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Factorial design in fermentation medium development for hyaluronic acid production by *Streptococcus zooepidemicus*

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ABSTRACT. Hyaluronic acid (HA) has biological functions of interest to medical and cosmetic industries. The optimization of a fermentation medium contributes to improve the microbial production of the polymer on a large scale and make the product more accessible to the market. Factorial design and response surface were used to evaluate the concentration of sucrose, yeast extract, glutamine, glutamate and oxalic acid on the HA production by *Streptococcus zooepidemicus* in shake flasks. Biomass, lactate and acetate production were also studied. Yeast extract showed to be the main significant variable for all the studied responses, showing positive effect. For the HA production, glutamine was also significant and presented a positive effect. The best condition for the polymer production was 50 g L^{-1} of yeast extract and sucrose and 0.6 g L^{-1} of glutamine, glutamate and oxalic acid. In this condition, the fermentation was carried out in bioreactor with pH controlled at 8 and 0.5 vvm. HA production in bioreactor was explained by logistic regression model and the maximum concentration asymptotic of HA was 0.860 g L^{-1}. An increase of 34% on polymer production was observed when compared to shake flask assay. Thus, it was possible to optimize the polymer production using statistical techniques.

Keywords: polymer; optimization; bioreactor; logistic regression; organic acids.

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Introduction

The hyaluronic acid (HA) is a polysaccharide that is present in all vertebrates as a main constituent of the extracellular matrix (Chen & Abatangelo, 1999). The polymer consists of repeated disaccharide units containing D-glucuronic acid and N-acetyl-glucosamine and presents several biological functions, such as joints lubrication, skin hydration and space filling (Kogan, Soltés, Stern, & Gemeiner, 2007). In addition, studies demonstrate that the polymer acts in several signaling pathway and regulation of pathologic processes, and also exhibits therapeutic potential in human diseases such as osteoarthritis, cancer, diabetes, cystic fibrosis, asthma and wound healing (Tripodo et al., 2015; Liang, Jiang, & Noble, 2016; Hermans et al., 2017).

Due to HA biological applications, the microbial production is preferred compared to extraction from rooster comb, umbilical cords and bovine humor vitreous. Animal-derived biochemical compounds are not recommended for human therapeutics because of viral contamination risks and other infectious agents (Yamada & Kawasaki, 2005). The microbial HA is mainly produced by *Streptococcus* that synthetizes the polymer as an extracellular capsule (Chong, Blank, Mclaughlin, & Nielsen, 2005).

Streptococci are lactic bacteria which required highly nutritive cultivation media for their growth (Chong et al., 2005). The typically used media included yeast extract, peptones, or hydrolyzed casein as nitrogen source and growth factors, as magnesium and phosphate salts (Armstrong, Cooney, & Johns, 1997). The diversity of combinatorial interactions of large number medium components with the cells metabolism do not permit a satisfactory detailed model elaboration to describe the HA production. Therefore, due to several variables which involve this process and the microorganism metabolic complexity, statistical techniques are used to optimize the compositions of fermentation media (Weuster-Botz, 2000).

Pan, Pereira, Silva, Vasconcelos, and Celligoi (2017) confirmed by central composite design the significant effect ($p < 0.05$) of the concentration of sugarcane molasses and yeast extract in the polymer production. Plackett-Burman design was used to estimate the different variables effect on the HA

production (Khue & Vo, 2013; Mohan, Balakrishnan, & Sivaprakasam, 2016). Patil, Kamalja, and Chaudhari (2011) employed a full factorial design and a central composite design to evaluate the concentration of glucose, soy peptone, $MgSO_4$ and K_2HPO_4. Using these statistical techniques, these authors obtained a 65% increase in HA production (Patil et al., 2011).

In addition, it has been described that amino acids and organic acids can influence in the metabolism of *Streptococcus*. The glutamine addition in the culture medium is known to increase HA production (Im, Song, Kang, & Kang, 2009; Aroskar, Kamat, & Kamat, 2013; Shah, Badle, & Ramachandran, 2013), because it favors the N-acetyl-glucosamine synthesis, forerunner unit for the polymer production (Chong et al., 2005). Aroskar et al. (2013) observed an increase of 0.7 to 1 g L^{-1} HA when added glutamine and arginine at the concentration of 2.0 g L^{-1}. Arginine was reported as an essential amino acid for microbial growth and HA production by *S. zooepidemicus* ATCC 35246 (Armstrong et al., 1997). Im et al. (2009) evaluated seventeen amino acids and twenty-three organic acids, and observed that only glutamine, glutamate and oxalic acid positively influence the polymer production.

In this context, the present study aimed to optimize the HA production by *S. zooepidemicus* ATCC 39920 using fractional factorial design and response surface methodology. Sucrose, yeast extract, glutamine, glutamate and oxalic acid were the explanatory variables studied. Fermentation in bioreactor was performed in the best condition obtained for the HA production in shake flasks and the variables responses were explained by logistic regression. The production of organic acids, lactate and acetate, and biomass synthesis were observed when the metabolic fluxes were analyzed under the experimental conditions.

Material and methods

Microorganism and medium

Streptococcus equi subsp. *zooepidemicus* ATCC 39920 was obtained from the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI). The strain was maintained in saline solution containing 50% glycerol and stocked 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 - Ref M210, HiMedia Laboratories, Mumbai, India). The flasks were incubated on an orbital shaker at 150 rpm, at 37°C, for 48 hours. For each fermentation process, the inoculum was standardized to 10% (v v^{-1}).

The fermentation media contained (g L^{-1}): sucrose, 10 - 50; yeast extract, 10 - 50; glutamate, 0 - 0.6; glutamine, 0-0.6; oxalic acid 0 - 0.6; K_2HPO_4, 2.5; NaCl, 2.0 and $MgSO_4.7H_2O$, 1.5.

Fractional factorial design

Fractional factorial design 2^{5-1} was performed to study the effect of sucrose, yeast extract, glutamine, glutamate and oxalic acid concentration on the HA production (Table 1). Hyaluronic acid, biomass, lactate and acetate production were evaluated as response variables. The factor level choice was based on the literature and previous work (Pan et al., 2015). Total of 20 experimental runs were carried out in random order, which included 16 factorial points and 4 central points. The experiment was carried out in 125 mL Erlenmeyers containing 25 mL fermentation medium at the concentrations described for each run (Table 1); initial pH 8.0 at 37°C and 100 rpm for 24 hours.

Bioreactor

The fermentation was evaluated under the HA production conditions optimized by fractional factorial designs in 4.5 L bioreactor (Tecnal, Brazil) containing 2 L of fermentation medium with 50 g L^{-1} of yeast extract and sucrose and 0.6 g L^{-1} of glutamate, glutamine and oxalic acid. Fermentation was performed at pH 8.0 that was controlled by 8 mol L^{-1} of NaOH solution. The temperature was maintained at 37°C, the rotation at 100 rpm and the aeration rate at 0.5 vvm. Samples were collected every 2 for 24 hours.

Analytical methods

Fermentation samples were centrifuged at 9956 g for 15 min. The biomass was determined by measuring the turbidimetry at λ = 600 nm and correlated to the biomass curve in g L^{-1}. For the HA, lactate and acetate quantifications, the culture supernatant samples were filtered through membranes with 0.45 µm pores (Millipore, São Paulo, State São Paulo, Brazil) and 20 µL was injected into a high-performance liquid chromatograph (HPLC) instrument (Shimadzu Corporation, Kyoto, Japan). The HA evaluation was performed in an OHpak SB-806MHQ 80 × 300 mm column (Shodex, Japan) at 40°C with a 0.1 M $NaNO_3$

mobile phase and 1 mL min.$^{-1}$ flow rate. Lactate and acetate were evaluated using an Aminex 7.8 × 300 mm HPX- 87H organic acid column (Bio-Rad, CA, USA) at 60°C. The mobile phase was composed of a 0.005 mol L^{-1} H_2SO_4 solution with a 0.7 mL min.$^{-1}$ flow rate. The peak elution profile was monitored with a Shimadzu RID - 10A refractive index detector (Shimadzu Corporation, Kyoto, Japan).

Statistical analysis

The data analysis was performed using the Software R (R Core Team, 2016) and package the rsm (Lenth, 2009). The fit quality of the models was evaluated by the determination coefficient R^2 and its statistical significance was assessed by the F-test. The models assumptions were evaluated. The homogeneity of residual variance was evaluated by the Breusch-Pagan test (Breusch & Pagan, 1979) or graphical analysis. The normality of the residues was evaluated by Shapiro-Wilk test (Shapiro & Wilk, 1965). At all statistical analyses, p-values of less than 0.05 were regarded as statistically significant.

Fractional factorial design

Fractional factorial designs allow the evaluation of a large number of factors with a reduced the size of experiments. Consequently, the goals of the experiment may be met with the least cost, shortest time, and most effective use of resources (Gunst & Mason, 2009).

For optimal point prediction, a polynomial function (Equation 1) was fitted to the fractional factorial design experimental results:

$$y_{ij} = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij}\, x_i x_j + \epsilon_{ij} \qquad (1)$$

where:

y_{ij} is the predicted response, β are unknown parameters that will be estimated from the data in the experiment, i and j range from 1 to k variables, x_i and x_j are the independent variables defined on a coded scale from -1 to +1, and ϵ_{ij} is the random error term. The parameter estimates in this regression model turn out to be related to the effect estimates (Montgomery, 2013). The parameters significance of polynomial models and their interactions were tested by analysis of variance (ANOVA) and Student's t-test.

Table 1. Fractional factorial design 2^{5-1} for the production of hyaluronic acid, lactate, acetate and biomass by *Streptococcus zooepidemicus* ATCC 39920 in different concentrations of sucrose (x_1), yeast extract (x_2), glutamate (x_3), glutamine (x_4) and oxalic acid (x_5).

Runs	Factor levels					Variable Responses			
	x_1	x_2	x_3	x_4	x_5	Hyaluronic acid (g L^{-1})	Lactate (g L^{-1})	Acetate (g L^{-1})	Biomass (g L^{-1})
1	-1	-1	-1	-1	+1	0.302	2.611	1.002	0.928
2	+1	-1	-1	-1	-1	0.320	3.547	0.836	1.097
3	-1	+1	-1	-1	-1	0.608	7.619	1.528	1.799
4	+1	+1	-1	-1	1	0.367	8.691	1.355	1.782
5	-1	-1	+1	-1	-1	0.290	3.578	0.995	1.053
6	+1	-1	+1	-1	+1	0.371	2.781	1.091	1.137
7	-1	+1	+1	-1	+1	0.380	9.688	1.581	1.713
8	+1	+1	+1	-1	-1	0.415	8.226	1.154	1.886
9	-1	-1	-1	+1	-1	0.350	3.175	0.870	1.020
10	+1	-1	-1	+1	+1	0.344	2.931	0.960	1.040
11	-1	+1	-1	+1	+1	0.547	8.484	1.467	1.745
12	+1	+1	-1	+1	-1	0.501	4.103	1.756	1.955
13	-1	-1	+1	+1	+1	0.578	3.821	1.887	1.006
14	+1	-1	+1	+1	-1	0.563	3.707	1.245	1.064
15	-1	+1	+1	+1	-1	0.512	8.054	1.239	1.923
16	+1	+1	+1	+1	+1	0.608	7.105	1.294	1.905
17	0	0	0	0	0	0.481	6.009	1.167	1.651
18	0	0	0	0	0	0.562	5.678	1.038	1.666
19	0	0	0	0	0	0.491	6.081	0.976	1.750
20	0	0	0	0	0	0.463	5.919	1.033	1.626

Symbols	Factors	Coded levels		
		-1	0	+1
(X_1)	Sucrose (g L^{-1})	10	30	50
(X_2)	Yeast extract (g L^{-1})	10	30	50
(X_3)	Glutamate (g L^{-1})	0	0.3	0.6
(X_4)	Glutamine (g L^{-1})	0	0.3	0.6
(X_5)	Oxalic acid (g L^{-1})	0	0.3	0.6

Logistic regression

The profiles of hyaluronic acid, biomass, lactate and acetate productions in bioreactor were modeled using the following non-linear logistic regression (Equation 2):

$$P = P_{max}/(1 + \exp[(x_0 - x)/k]) \quad (2)$$

where:

P_{max} is the maximum asymptotic value of biomass, hyaluronic acid, lactate or acetate production (g L^{-1}), k is the numeric scale parameter on the input axis, and x_0 is the x value at the inflection point of the curve. The parameters significance were tested by Student's t-test.

Results and discussion

Fractional factorial design

The concentration of carbon and nitrogen sources and other fermentation medium supplements have already been described as important variables for HA productions (Im et al., 2009; Shah et al., 2013; Pan et al., 2017). Through fractional factorial design 2^{5-1} the concentration of sucrose (X_1), yeast extract (X_2), glutamate (X_3), glutamine (X_4) and oxalic acid (X_5) were evaluated for the HA, lactate, acetate and biomass production (Table 1).

For each variable response a model was proposed and the assumptions and adequacy of these were satisfied (Table 2 - 5). The models proposed present homogeneity of variance (Breusch-Pagan test, $p > 0.05$) and normality (Shapiro-Wilk test, $p > 0.05$) of the residues. Yeast extract was the factor that presented higher positive effect for biomass synthesis (Table 2). The other factors studied were not significant for biomass synthesis at the 5% level of significance. The proposed model explained 91.53% of the variability in the biomass response. However, the lack-of-fit was significant indicating that the region presents curvature. Armstrong et al. (1997) justify that organic nitrogen sources supply the need of essential amino acids to *Streptococci*, besides containing a large proportional of carbon necessary for microbial synthesis. Corroborating with this result, yeast extract was also the most significant factor for the HA and organic acids production, being its effect positive for all variables responses.

For the HA production (Table 3), the glutamine and its interaction with glutamate were also significant at the 5% significance level. The variables that present higher estimated effect were yeast extract (0.114 g L^{-1}), followed by glutamine (0.106 g L^{-1}). The interactions between yeast extract and glutamate, oxalic acid and glutamine and oxalic acid and glutamate were significant at the 10% significance level.

Table 2. Estimates of the model parameters for biomass production by *S. zooepidemicus* ATCC 3992 from the fractional factorial designs.

Factors	Estimate	Standard error	t-value	p-value
Intercept	1.487	0.026	56.352	< 0.0001*
x_1	0.043	0.030	1.442	0.168
x_2	0.398	0.030	13.479	< 0.0001*

Regression: $p < 0.0001$; $R^2 = 0.9153$; $R^2_{adjusted} = 0.9054$; lack-of-fit: $p < 0.0001$; *significant factors ($p < 0.05$); x_1 = sucrose; x_2 = yeast extract.

Table 3. Estimates of the model parameters for hyaluronic acid production by *S. zooepidemicus* ATCC 3992 from the fractional factorial designs.

Factors	Estimate	Standard error	t-value	p-value
Intercept	0.448	0.013	34.507	< 0.0001*
x_2	0.057	0.015	3.950	0.0022 *
x_3	0.018	0.015	1.213	0.25053
x_4	0.053	0.015	3.675	0.00366*
x_5	0.002	0.015	0.155	0.87974
x_2:x_3	-0.031	0.015	-2.142	0.05537
x_3:x_4	0.035	0.015	2.418	0.03411*
x_4:x_5	0.029	0.015	1.985	0.07260
x_3:x_5	0.029	0.015	2.031	0.06716

Regression: $p = 0.0037$; $R^2 = 0.8170$; $R^2_{adjusted} = 0.6839$; lack-of-fit: $p = 0.2970$; *significant factors ($p < 0.05$); x_2 = yeast extract; x_3 = glutamate; x_4 = glutamine; x_5 = oxalic acid.

Table 4. Estimates of the model parameters for lactate production by *S. zooepidemicus* ATCC 3992 from the fractional factorial designs.

Factors	Estimate	Standard error	t-value	p-value
Intercept	5.590	0.092	60.488	<0.0001*
x_1	-0.371	0.103	-3.592	0.00706*
x_2	2.239	0.103	21.665	<0.0001*
x_3	0.363	0.103	3.508	0.00798*
x_4	-0.335	0.103	-3.244	0.01181*
x_5	0.257	0.103	2.483	0.03796*
x_1:x_2	-0.344	0.103	-3.327	0.01043*
x_1:x_4	-0.340	0.103	-3.289	0.01104*
x_2:x_3	0.160	0.103	1.544	0.16114
x_2:x_4	-0.475	0.103	-4.594	0.00177*
x_2:x_5	0.489	0.103	4.734	0.00148*
x_5:x_3	-0.278	0.103	-2.688	0.02758*

Regression: $p < 0.0001$; $R^2 = 0.9865$; $R^2_{adjusted} = 0.9680$; lack-of-fit: $p = 0.0560$; *significant factors ($p < 0.05$); x_1 = sucrose; x_2 = yeast extract; x_3 = glutamate; x_4 = glutamine; x_5 = oxalic acid.

Table 5. Estimates of the model parameters for acetate production by *S. zooepidemicus* ATCC 3992 from the fractional factorial designs.

Factors	Estimate	Standard error	t-value	p-value
Intercept	1.224	0.044	28.075	<0.0001*
x_1	-0.055	0.049	-1.126	0.28213
x_2	0.155	0.049	3.190	0.00778*
x_3	0.044	0.049	0.911	0.38009
x_5	0.063	0.049	1.302	0.21747
x_1:x_5	-0.100	0.049	-2.046	0.06336
x_2:x_3	-0.149	0.049	-3.063	0.00985*
x_3:x_5	0.089	0.049	1.828	0.09248

Regression: $p = 0.0121$; $R^2 = 0.7201$; $R^2_{adjusted} = 0.5569$; lack-of-fit: $p = 0.0626$; *significant factors ($p < 0.05$); x_1 = sucrose; x_2 = yeast extract; x_3 = glutamate; x_5 = oxalic acid.

The increase in the polymer production through the addition of glutamine in the cultivation medium is due to this amino acid act as amino group donor of fructose 6-phosphate in presence of amidotransferase enzyme which yields glucosamine 6-phosphate (Shah et al., 2013). Im et al. (2009) observed an 18% of increase in the HA production with addition of glutamine, glutamate and oxalic acid to the fermentation medium. The model proposed for the HA production explained 81.7% of the variability of the obtained data and the F-test ($p < 0.05$) was significant and indicated that this is an adequate model. The contour curve (Figure 1) shows that, for all variables, the production of HA increased with the concentration. Among the conditions studied, the best for HA production was 50 g L^{-1} of yeast extract and 0.6 g L^{-1} of glutamine, glutamate and oxalic acid. Sucrose did not present significant effect in the hyaluronic acid production at the 5% level of significance. Pires and Santana (2010) and Pan et al. (2015) observed that glucose carbon source did not exert influence on the polymer production in shake flasks as well.

Lactate and acetate are the main metabolites produced by *Streptococcus* in aerobic cultivation (Chong & Nielsen, 2003). The production of lactate and acetate in the assays range from 2.611 to 9.688 g L^{-1} and 0.836 to 1.887 g L^{-1}, respectively. The ANOVA of the model obtained to describe the lactate synthesis showed determination coefficient (R^2) of 0.99 and it was significant at the 5% significance level. Yeast extract, glutamate, oxalic acid and the interaction between yeast extract and oxalic acid were significant ($p < 0.05$) and showed positive effect for the lactate synthesis (Table 4). The other significant variables of the model presented a negative effect. The interaction between yeast extract and glutamate was not significant; however, it was essential to satisfy the model assumptions.

The significant variables at the 5% level for the acetate production were yeast extract and the interaction between yeast extract and glutamate (Table 5). At the 10% of significance level, the interaction between sucrose and oxalic acid and glutamate and oxalic acid were significant. The main effects of sucrose, glutamate and oxalic acid remained in the model because they were present significantly in the interactions between the compounds. The model for acetate production obtained determination coefficient of 0.72.

Summing up, to produce metabolites and biomass, the yeast extract was the significant variable with the greatest positive effect. The carbon source showed a significant negative effect for the lactate production. Glutamine had a significant effect on the production of HA and lactate. However, the effect was positive for production of HA and negative for lactate. This may indicate that the amino acid favors the carbon flow for the polymer production. Glutamate and oxalic acid also showed a significant positive effect in the lactate

production. The interaction glutamate and glutamine was significant ($p < 0.05$) for HA production. Interactions sucrose and yeast extract, sucrose and glutamate, yeast extract and glutamine, extract yeast and oxalic acid and glutamate and oxalic acid to lactate production, and yeast extract and glutamate to acetate production. The analyzed variables are molecules that participate in the modulation of the metabolic pathway of the studied microorganism.

Although assays in shake flask are the most used when applying statistical designs for the biotechnological processes optimization, they have some limitations such as unable to control the pH and aeration (Liu, Wang, Du, & Chen, 2008; Gamboa-Suasnavart et al., 2013; Mohandas, Anisha, Chennazhi, & Jayakumar, 2015; Pan et al., 2017). The final pH ranged from 4.44 to 4.76 in the assays in shake flask by fractional factory design. The decrease in pH may have caused the inhibition of microbial growth and metabolites production. Therefore, fermentation in a bioreactor was performed under the best conditions obtained for the HA production in shake flask. According to results, it was defined that the best condition for the HA production by *S. zooepidemicus* was in medium containing 50 g L^{-1} of sucrose and yeast extract and 0.6 g L^{-1} of glutamate, glutamine and acid oxalic. The value predicted by the model in this condition was 0.640 g L^{-1} of HA.

Bioreactor

The fermentation in bioreactor was performed using the culture medium optimized by the fractional factorial assay. The production of HA, biomass, lactate and acetate were modeled using logistic regression (Equation 2). The proposed models (Equation 2; Table 6) showed high agreement between predicted and experimental data with R^2 values higher than 0.99. The p-values ($p < 0.001$) from Fisher's F-test also indicated the consistency of the models to describe the experimental data. The residues analysis showed homogeneity of variance and normality (Shapiro-Wilk test, $p > 0.05$) of the residual, taking into account the assumptions of the model. The kinetic parameters defined by equation 2 were statistically significant ($p < 0.001$) for biomass and production of hyaluronic acid, lactate and acetate.

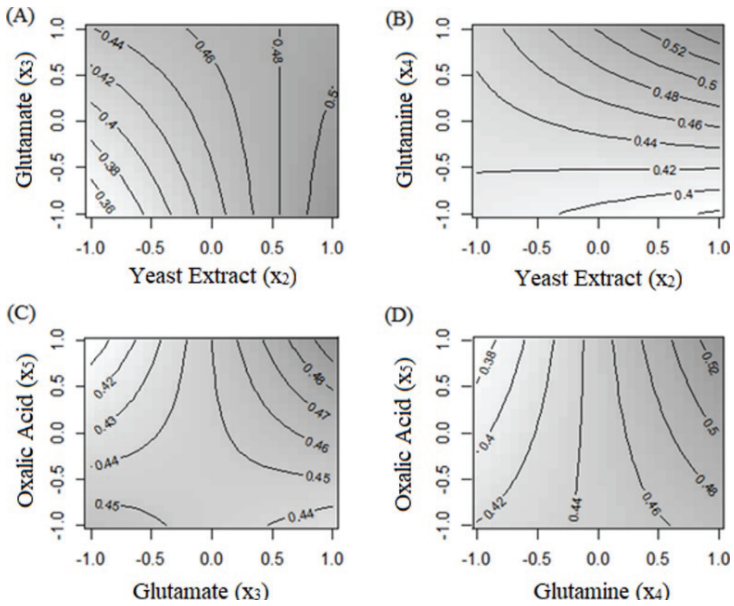

Figure 1. Contour curve of hyaluronic acid production by *Streptococcus zooepidemicus* ATCC 39920 regarding concentration of (a) glutamate (x_3) and yeast extract (x_2); b) glutamine (x_4) and yeast extract (x_2); c) oxalic acid (x_5) and glutamate (x_3); d) oxalic acid (x_5) and glutamine (x_4). Variables when fixed were in the central point.

Table 6. Parametric estimations corresponding to logistic regression (Equation 2) applied to the production of hyaluronic acid, lactate, acetate and biomass in bioreactor by *S. zooepidemicus* ATCC 39920.

Parameters	Hyaluronic acid	Lactate	Acetate	Biomass
P_{max}	0.860 ± 0.017	17.958 ± 0.398	2.503 ± 0.060	4.525 ± 0.123
K	1.712 ± 0.151	1.468 ± 0.159	1.114 ± 0.171	1.500 ± 0.223
x_0	13.036 ± 0.179	13.855 ± 0.186	12.532 ± 0.198	11.513 ± 0.260
R^2	0.996	0.994	0.991	0.988
p-value	< 0.001	< 0.001	< 0.001	< 0.001

The maximum asymptotic HA concentration was 0.860 g L^{-1} (Figure 2A). There was a 34% increase in the polymer production compared with predict value in shake flask (0.640 g L^{-1}). This increase in the HA production can be explained by the pH control, as in shake flask the pH decreased during the fermentation. Previous work has shown that the optimal pH for the polymer production by *S. zooepidemicus* ranges from 7 to 8 (Johns, Goh, & Oeggerli, 1994; Liu et al., 2008; Pan et al., 2015; Zakeri & Rasaee, 2016). Pan et al. (2017) evaluated the HA production in bioreactor with controlled and uncontrolled pH in sugarcane molasses medium. The authors obtained an increase in the polymer production of 2.86 times with controlled pH at 8.0. The aeration also influences polymer production (Jagannath & Ramachandran, 2010; Oliveira, Ogrodowski, Macedo, Santana, & Gonçalves, 2013). The positive effect of alkaline pH and aeration on the HA production might be due to the exposition of the microorganisms to the stress condition in which the cells produce the capsule as a protective mechanism (Hasegawa, Nagatsuru, Shibutani, Yamamoto, & Hasebe, 1999; Liu et al., 2008; Oliveira et al., 2013).

The synthesis of organic acids and biomass also increased in bioreactor compared to the shake flask assays (Figure 2B). After 8 hours of lag phase, cells started exponential growth up to 14 hours, and biomass concentration reached maximum asymptotic concentration of 4.525 g L^{-1}. The maximum production of lactate and acetate were 17.985 and 2.503 g L^{-1}, respectively (Figure 2C and D).

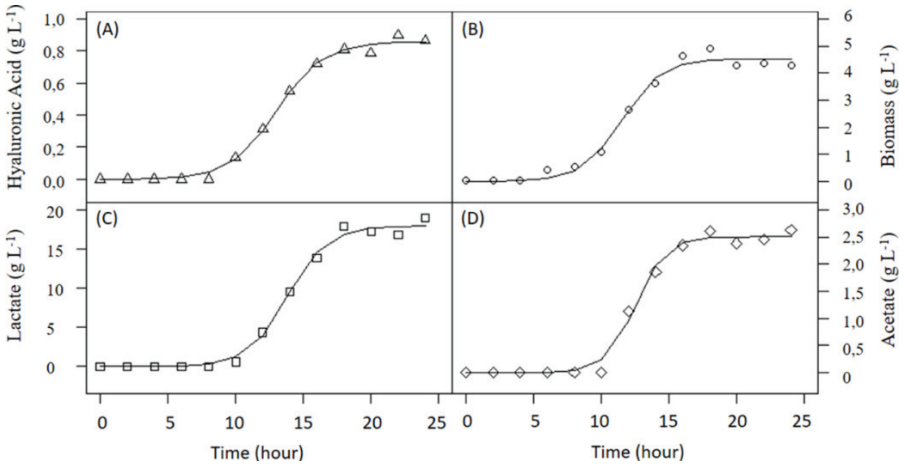

Figure 2. Production of hyaluronic acid (A), biomass (B), lactate (C) and acetate (D) by *S. zooepidemicus* ATCC 39920 in bioreactor.

Conclusion

In this study, the applied statistical tools proved to be efficient for optimizing the hyaluronic acid production by *S. zooepidemicus*. The best condition for the polymer production was 50 g L^{-1} of sucrose and yeast extract and 0.6 g L^{-1} of glutamine, glutamate and oxalic acid. Yeast extract was the significant explanatory variable that exhibited higher positive effect on the hyaluronic acid production and synthesis of lactate, acetate and biomass. In bioreactor, using the optimized condition, fixed pH 8.0 and 0.5 vvm, the variable responses were modeled using logistic regression. The results showed high agreement between predicted and experimental data ($R^2 > 0.99$). The maximum asymptotic hyaluronic acid concentration was 0.860 g L^{-1}. Thus, the hyaluronic acid production was increased by media fermentation optimization and its production kinetic was modeled using logistic regression. The results suggest that statistical methods can be used in the fermentation industry to increase production.

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