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Original paper

Optimization and kinetics studies on exopolysaccharide production using Klebsiella oxytoca ICCF 419 strain, and glucose as substrate

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Abstract The present work aimed to investigate the efficiency of kinetic modeling and Response Surface Methodology (RSM), by employing a Central Composite Rotatable Design (CCRD) and to evaluate the effects of carbon and nitrogen sources, inoculation volume, and bioprocess time on an exopolysaccharide (EPS) producer, *Klebsiella oxytoca* ICCF 419, in batch fermentation with shaken flasks. Among the kinetic models tested, logistic equations were found to fit accordingly, with a correlation coefficient (R^2) of 0.999. The interaction between glucose and corn steep liquor was found to have a significant impact on the EPS production, and glucose (2% w/v), and a 5% v/v inoculum of 24 hours age, are sufficiently to positively affect the bioprocess, if it is desired only a maximum cell growth, but not associated with the polymer synthesis. The optimal formula predicted by the RSM design was experimentally validated by the following results: 12.16 g EPS/L and 1.65 dry biomass/L, after 48 hours fermentation.

Keywords Bacterial exopolysaccharide (EPS), *Klebsiella oxytoca*, microbial kinetics, Central Composite Rotatable Design (CCRD), bioprocess optimization.

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Introduction

Chemically synthesized polymers generate a negative impact on the ecosystems, being non-degradable, toxic for humans and animals, and difficult to dispose of, aspects which lead to environmental pollution and human safety risks. Discovery of new bio-based polymers, environmentally friendly natural polysaccharides especially, shows a growing interest among researchers (KUMAR et al, 2007; DUBÉ and SALEHPOUR, 2014).

Bacterial polysaccharides, as natural polymers and renewable resources emerged as industrially important materials, due to the recent development of greener technologies. These polymeric compounds are proving advantages over the gums produced by plants or algae, attracting the interest due to their novel characteristics and their extensive application potential in food, cosmetics and pharmaceuticals, biomedicine, oil industries and other (BUENO and GARCIA-CRUZ, 2006; KUMAR et al, 2007; BADEL et al, 2011).

Microbial EPSs are exploited for the industrial applications, because they have a structural variability that offers physico-chemical and biological properties, a better quality and a high level of purity over the polysaccharides of plant origin. Some examples of application fields, where EPSs were successfully introduced are: medicine – EPS with therapeutic effects (antitumor, antioxidant, antibacterial, antiulcer, immunomodulatory, antidiabetic, antiviral and cholesterol-lowering activities), cosmetics and pharmacology – i.e cellulose as stabilizer of emulsions, acoustic membrane or artificial skin, food industries – EPSs as thickening, stabilizing and emulsifying agents (xanthan, gellan or curdlan – additives, gelrite – gelling agent, emulsan), in heavy metal removal and enhanced oil recovery, and EPSs as sources of monosaccharides (L-fucose, L-rhamnose, L-altrose, D-mannose (i.e clavan). (KUMAR et al, 2007; BADEL et al, 2011; AHMAD et al, 2015; AHMAD and ALKARFY, 2015; ALMALKI, 2019) Among the polysaccharides produced by species belonging to the genus *Klebsiella*, a report by Nakanishi et al (2001) reminds of their antistatic properties, stabilizing activity suited for blending them in cosmetics, or film forming with high break elongation properties. Furthermore, Leone et al (2007) presents the *K. oxytoca* strains as producing EPSs of environmental and pharmaceutical interest, but also being nitrogen-fixing organisms.

The first experimental design was originally developed by Fisher in the 1930s and corresponded to the analysis of variance and factorial design with application in agricultural and biological research. Box and Wilson discussed about response surface methodology (RSM) later in the 1950s, with regard to some chemical experimentations. Over time, the research evolved and statistical experimental designs provided a large amount of information as an efficient and more economical approach to develop and optimize various bioprocesses, considering the identification of the most significant factors and interactions for the culture cultivation (AHMAD et al, 2015; MÄKELÄ M, 2017).

The present work was aimed to evaluate and improve the performance of fermentative production using *K. oxytoca* ICCF 419. In view of this, statistical experimental design and mathematical modeling (CCRD-RSM, ANOVA, logistic and Gompertz models) were applied to study the effects of four variables and their interactions, and to estimate the kinetic parameters of the bioprocess. This is one of the first reports on the kinetics of the EPS producer, *K. oxytoca* ICCF 419 strain.

Materials and Methods

Microorganism and EPS biosynthesis

K. oxytoca ICCF 419 strain was recently isolated from bitter cucumber (*Momordica charantia*) roots, by using nutrient agar and King's B medium agar plates. After some initial screening experiments for EPS production, it was selected for further studies and subcultured periodically on Bacto Emerson agar. The microorganism is deposited in the Culture Collection of Industrial Importance Microorganisms (CMII), belonging to the National Institute for Chemical-Pharmaceutical R&D, ICCF Bucharest.

The strain was grown in FP medium (ICCF formula), by incubating the Erlenmeyer flasks of 500 mL capacity with a total working volume of 100 mL, at 33-34°C, 220 rpm. The medium components were (% w/v): glucose 4.0, corn steep liquor 1.5, KH_2PO_4 0.2, $MgSO_4$ 0.05, NaCl 0.2 and citric acid 0.1, with an initial pH of 7.5. Different concentrations of 24 hours (hrs) inoculums (5, 10 and 15% v/v) were used for the basal fermentation medium, and then incubated at 33-34°C, 220 rpm, for 26, 52 and 78 hrs.

Bacterial growth was estimated by measuring optical density of cultures with a UV-VIS spectrophotometer Helios (Gamma), at $\lambda = 540$ nm, and then converted to dry weight, which was obtained by harvesting cultures at 4°C, centrifugation at 8000 rpm for 15 minutes and vacuum-drying at 105°C for 10 hrs, in a Memmert oven.

Glucose concentration was measured accordingly to the iodometric method of Schoorl.

The productivity of EPS was expressed in terms of weight (grams per liter of culture medium) after isolation by ethanol p.a. precipitation (three volumes) of the cell free supernatant, and vacuum-drying to constant weight, at 35-85°C, for 8-10 hrs.

Kinetic modeling

The modeling of EPS synthesis, microbial growth and substrate consumption kinetics was utilized to describe the fermentation process, according to the following equations:

Logistic model (DHANASEKAR et al, 2003):

$$X(t) = \frac{X_0 e^{\mu_{max} t}}{1 - \left[\frac{X_0}{X_{max}}\right]\left(1 - e^{\mu_{max} t}\right)} \qquad (1)$$

Gompertz model (THOMSON and OLLIS, 1990; ZWIETERING et al, 1990):

$$X(t)=X_{\max}\exp\left\{-\exp\left[\frac{\mu_{\max}e}{X_{\max}}(\lambda-t)+1\right]\right\} \quad (2)$$

$$P(t)=P_{\max}\exp\left\{-\exp\left[\frac{\mu_{\max-P}e}{P_{\max}}(\lambda-t)+1\right]\right\} \quad (3)$$

$$S(t)=S_m-S_m\exp\left\{-\exp\left[\frac{\mu_{m-S}e}{S_m}(\lambda-t)+1\right]\right\} \quad (4)$$

Logistic Luedeking-Piret model (ZWIETERING et al, 1990; LUEDEKING and PIRET, 2000):

$$P(t)=P_0+\alpha\left[\frac{X_0e^{\mu_{max}t}}{1-\frac{X_0}{X_{max}}\left(1-e^{\mu_{max}t}\right)}-X_0\right]+\beta\left(\frac{X_{max}}{\mu_{max}}\right)\ln\left[1-\frac{X_0}{X_{max}}\left(1-e^{\mu_{max}t}\right)\right] \quad (5)$$

$$S(t)=S_0-\gamma\left[\frac{X_0e^{\mu_{max}t}}{1-\frac{X_0}{X_{max}}\left(1-e^{\mu_{max}t}\right)}-X_0\right]-\eta\left(\frac{X_{max}}{\mu_{max}}\right)\ln\left[1-\frac{X_0}{X_{max}}\left(1-e^{\mu_{max}t}\right)\right] \quad (6)$$

where X is the instantaneous biomass concentration, X_0 - initial biomass concentration, X_{max} - maximum biomass concentration, P - instantaneous product concentration, P_0 initial product concentration, S - instantaneous substrate concentration, S_0 - initial substrate concentration, α, β, γ and η - empirical constants depending on the fermentation conditions.

Experimental values were fitted to the models above by using the computational Matlab programming (R2017a, MathWorks, Natick, MA), and initial values of the parameters were obtained from model linearization, according to Mohammad et al. (1995). Kinetic coefficients were calculated using sequential quadratic programming, Design Software - Expert 9 (Stat-Ease, Inc., Minneapolis, U.S.A.).

Experimental design by RSM-CCRD and statistical analysis

The variables, namely fermentation time (hrs), inoculation volume, and concentrations of glucose and corn steep liquor were selected to investigate the optimization parameters of EPS production by statistical approach based on CCRD-RSM and using Design-Expert Version 9.0.6.2. Software (State Ease, Minneapolis, MN). The process parameters were initially evaluated by Taguchi method, in a previous study of EPS screening.

The selected independent variables for the EPS production, cell growth and substrate utilization were evaluated at three different levels. The final equations in terms of coded factors are:

a) Polysaccharide *= + 1.01 - 0.012 * A + 0.034 * B + 0.029 * C - 0.096 * D - 0.046 * AB + 8.094E-003 * AC + 0.013 * AD - 0.040 * BC - 0.040 * BD + 0.095 * CD - 0.23 * A^2 - 0.036 * B^2-0.10 * C^2 - 0.14 * D^2;*

b) Biomass *= + 0.44 + 0.11 * A - 0.045 * B + 0.13 * C + 0.035 * D - 0.079 * AB + 0.074 * AC + 4.312E-003 * AD -0.049 * BC + 0.021 * BD - 0.028 * CD - 0.012 * A^2 - 0.071 * B^2 + 0.097 * C^2 + 0.057 * D^2;*

c) Substrate *= + 0.14 - 0.18 * A - 0.048 * B - 0.56 * C + 0.61 * D + 0.091 * AB + 0.068 * AC - 0.021 * AD + 0.035 * BC - 0.037 * BD - 0.48 * CD + 0.32 * A^2 + 0.028 * B^2 + 0.29 * C^2 + 0.24 * D^2;*

where the actual factor significance is: A - incubation time (hrs), B - inoculum volume (% v/v), C - glucose (% w/v) and D - corn steep liquor (% w/v).

The CCRD design consisted of 30 experimental runs and the experimental results were performed as average values derived from duplicates samples. Analysis of variance (ANOVA) was employed to study the significance of designed models, evaluating statistical parameters such as p-values, F-values, correlation coefficients (R^2), mean square error (MSE), and root mean square error (RMSE). Response surface (3D) and contour plots (2D) were obtained based on the models equations in order to investigate the optimal parameters and to visualize their interactions, for a better EPS production.

Results

Kinetic modeling

Results of EPS synthesis, cell growth and substrate consumption were fitted by using the Logistic, Gompertz and Logistic Luedeking-Piret models, and the model with best fit was selected on the basis of the highest correlation coefficient (R^2>0.99). The parameters of the models are given in Table 1.

Table 1. Statistical coefficients

Response	Model	RMSE	MSE	R^2
Biomass	Logistic	0.004	0.004	0.999
	Gompertz	0.005	0.006	0.999
	Logistic Luedeking-Piret (LLP)	0.009	0.011	0.997
Crude polysaccharide content	Logistic	0.011	0.012	0.999
	Gompertz	0.016	0.014	0.998
	Logistic Luedeking-Piret (LLP)	2.936	3.242	0.995
Substrate concentration	Logistic[3]	0.081	0.088	0.996
	Gompertz	0.071	0.078	0.997
	Logistic Luedeking-Piret (LLP)	0.002	0.003	0.999

The batch experimental data of biomass, EPS content and glucose was obtained as an average of duplicates and plotted against time, as shown in Fig. 1.

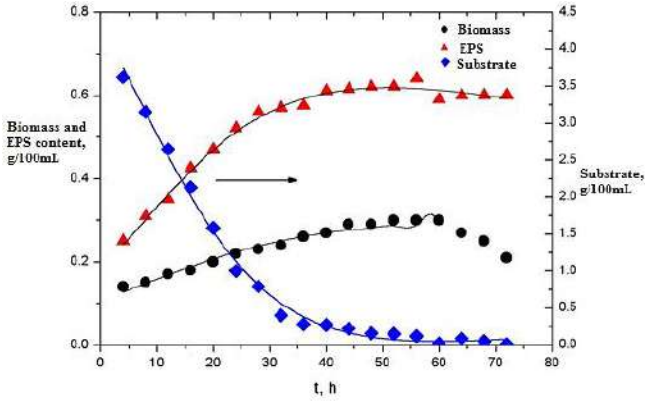
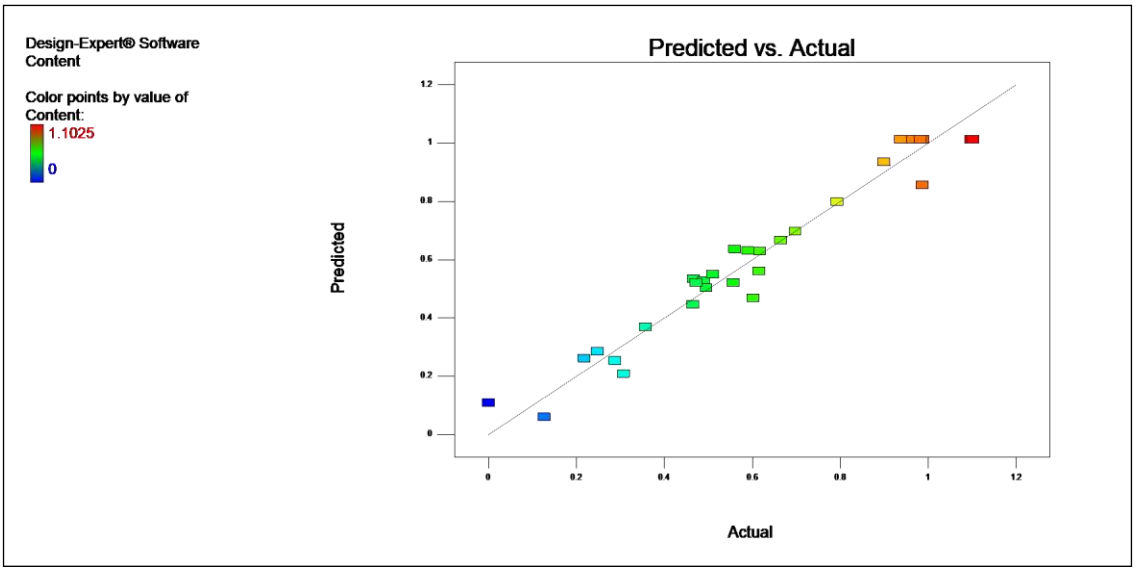


Figure 1. Kinetic modeling for the *K. oxytoca* ICCF 419 – Logistic Luedeking-Piret model

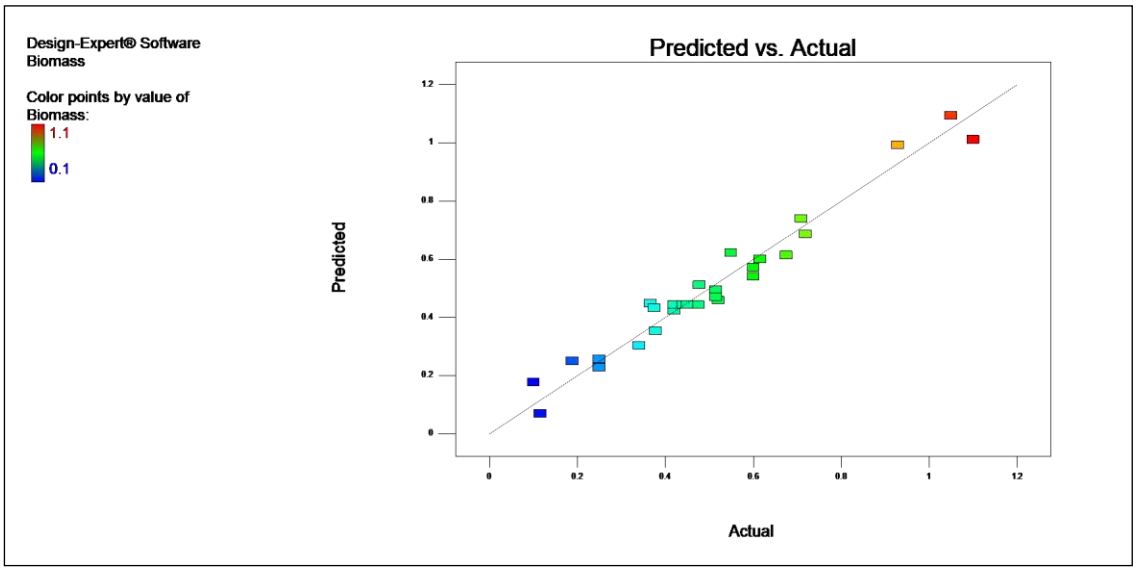
Investigation of fermentation parameters effects using RSM-CCRD

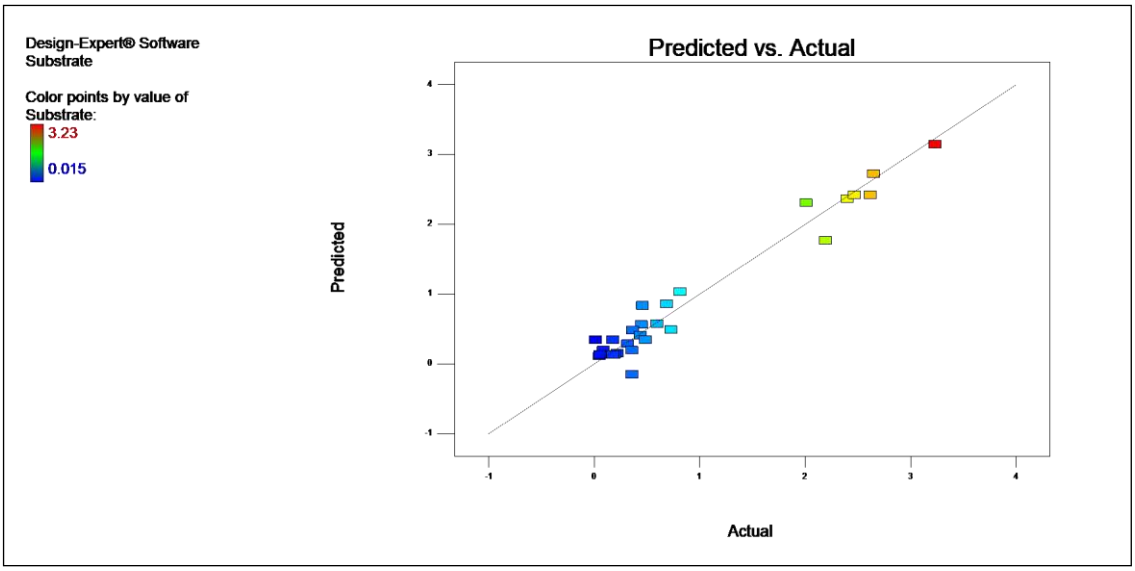
Experiments were carried out to examine the effect of four different process parameters on EPS formation, cell growth and substrate utilization in the fermentation broth. The experimental values were in agreement with the predicted response values, as shown in Fig. 2.

a)



b)





c)

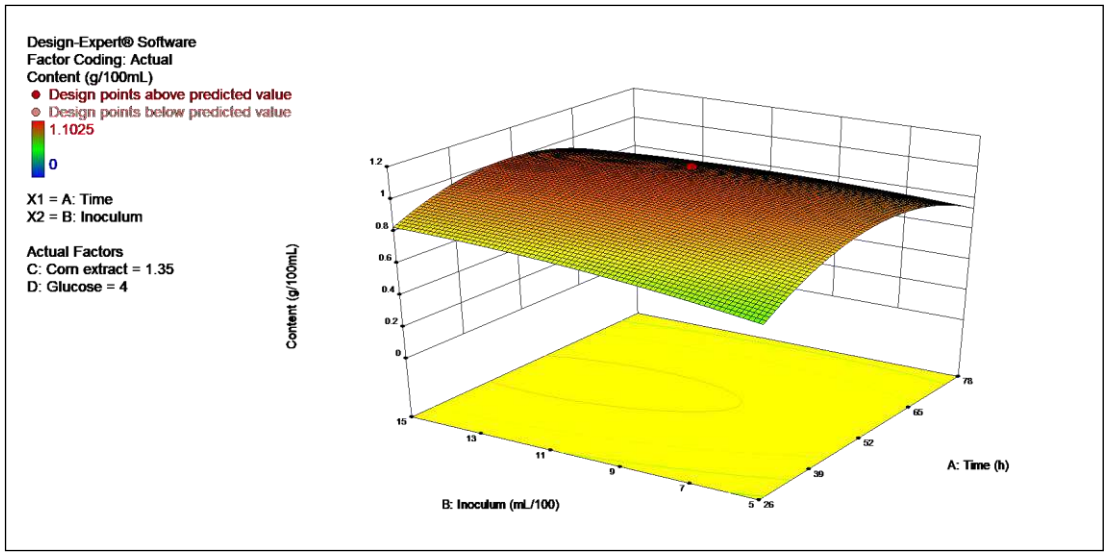
Figure 2. Predicted vs experimental values of: **a)** EPS production; **b)** Biomass growth; **c)** Substrate consumption

The adequacy of the models was examined through ANOVA and the results are given in Table 2.

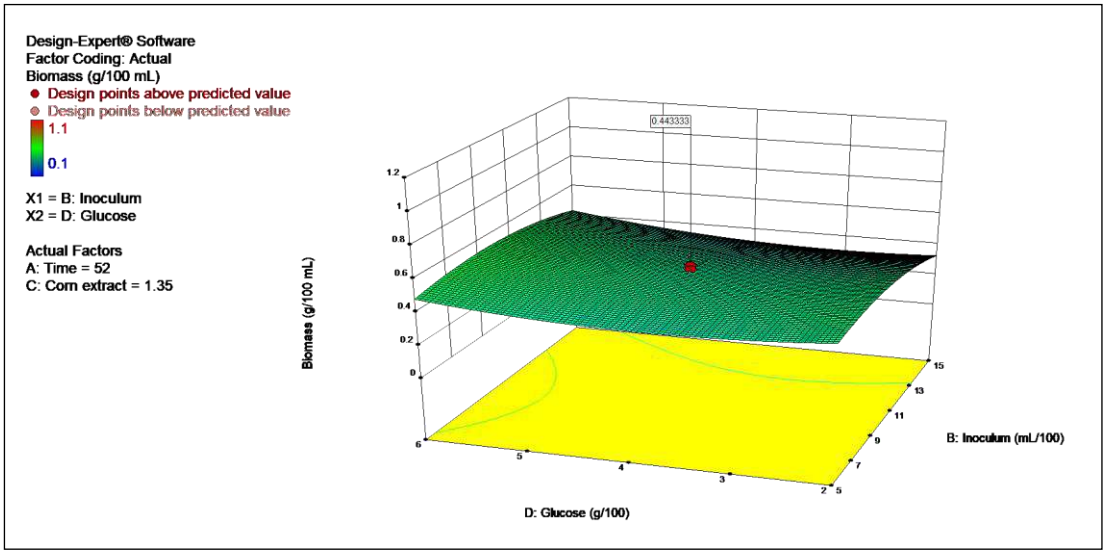
Table 2. Analysis of variance for the response surface Quadratic model

Polysaccharide			**Biomass**			**Substrate**		
Source	**F value**	**p-value**	**Source**	**F value**	**p-value**	**Source**	**F value**	**p-value**
Model	**22.52**	**< 0.0001**	**Model**	**26.67**	**< 0.0001**	**Model**	**25.70**	**< 0.0001**
A-Time	0.48	0.5001	**A**-Time	68.01	< 0.0001	**A**-Time	10.82	0.0050
B-Inoculum	3.66	0.0749	**B**-Inoculum	11.59	0.0039	**B**-Inoculum	0.74	0.4045
C-Corn extract	2.71	0.1207	**C**-Corn extract	96.78	< 0.0001	**C**-Corn extract	100.59	< 0.0001
D-Glucose	28.89	< 0.0001	**D**-Glucose	6.89	0.0191	**D**-Glucose	122.11	< 0.0001
AB	4.47	0.0516	**AB**	23.80	0.0002	**AB**	1.80	0.1999
AC	0.14	0.7159	**AC**	20.68	0.0004	**AC**	1.01	0.3310
AD	0.33	0.5724	**AD**	0.071	0.7941	**AD**	0.094	0.7638
BC	3.40	0.0852	**BC**	9.14	0.0086	**BC**	0.26	0.6174
BD	3.40	0.0852	**BD**	1.62	0.2219	**BD**	0.30	0.5925
CD	18.81	0.0006	**CD**	2.99	0.1043	**CD**	50.51	< 0.0001
A^2	193.65	< 0.0001	**A^2**	0.92	0.3537	**A^2**	36.94	< 0.0001
B^2	4.73	0.0460	**B^2**	32.68	< 0.0001	**B^2**	0.29	0.6006
C^2	36.68	< 0.0001	**C^2**	61.71	< 0.0001	**C^2**	31.78	< 0.0001
D^2	72.40	< 0.0001	**D^2**	20.96	0.0004	**D^2**	20.52	0.0004

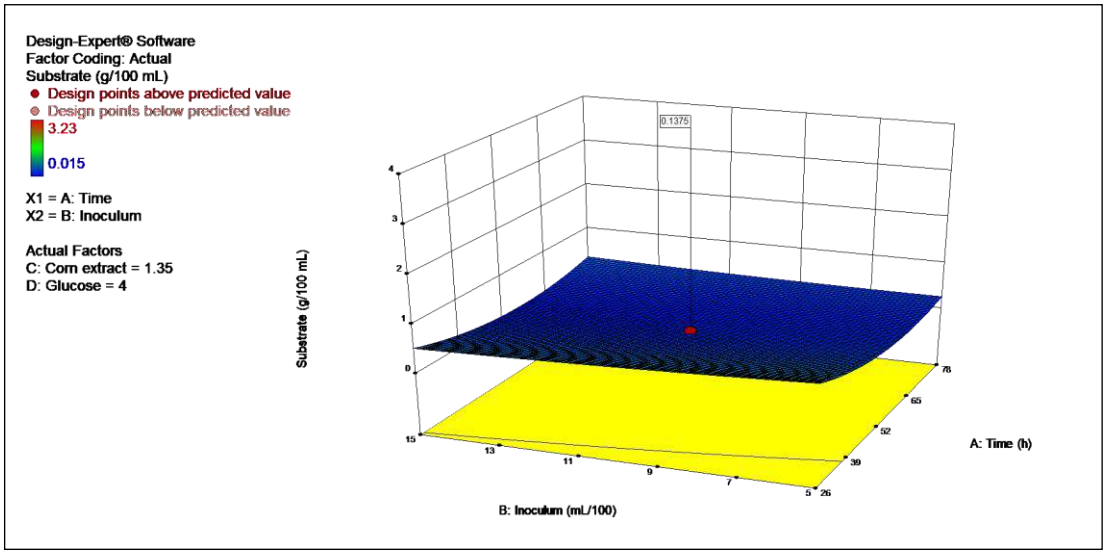
The 3D plots are shown in Fig. 2, in which the main interactions affecting the EPS formation, biomass growth and substrate utilization are presented to be as between corn extract and glucose, and corn extract an time.



a)



b)



c)

Figure 3. RSM 3D profile of the: **a)** EPS production and the CD factors interaction (corn extract and glucose); **b)** Biomass growth and the AC factors interaction (time and corn extract); **c)** Substrate utilization and the CD factors interaction (corn extract and glucose)

Discussions

Carbon (sucrose, glucose, lactose, maltose, whey, starch, mannitol, sorbitol or sugar concentrates, methanol, and C_9 to C_{16} n-alkanes) and nitrogen sources (ammonium sulfate, peptone, sodium nitrate, urea, and yeast extract) are used to produce microbial polysaccharides, and they have effects on the yield of the EPS, or even on its molecular size. Organic nitrogen is preferred for higher specific growth rates and polymer synthesis, due to their growth factors (DUMITRIU, 1998).

Previous reports mentioned that a limitation in the nitrogen source, and an excess of carbon is recommended for the EPS producing *Klebsiella* spp. This means that a higher C/N ratio sustains the biopolymer production (DUMITRIU, 1998). In the case of a limitation in carbon source (i.e glucose), the C/N value will be lower, the EPS will be still produced, with the advantage of a reduced accumulation of by-products (SUTHERLAND, 1982; RAMIREZ-CASTILLO and URIBELARREA, 2004).

Therefore, the medium composition and the relation between carbon and nitrogen sources constitute significant parameters that influence the EPS biosynthesis.

A logarithmic cell growth with a minimum value as 1.4 g dry biomass/L was observed in the first 42 hrs, followed by the stationary phase, with a maximum experimental at 3.16 g dry biomass/L after 52 hrs, and a decreased value registered in the decline phase after 60 hrs of fermentation.

The strain constantly produces EPS during the exponential phase, with a maximum of 6.4 g crude product/L (at 30 hrs of fermentation), and when it enters in decline phase, the EPS content in the fermentation broth starts to decrease, which may lead to the idea that a hydrolysis could be involved.

An immediate decrease of glucose in the fermentation broth, and an inverse proportionality relation between substrate, and cell growth or EPS production wereobserved when the strain started to exponential multiply. Glucose was consumed during the period from 8 and 40 hrs of fermentation, reaching a minimum value of 2.7 g/L, and assuming that this was the moment when the cells were metabolically inactive, and started the autolysis.

Analyzing the statistical coefficients presented in Table 1, the kinetic models used to study the bioprocess parameters, it could be affirmed that Logistic equations are fitting well (R^2> 0.99). The Logistic Luedeking-Piret (LLP) and Gompertz models are comparable and recommended to use for an entire time-domain data analysis, than using the Monod model, which has a linear allure and allows only a linear growth domain analysis (up to 52 hrs and 44 hrs of fermentation – from previous results). Therefore, applying Logistic models offers the closest interpretation of the present experimental data. The results showed a correlation coefficient (R^2) of 0.999.

Experimental results obtained for CCRD design showed the highest concentration of crude EPS (11.03 g/L) for run 2. However, the most profitable formula due to the resources and time involved in fermentation was found to be run 30, with an EPS content of 9.87 g/L, and the following culture conditions: incubation time 26 hrs, inoculation volume of 15% (v/v), corn extract 0.7% (w/v) and glucose 2% (w/v). Higher concentrations of polysaccharide were obtained as an average of 10 g/L (for runs 2, 21, 22, 23, 24 and 26), and the following cultivation conditions: 52 hrs of bioprocess, inoculation volume of 10% (v/v), corn extract 1.35% (w/v) and glucose 4% (w/v). The lowest production values were measured as an average of 2.37 g/L (for runs 1, 5, 7, 18 and 20), at different conditions, such as incubation time (i.e after 104 hrs of fermentation, when most likely a hydrolysis was involved - run 18) or high concentration of carbon source (6% w/v), and low concentration of nitrogen source (0.7% w/v) with an extreme value of C/N as 85.6 (run 20).

The highest values of dry biomass were determined for runs 17, 28 and 19, as 11, 10.49 and 9.29 g/L, with a corresponding EPS content of 4.9, 6.65 and 5.9 g/L, which demonstrates a condition of inverse proportionality between biomass and EPS production, regardless of time, inoculation or carbon source concentration. One of the bioprocess parameters that affects biomass growth, in terms of decreasing it as the bioprocess is prolonged, was found to be the fermentation time. A lower carbon source (2% w/v), with a small inoculation volume (5% v/v) and a longer incubation time (78 hrs) is desirable for cell growth, but not for EPS synthesis. Also, an initial glucose concentration of 8% (w/v) is not recommended for the EPS formation using the ICCF 419 strain. Lower values of biomass were measured for runs 9, 29, 27, and 13, as 1.88, 1.15, and 2.5 g/L. A larger volume of inoculum (20% v/v) and a glucose concentration of 4% (w/v) affect biomass by decreasing it, but does not interfere with the EPS content (9 g/L). Comparing runs 13 and 27, with initial culture conditions, such as glucose 2% (w/v) and corn extract 0.7% (w/v), different inoculation volume (15 and 5% v/v), and fermentation times (78 hrs and 26 hrs), it was found that the biomass value was higher after 26 hrs (4.66 g/L), while after 78 hrs it decreased to 2.5 g/L, despite using a concentrated inoculum. However, the EPS content increased from 4.66 g/L (26 hrs of bioprocess) to 6.98 g/L (78 hrs of bioprocess), which confirms the inverse proportionality of biomass and polysaccharide production.

Approximately a total consumption of glucose (<1 g/L in the fermentation media) used as a carbon source and, implicitly, as a substrate for polysaccharide production was determined for runs containing an initial concentration of 2 and 4% (w/v), while 6 and 8% (w/v) are not recommended when the aim is to produce higher yields of EPS.

Analysis of variance was conducted, taking into consideration polysaccharide content, biomass growth and substrate utilization as responses, and the quadratic model was found to fit well (Table 2).

Therefore, in the case of *polysaccharide synthesis*, the calculated ANOVA results were as following: the F-value of 22.52 implies that the quadratic model is significant and there is only a 0.01% chance that this could occur due to noise; a value of "Prob>F" less than 0.05 indicates that model terms are significant and the "Lack of Fit F-value" of 1.81 implies that the model fits and there is only a 26.53% chance that this could occur due to the noise.

Terms such as D, CD, *A^2*, B^*2*, C^*2*, and D^*2* were significant, which means that glucoseitself was significantly

effective on the EPS production, with a p-value < 0.0001; also, the interaction between corn extract and glucose influenced the EPS content, with a p-value of 0.0006. However, the corn extract itself was not significantly effective, registering a p-value of 0.1207.

The model had high values for R^2, R^2(adj) and R^2(pred) of 0.95, 0.91 and 0.78, indicating that the experimental results were fitted accordingly. Also, the value of 15.43 corresponding to the "Adeq Precision" indicates adequate signal to use these model in further spatial designing.

In the case of *cell growth*, the F-value of 26.67 implies that the quadratic model is significant and the "Lack of Fit F-value" of 15.44 means that it fits and there is only a 0.37% chance that this could occur due to the noise (Table 2). ANOVA evaluation showed that terms such as A, B, C, D, AB, AC, BC, B^2, C^2, and D^2 were significant, which means that all factors themselves effectively influence the biomass (p-value <0.0001); also, the interactions between fermentation time and inoculum volume or corn extract influenced the biomass production (p-values of 0.0002 and 0.0004).

High values for R^2, R^2 (adj) and R^2(pred) of 0.96, 0.93 and 0.78 were obtained, indicating that the experimental results were fitted accordingly. The "Pred R-Squared" value of 0.78 is in a reasonable agreement with the "Adj R-Squared" value of 0.93, because the difference is less than 0.2. Also, the value of 22.30 corresponding to the "Adeq Precision" indicates adequate signal to use the models in further spatial designing.

In the case of *substrate utilization*, the F-value of 25.70 implies that the quadratic model is significant and the "Lack of Fit F-value" of 40.04 demonstrates that the model fits and there is only a 0.04% chance that this could occur due to the noise (Table 2). ANOVA analysis showed that terms, such as A, C, D, CD, A^2, C^2, and D^2 were significant, which means that fermentation time, corn extract, and carbon source themselves effectively influence the substrate consumption (p-values of 0.005 and <0.0001); also, the interaction between corn extract and glucose (p-value <0.0001) was significant.

High values for R^2, R^2 (adj) and R^2 (pred) of 0.96, 0.92 and 0.77 indicate that the experimental results were fitted accordingly. The "Pred R-Squared" value of 0.77 is in reasonable agreement with the "Adj R-Squared" value of 0.92, because the difference is less than 0.2. Also, the value of 25.70 corresponding to the "Adeq Precision" indicates adequate signal to use the quadratic model in further spatial designing.

As represented in Fig. 2, the experimental data were three-dimensionally plotted, and the main factors interactions affecting the EPS production, biomass growth and glucose consumption were evaluated.

The EPS content was maximized when substrate was added in an initial concentration of 40 g glucose/L, while the corn extract concentration was 1.35% (w/v), which means that the interaction between carbon and nitrogen sources contributes to the product biosynthesis.When other concentrations were used, lower values of the EPS content were registered. Inoculation volume was 10% (v/v), and fermentation time was 52 hrs.

If we consider the biomass profile, it was observed that for obtaining a sufficient cell growth and an optimum EPS production was very important to use an inoculation volume of 10% (v/v), and to maintain the C/N values between 28 and 30. Lower levels of biomass were produced when the inoculation volume was higher than 15% (v/v), and the lowest concentration of 1.15 g dry biomass/L was obtained in the case of a 20% (v/v) inoculum. A higher inoculum concentration and a longer time for the bioprocess will lead to a decrease in the biomass content. In order to obtain higher values of dry biomass, but also considering the EPS production, it is recommended to use as initial cultivation conditions, the followings: an inoculum of 10% (v/v), 52 hrs of fermentation time, glucose 4% (w/v), and corn extract 1.35% (w/v). In the case of a smaller inoculation volume (5% v/v), a higher concentration of biomass will be obtained only if the bioprocess time is extended more than 70 hrs. The interaction between time and inoculation volume is significantly effective on biomass production, but it strictly depends on the concentration of carbon and nitrogen sources in the fermentation media (there is a direct proportionality relation, as their concentrations incubation time are increasing, higher will be the biomass yield).

The main interaction which affected the substrate consumption was between carbon and nitrogen source, and taking into account the incubation time and inoculum volume.

The optimum conditions to have a substrate consumption close to zero, are as following: glucose 4% (w/v), corn extract 1.35% (w/v), inoculum volume of 10% (v/v), and fermentation time of 52 hrs. An inverse proportionality relation between bioprocess time and glucose utilization is shown, but at the same time, if higher carbon concentrations are used, this will impact more the cellular growth than EPS production.

Following the RSM modeling and data interpretation, bioprocess parameters were estimated considering a medium composed of glucose 2.78 and corn extract 1.09 (%w/v), and 15% (v/v) inoculum, 48 hrs of fermentation, with a C/N value of 25.42. The predicted values were: 10.6 g/L for the crude EPS content, 2.9 g/L for dry biomass and 1.4 g/L substrate in the fermentation broth. To validate the prediction of the models, additional experiments were performed at optimized conditions, and the results were close to the expectations: 12.16 g EPS/L, 1.65 g dry biomass/L and 1.8 g glucose/L.

If we compare with the available data, Qiang et al (2013) reported a maximum yield of 15.05 g EPS/L produced by a *Klebsiella* strain, after employing RSM optimization with sucrose (31.93 g/L) as substrate, and a maximization in the EPS production was observed when KNO_3 (3 g/L) was added as nitrogen source. Saad et al (2017) reported a maximum EPS of 7.88 g/L yield produced by a *K. oxytoca* strain, when sucrose was used in an RSM optimization study.

Conclusions

There is an increased demand for microbial EPSs used in foods, medicines and industrial products, and in this view, isolation of new biopolymers and testing them for innovative applications has found research support (POLI et al, 2010).

The RSM-CCRD results obtained by using the newly-isolated *K. oxytoca* ICCF 419 strain gave promising results for further optimization and application studies in order to maximize the EPS yield produced when glucose is used as substrate. It was found that a higher C/N ratio is favorable for the microbial EPS synthesis, initial concentrations of glucose and corn steep liquor, such as 27.8, and 10.9 g/L, respectively (C/N = 25) resulted in 12.16 g EPS/L, and 1.65 g dry biomass/L, after 48 hours of fermentation. The bioprocess kinetics suggested that Logistic models could be successfully applied (R2 = 0.999) to study EPS production, biomass growth and substrate utilization.

Further studies are needed to maximize the EPS yield by using a cost effective cultivation formula.

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