and acetate (Sigma-Aldrich Brasil Ltda) were used as standard.

Factorial designs

Hyaluronic acid production by *Streptococcus zooepidemicus* ATCC 39920 were performed through the control of the fermentation variables by factorial design and analysis of the response surface method. A 3-factor, 3-level (-1; 0; +1) Box-Behnken design (MONTGOMERY, 2012), with three replicates, at the central point, summarizing 15 experimental runs (Table 1), was used to study pH, temperature and agitation conditions for hyaluronic acid production. Glucose and yeast extract concentration was fixed at 20 g L^{-1} and 10 g L^{-1}, respectively. Within the best

conditions of pH, temperature and agitation, defined in the Box-Behnken design, a full 3^2 design plus central points was used to evaluate the concentration of glucose and yeast extract (Table 3). Analysis of variance (ANOVA) and multiple regression analyses were performed at 5% significance level with Statistica 9.0 software (2009).

Results and discussion

Table 1 shows the results of the production of hyaluronic acid and organic acids obtained from *S. zooepidemicus* at different pH, temperature and agitation conditions. Results demonstrate that the highest production of hyaluronic acid was 0.437 g L^{-1} at pH 8.0, 37°C and 100 rpm (run 6; Table 1). When glucose consumption is taken into account, maximum productivity was also observed at pH 8.0, reaching 0.062 g g^{-1}, at 34°C and 150 rpm. This effect may have been due to the exposure of the microorganism to stress condition in which it produced hyaluronic acid capsule as a barrier against acidic or alkaline pH of the medium (PIRES; SANTANA, 2010). Similarly, Liu et al. (2008), using bioreactor, reported an increase of hyaluronic acid production when *S. zooepidemicus* WSH-24 was intermittently exposed to alkaline stress (pH 7.0 – 8.5) at 37°C.

In current study, low polymer production rate was reported with pH 6.0 (runs 1, 3, 5, 7; Table 1). Since there was no production of lactate in these assays, a shift in the carbon flux pathway was detected towards the homolactic metabolism for the synthesis of formate and acetate. In addition, there was no production of lactate with pH 7.0 and 200 rpm (runs 11 and 12). According to Garrigues et al. (2001), the deviation to homolactic metabolism occurred to compensate the reduction of cellular energy production (ATP).

It must be underscored that temperature increase from 34 to 40°C caused a decrease in the production of the polymer from 0.409 to 0.240 g L^{-1} (runs 9 and 10; Table 1), showing that the parameter affected the production of hyaluronic acid. The significant impact of temperature on the production of the hyaluronic acid by *S. zooepidemicus* ATCC 43079 was reported by Khue and Vo (2013) who registered increase of hyaluronic acid production at 40°C. In another study, 37°C was considered the optimal temperature for the growth and hyaluronic acid production by *Streptococcus equi* mutant (KIM et al., 1996). In current study, when pH and temperature were fixed, the changes in the agitation had no effect on the production of hyaluronic acid which might be visualized when run 5 (0.168 g L^{-1}) and 7 (0.112 g L^{-1}) and run 6 (0.437 g L^{-1}) and 8 (0.434 g L^{-1}) were compared.

The analysis of variance (Table 2) showed that pH ($p = 0.00002$) and the temperature ($p = 0.046$) were the significant variables and that the interaction of temperature and agitation ($p = 0.043$) was important in the process. The quadratic model explains 98% of the experimental data and lack-of-fit was not significant ($p = 0.062$).

Based on the experimental results, a canonical equation was developed to estimate the hyaluronic acid production (Equação 1):

$$Y = 0.400 + 0.159x_1^* - 0.117x_1^{2*} - 0.028x_2^* - 0.073x_2^{2*} - 0.004x_3 + 0.005x_3^2 + 0.004x_1x_2 + 0.013x_1x_3 + 0.04x_2x_3^* \quad (1)$$

Table 1. Hyaluronic acid and organic acid production by *Streptococcus zooepidemicus* ATCC 39920 at different pH, temperature and agitation.

Run (standard order)*	Factor levels			Response			
	x_1	x_2	x_3	Hyaluronic acid (g L^{-1})	Lactate (g L^{-1})	Formate (g L^{-1})	Acetate (g L^{-1})
1	-1	-1	0	0.054	0.000	1.635	1.085
2	+1	-1	0	0.387	2.991	0.000	1.003
3	-1	+1	0	0.024	0.000	0.000	1.876
4	+1	+1	0	0.372	2.752	0.000	1.176
5	-1	0	-1	0.168	0.000	0.000	0.585
6	+1	0	-1	0.437	3.025	0.000	0.945
7	-1	0	+1	0.112	0.000	0.000	1.922
8	+1	0	+1	0.434	3.870	1.878	1.177
9	0	-1	-1	0.409	2.061	0.000	0.634
10	0	+1	-1	0.240	2.662	0.000	0.758
11	0	-1	+1	0.344	0.000	0.000	1.744
12	0	+1	+1	0.336	0.000	0.203	2.800
13	0	0	0	0.410	2.554	0.921	0.767
14	0	0	0	0.399	2.788	0.000	0.816
15	0	0	0	0.390	3.104	0.461	0.991

Code	Factors	Levels		
		-1	0	+1
(X_1)	pH	6.0	7.0	8.0
(X_2)	Temperature (°C)	34	37	40
(X_3)	Agitation (rpm)	100	150	200

(*)Assays were randomized.

Table 2. Analysis of variance (ANOVA) of hyaluronic acid production by *Streptococcus zooepidemicus* ATCC 39920 by quadratic model.

Source	Sum of Square	Degrees of Freedom	Mean Square	F	p-Value
pH (L)	0.202324	1	0.202324	228.3834	0.000023*
pH (Q)	0.050961	1	0.050961	57.5247	0.000632*
Temperature (L)	0.006157	1	0.006157	6.9501	0.046181*
Temperature (Q)	0.019643	1	0.019643	22.1726	0.005295*
Agitation (L)	0.000114	1	0.000114	0.1288	0.734303
Agitation (Q)	0.000110	1	0.000110	0.1247	0.738371
x_1x_2	0.000053	1	0.000053	0.0601	0.816155
x_1x_3	0.000706	1	0.000706	0.7969	0.412919
x_2x_3	0.006437	1	0.006437	7.2662	0.043011*
Error	0.004429	5	0.000886	-	-
Total SS	0.287706	14	-	-	-

Lack-of-fit: p = 0.062386; R^2 = 0.9846; *Significance at $p < 0.05$; L = linear; Q = quadratic.

where:

Y is the response (hyaluronic acid production);

x_1, x_2 and x_3 are coded rates respectively of pH, temperature and agitation.

The coefficient of determination (R^2) was 0.9846 and indicated that the proposed model may be used for prediction. The response surface (Figure 1) showed maximum production of hyaluronic acid at pH 8.0 and 37°C. The agitation was set at 100 rpm.

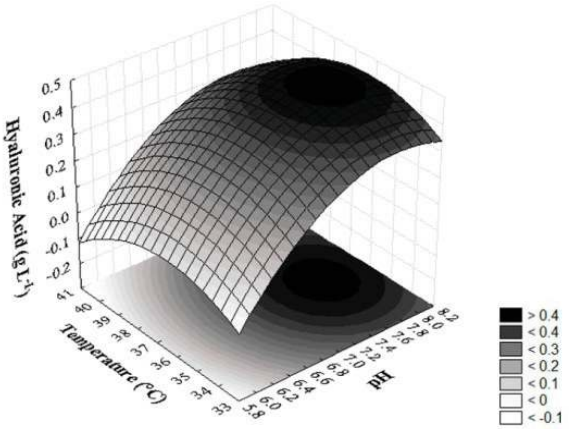

Figure 1. Response surface plots showing the effect of pH and temperature on the production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 39920. The agitation was set at 100 rpm.

Since pH 8.0, 37°C and 100 rpm were the best conditions for the production of hyaluronic acid, the next step was to investigate the suitable concentrations of glucose and yeast extract for hyaluronic acid production. The results are shown in Table 3.

According to the results, the highest production of hyaluronic acid was 0.787 g L^{-1} using 30 g L^{-1} glucose and 30 g L^{-1} yeast extract (run 9; Table 3) with an approximately 80% increase of the polymer production when compared to the first experimental design (run 6; Table 1). Runs 3 (0.561 g L^{-1}) and 6 (0.647 g L^{-1}) also featured high productions rates (Table 3), in which the yeast extract concentration was 30 g L^{-1}. The increase in glucose concentration from 10 g L^{-1} (run 3) to 20 g L^{-1} (run 6) and 30 g L^{-1} (run 9) produced more hyaluronic acid. In these assays the concentration of organic acids remained constant.

Pires and Santana (2010) evaluated the initial glucose concentration from 0 to 45 g L^{-1} for the production of hyaluronic acid by *S. zooepidemicus* ATCC 39920 and showed that the production of the polymer was not influenced by glucose concentration over 5 g L^{-1} and the lactate synthesis remained constant from 10 g L^{-1}. Using a bioreactor, Don and Shoparwe (2010) evaluated the effect of glucose in concentrations ranging between 10 and 60 g L^{-1}, whilst a higher production of hyaluronic acid in a medium containing 40 g L^{-1} glucose was reported. In a recent work published by our group, it was found that glucose is one of the best carbon sources for the production of hyaluronic acid, followed by sucrose (PAN et al., 2015). However, within the same conditions, the production of hyaluronic acid was 0.429 g L^{-1}, or rather, lower than the one obtained in current assay (0.787 g L^{-1}). Reduction in the production of the polymer observed by Pan et al. (2015) in the same growing conditions may be due to difference in the viability of the inoculum.

The increase of yeast extract concentration caused a higher production of hyaluronic acid and lactate, and suggested a diversion of the carbon flux for the homolactic pathway and the production of the polymer. This effect could be observed when the glucose concentration was fixed at 30 g L^{-1} (run 7, 8, 9; Table 3). The increase of yeast extract concentration from 10 to 20 g L^{-1} increased the production of hyaluronic acid from 0.278 to 0.580 g L^{-1}, respectively (run 7 and 8; Table 3). The same result was observed for lactate. Further, when run 8
and 9 were compared (Table 3), the increase of yeast extract concentration from 20 to 30 g L^{-1} caused a 35% increment in the production of the polymer and 11.1% for the lactate. The acetate synthesis was also lower in concentration 10 g L^{-1}

of yeast extract, which reduced the formation of ATP and impaired the production of hyaluronic acid. Chen et al. (2009) evaluated the influence of yeast extract concentration on the production of hyaluronic acid and concluded that the initial concentration of yeast extract between 5 g L^{-1} and 10 g L^{-1} led to an 11% increase in the hyaluronic acid production.

The analysis of variance (ANOVA) (Table 4) showed that only the yeast extract was significant ($p = 0.0006$). This result corroborates specialized literature that showed the significant effect of nitrogen sources on the production of hyaluronic acid using yeast extract, soy peptone and meat extract (AROSKAR et al., 2012; IM et al., 2009; KHUE; VO, 2013; KOTRA et al., 2013; PATIL et al., 2011). Lack-of-fit was not significant ($p = 0.379$) and the determination coefficient (R^2) was 0.94%, implying that 94% of the variance of the experimental data may be explained.

The final empirical model for hyaluronic acid production in terms of glucose and yeast extract concentration in coded units was as follows (Equation 2):

$$Y = 0.608 + 0.059x_1 - 0.043x_1^2 + 0.200x_2^* - 0.144x_2^{2*} + 0.029x_1x_2 \quad (2)$$

where:

Y is the response (hyaluronic acid)

x_1 and x_2 are respectively the glucose and yeast extract.

The three-dimensional response surface (Figure 2) showed that polymer production was enhanced by increasing the concentration of yeast extract in the glucose conditions under analysis.

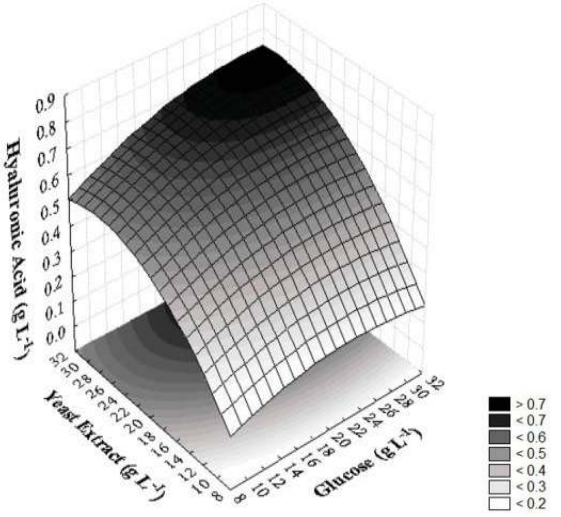

Figure 2. Response surface showing the effect of glucose and yeast extract concentration on the production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 39920.

Table 3. Effect of glucose and yeast extract concentration on the production of hyaluronic acid and organic acids by *Streptococcus zooepidemicus* ATCC 39920.

Run (standard order)*	Factor levels		Response			
	x_1	x_2	Hyaluronic acid (g L^{-1})	Lactate (g L^{-1})	Formate (g L^{-1})	Acetate (g L^{-1})
1	-1	-1	0.167	1.145	0.290	0.845
2	-1	0	0.560	3.846	1.241	1.370
3	-1	+1	0.561	5.531	0.000	1.496
4	0	-1	0.350	1.215	0.826	0.636
5	0	0	0.666	3.890	0.193	1.128
6	0	+1	0.647	5.982	0.000	1.334
7	+1	-1	0.278	1.332	0.000	0.819
8	+1	0	0.580	4.984	0.000	1.371
9	+1	+1	0.787	5.581	0.000	1.316
10	0	0	0.565	4.496	0.000	1.245
11	0	0	0.584	4.594	0.000	1.263

Code	Factors	Levels		
		-1	0	+1
(X_1)	Glucose (g L^{-1})	10	20	30
(X_2)	Yeast Extract (g L^{-1})	10	20	30

(*)Assays were randomized.

Table 4. Analysis of variance (ANOVA) of hyaluronic acid production by *Streptococcus zooepidemicus* ATCC 39920 by quadratic model.

Source	Sum of Square	Degrees of Freedom	Mean Square	F	p-Value
Glucose (L)	0.021164	1	0.021164	5.01024	0.075370
Glucose (Q)	0.004725	1	0.004725	1.11852	0.338634
Yeast Extract (L)	0.239929	1	0.239929	56.79811	0.000651*
Yeast Extract (Q)	0.033097	1	0.033097	7.83505	0.038033*
$x_1 \times x_2$	0.003314	1	0.003314	0.78455	0.416313
Error	0.021121	5	0.004224		
Total SS	0.333427	10			

Lack-of-fit: $p = 0.379102$; $R^2 = 0.93665$ *Significance at $p < 0.05$; L = linear; Q = quadratic.

Conclusion

Results revealed that the production of hyaluronic acid by *S. zooepidemicus* ATCC 39920 could be improved by adjusting the physical and nutritional parameters of the fermentation process. The optimization of temperature, pH and agitation by the factorial design resulted in the maximum production of 0.787 g L^{-1}, obtained at 37°C, pH 8.0 and 100 rpm, in a medium containing 30 g L^{-1} glucose and 30 g L^{-1} yeast extract. The evaluation of organic acids demonstrated that at pH 6.0 there was no production of lactate and the increment of yeast extract increased the acid synthesis.

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