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Original paper

Investigation of culture conditions by Response Surface Methodology and kinetic modeling for exopolysaccharide production by Klebsiella oxytoca ICCF 419 strain, using lactose as substrate

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Abstract The objective of this work was to optimize the bioprocess parameters, using *Klebsiella oxytoca* ICCF 419 to obtain an exopolysaccharide based on lactose as substrate. A kinetic study was employed and Logistic and Gompertz models were applied to describe the polysaccharide production, in relation with biomass growth and substrate consumption. The RSM methodology based on Central Composite Rotatable Design was used to evaluate and optimize the effect of lactose, corn extract, KH_2PO_4 and citric acid concentrations as independent variables on the polysaccharide production, biomass growth and substrate consumption as the response functions. The interaction effects and optimal parameters were obtained using Design Expert Software (version 9.0.6.2). The significance of the variables and their interactions was tested by means of ANOVA analysis with a 99% confidence level. The optimum culture conditions were determined and the model prediction was compared with experimental results. At an initial value of 23.45 for the C/N in the fermentation medium, the strain produces 17.41 g/L of crude polysaccharide and 2.53 g/L dry biomass. The EPS production was significantly influenced by lactose, corn extract and KH_2PO_4, while the citric acid had no influence. The biomass growth was influenced by the corn extract, KH_2PO_4 and citric acid.

Keywords *Klebsiella oxytoca* ICCF 419 strain, KH_2PO_4.

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Introduction

The natural environment represents a valuable resource of microorganisms for the polysaccharide biosynthesis, while bacteria are considered as a significant source of bioactive natural products (SEKUROVA et al, 2019). The need for new therapeutic agents, with low toxicity, obtained from renewable resources which can replace the petroleum-based ones, contributed in the last decades to the discovery of novel compounds (VENTORINO et al, 2019; YADAV et al, 2019). Microbial exopolysaccharides (EPSs), as secondary metabolites, advantageous for environmental adaptation and bacteria survival, represent a class of superior biopolymers with an extensive range of industrial applications (pharmaceuticals, nutraceuticals and cosmetics, chemicals, biomedicine, food and agriculture, packaging industries, environmental protection), which present multiple advantages over their similar chemical derived compounds, such as bioactive functions, biodegradability, biocompatibility and non-toxicity. These natural polymers are well known for their beneficial effects on human health, such as immune (including the reduction of oxidative stress in relation with anti-inflammatory action and human microbiota) and digestive functions, and for their important role in nutraceuticals formulation, if we consider their contribution to the growth of probiotic organisms (BUENO AND GARCIA-CRUZ, 2006; VU et al, 2009; SANCHEZ et al., 2015; DAM et al, 2017; SEKUROVA et al, 2019; VAMANU E, 2019; VENTORINO et al, 2019). EPSs represent a good alternative to those obtained from plants, animals or seaweed, because their biosynthesis can be easily conducted in controlled conditions (VENTORINO et al, 2019). Natural molecules are produced in small yields, and this is why classical approaches of fermentation processes (upstream, downstream) need to be improved (YADAV et al, 2019), in order to ensure a constant or a higher yield; an increased understanding of the biotechnological process could be achieved by using kinetics and optimization studies.

There is a need to mathematically model the microbial growth in the fermentation, to describe the behavior of microorganisms and product formation under different physical or chemical conditions, and further elucidate the larger scale fermentation strategies (TELEKEN et al, 2010). In this context, mathematical biology has gained attention in the past years, and kinetic models are employed to solve various problems encountered in industrial bioprocesses, facilitating data analysis (GANDURI and PODHA, 2014; RAJENDRAN and THANGAVELU, 2012).

The mathematical modeling of the fermentation process provides information on the kinetics and metabolic nature of microorganisms, but also allows controlling and optimizing production during biosynthesis. The prediction of the production can have practical consequences as biomass can be produced much faster, and even in a continuous manner.

In the literature, Gompertz, Logistic, Richards, Stannard, Weibull and Morgan–Mercer–Flodin based models are used to fit well the experimental data (ALIREZA, 2008; BARANYI and ROBERTS, 1994; CHENG et al, 2010; COBAN and DEMIRCI, 2016; DODIĆ et al, 2012; FAN et al, 2004; FERCHICHI et al., 2005; GOMPERTZ, 1825; KHANNA et al, 2011; MORGAN et al, 1975; PEARL and REED, 1920; PHUKOETPHIM et al, 2017; RICHARDS, 1959; SINGHASUWAN et al, 2015; STANNARD et al, 1985; VÁZQUEZ et al, 2004; WEIBULL, 1951a; ZWIETERING et al, 1990).

Among these, Logistic and Gompertz are the most frequently models used to predict microbial growth and product formation in fermentations (COBAN and DEMIRCI, 2016). The logistic function model was originally utilized to model the biomass growth and it was applied to calculate batch kinetics of microbial growth for polysaccharide production. Luedeking–Piret and Gompertz equations were used to correlate the product formation to microbial growth and substrate consumption, and also to model the substrate consumption in relation with biomass production (KLIMEK and OLLIS, 1980; THOMSON and OLLIS, 1980; BOA and LEDUY, 1987; Mohammad et al, 1995). Predictions of the fermentation kinetics parameters have been also reported in the past few years, including: i.e. cell growth for *Xanthomonas campestris* (ZAKERI et al, 2015), *Enterobacter* sp. (MARQUES et al, 2016), *Klebsiella pneumoniae* (CHENG et al, 2015), *Lactobacillus delbrueckii* ssp. *bulgaricus* (GORANOV et al, 2015), *Sphingomonas paucimobilis* (WANG et al, 2006), *Kocuria marina* (MITRA and DUTTA, 2018), *Streptococcus* sp. (TARRAH et al, 2018), *Pediococcus acidilactici* (CHOWDHURY et al, 2007), *Rhizoppus arrhizus* (RAJENDRAN and THANGAVELU, 2012), *Aureobasidium pullulans* (GANDURI and PODHA, 2014), and described by Logistic or Gompertz models; EPSs synthesis – xanthan (ZAKERI et al, 2015), gellan (WANG et al, 2006), pullulan (CHAO et al, 2017), bioflocculant EPS (GUO et al, 2018), described by the Luedeking–Piret model; substrate utilization – vegetable sugar juices (SHARMA and MISHRA, 2014), lactose from cheese whey (MITRA and DUTTA, 2018), lactose from whey (ARIYANTI and HADIYANTO, 2013), described by Logistic, Gompertz or Monod models.

Along with the kinetics studies, bioprocess parameters optimization aims to maximize the yield and quality of the product (KUMAR et al, 2007; SIRAJUNNISA et al, 2016; SING et al, 2017). In this context, statistical approaches and experimental designs are effective, because the limitations of conventional methods can be efficiently eliminated (LIU et al, 2005; MAHAJAN et al, 2010). Response Surface Methodology (RSM) is frequently utilized as an optimization method, with applications in the development and formulation of new products. RSM offers complex information based on various designs that fit empirical models to experimental results, assessing the effects of the independent variables, but also of their interactions (BAŞ and BOYACI, 2007; AHMAD et al, 2015; MANAGAMURI et al, 2017). Industrial optimization processes are addressing to the potential problems using the RSM approach, which provides a panorama of them. Its popularity has been reported in various studies during the past years, including for the EPSs biosynthesis, such as xanthan, gellan, dextran, wellan, cellulose, hyaluronic acid, levan and many others, including polysaccharides produced by *K. oxytoca*, bioflocculants obtained from *K. pneumoniae* (ZHONG et al, 2014; Marques et al, 2016; YU et al, 2016).

This research presents a preliminary study, the main purpose of which was to obtain a microbial exopolysaccharide using a bacterial strain, *Klebsiella oxytoca* ICCF 419, recently isolated from bitter cucumber roots, and to investigate the effects generated by some key factors in the biosynthesis

process, to improve the product yield, when lactose is used as the main carbon source and substrate.

Materials and Methods

Bacterial strain and batch fermentation

A bacterial strain of Klebsiella oxytoca (K. oxytoca) ICCF 419, belonging to the CMII-ICCF-WFCC 232 Culture Collection[1], was recently isolated from a sample of bitter cucumber root nodules (*Momordica charantia*), extracted from fresh garden soil. The microorganism was stored as slant cultures in nutrient agar, King's B[2] medium and Bacto Emerson agar (BE)[3], at 4°C. Slant cultures grown in BE were transferred to 10 mL of the seed medium, IPS (an ICCF medium)[4], in Erlenmeyer flasks of 500 mL capacity, with a total working volume of 100 mL. IPS medium consisted of (% w/v): glucose 1.0, corn steep liquor 1.5, KH_2PO_4 0.2, $MgSO_4$ 0.05 and NaCl 0.2. Inoculum cultures were incubated at 33-34°C, 220 rpm. Fermentation was carried out in 500 mL conical flasks, each containing 100 mL of the sterile production medium. The basal medium LAC (an ICCF original formula) was inoculated with 10% (v/v) of 24 hrs inoculum cultures and then incubated at 33-34°C, 220 rpm, for 64 hrs. The EPS production medium was composed of (% w/v): lactose 3.0, corn steep liquor (as a waste product from industry) 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.

The rate of substrate consumption was determined by measuring free reducing sugars expressed as glucose (% w/v) in the fermentation broth, at the end of the bioprocess (Luff-Schoorl method). Samples with lactose were measured separately due to an increased viscosity, after an HCl (20%) hydrolysis, at 67°-70°C, for 10 minutes (adapted after the method of xanthan hydrolysis, in European Pharmacopoeia 9th Ed.).

Downstream process followed an adapted methodology (MOSCOVICI et al, 2009). To determine the crude EPS content, the microbial cells were first removed by centrifugation (8000 rpm/30 min/4°C) and filtration, using a celite precoat. Highly viscous samples were first diluted with distilled water (1:1, v/v). Then, the crude polysaccharide was precipitated, under vigorous shaking by adding 5 g/L of KCl and cold ethanol (99.5%), 1:3 (v/v) and the mixture was allowed to settle overnight, at 4°C, to ensure the complete precipitation. Then, the alcoholic supernatant was centrifuged (3000 rpm/15 min/4°C) and the precipitate was dried to constant weight, in a vacuum oven (Memmert) at 85°C, for 8-10 hrs.

Cell growth was measured by optical density and converted to dry weight, after centrifugation (8000 rpm/ 15-30 min/4°C) and vacuum-drying, at 105°C for 10 hours.

Optimization of fermentation parameters using RSM

Effects of the independent variables on the bioprocess and their interactions were studied, to find their optimum, lower and upper values in maximizing the EPS production.

A central composite rotatable design (CCRD) for four independent variables was used to obtain a combination of values that optimizes the response within the region of three-dimensional observation spaces, which allows designing a minimal number of experiments (MOGHANNEM et al, 2018; MONTGOMERY, 2019).

The experiments were designed using the Design Expert Version 9.0.6.2. Software (State Ease, Minneapolis, MN).

The second-order polynomial coefficients were computed in order to estimate the responses of the dependent variable and the response surface plots were also obtained by the use of the Design Expert Version 9.0.6.2. Software package.

Variables such as carbon source (lactose – 3.0, 5.5 and 8.0 % w/v) and nitrogen source (liquid corn extract – 1.15, 2.075 and 3.0 % w/v), KH_2PO_4 (0.1, 0.2 and 0.3 % w/v) and citric acid (0.1, 0.2 and 0.3 % w/v) were studied to optimize the EPS production, using the *K. oxytoca* ICCF 419 strain in shaken culture fermentations.

A central composite factorial design of $2^4 = 16$ plus 6 centred points and (2 x 4 = 8) star points, leading to a total of 30 experiments, was performed by using the Design Expert.

The F-test in ANOVA analysis was used to check the statistical significance of models equations and *p*-values lower than 0.05 were considered as significant. Verification of experiments was used to validate the results under the optimized experimental conditions.

Results were compared with predicted values from the model, with determined minor standard deviation and a representative coefficient of variation. All experimental variants were made in duplicates.

The final equations in terms of coded factors were:

(1) ***EPS*** *= +1.49-0.052*A-0.078*B+0.10*C+0.029*D+ 0.26*AB+0.061*AC-1.406E-003*AD+0.050*BC-0.025*BD-0.057*CD,*

(2) ***Biomass*** *= +0.43-0.025*A-0.17*B+0.089*C+0.12*D+ 0.091*AB+0.013*AC-0.024*AD-0.018*BC-0.18*BD-0.044*CD,*

(3) ***Substrate*** *= +0.10+0.39*A-0.28*B+0.014*C+0.024*D-0.34*AB-0.071*AC+8.812E-003*AD+0.12*BC-8.063E-003*BD-0.049*CD+0.17*A^2+0.26*B^2+0.069*C^2+ 0.031*D^2,*

where the actual factor significance is: **A** – lactose (% w/v), **B** – corn steep liquor (corn extract) (% w/v), **C** – KH_2PO_4 (% w/v) and **D** – citric acid (% w/v).

Kinetics models for EPS production, biomass growth and substrate consumption

A modified Gompertz model and a Logistic model were proposed to predict the microbial growth, polysaccharide production and substrate utilization in the fermentation process. A logistic incorporated modified Luedeking-Pirret model was also employed. The values were calculated using the computational Matlab programming (R2017a, MathWorks, Natick, MA), and initial values of the parameters were obtained from model linearization, according

[1] http://cfarm.ncpri.ro/sct_1/page_58/culture_collection_of_industrial_importance_microorganisms_-_cmii.htm

[2] King's B medium was originally described by King EMS et al, 1954, but the medium used for the *Klebsiella* ICCF 419 strain was modified, according to the formula listed in: http://cfarm.ncpri.ro/files/cmii-iccf/CMII-ICCF.pdf

[3] https://www.atcc.org/~/media/6D65EC60358F46DFA71728D028DEBB8C.ashx

[4] IPS medium – modified formula of an ICCF original medium, IPS broth (SOARE MG et al, 2017)

to Mohammad et al. (1995). Kinetic coefficients were calculated using sequential quadratic programming, Design Software - Expert 9 (Stat-Ease, Inc., Minneapolis, U.S.A.).

According to Zwietering et al. (1990), the mathematical equations are: (1) for the modified Gompertz model and (2) for the modified logistic model:

$$A_t = A_m \exp\left\{-\exp\left[\frac{B_m e}{A_m}|\lambda - t| + 1\right]\right\} \quad (1)$$

$$A_t = \frac{A_m}{1 + \exp\left[\frac{4B_m}{A_m}|\lambda - t| + 2\right]} \quad (2)$$

where A_t is the concentration of the studied factor at "t" time, A_m is the maximum concentration of the studied factor, B_m the production or consumption rate of the studied factor, λ is the duration of the lag phase and t the sampling time.

The EPS formation is described by the Leudeking–Piret kinetics (ZWIETERING et al, 1990) expressed as equation (3) and depending on both, the instantaneous biomass concentration X and growth rate dX/dt, in a linear manner:

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \quad (3)$$

where α and β are empirical constants depending on the fermentation conditions.

Solving the equation (3) based on the logistic equation, the logistic incorporated Luedeking–Piret model for the EPS production is:

$$P(t) = P_0 + \alpha\left[\frac{X_0\exp(\mu_m t)}{1 - \frac{X_0}{X_m}\left(1 - \exp(\mu_m t)\right)} - X_0\right] + \beta\frac{X_m}{\mu}\ln\left[1 - \frac{X_0}{X_m}\left(1 - \exp(\mu_m t)\right)\right] \quad (4)$$

The kinetics of substrate concentration in relation with time is descrbed by the following equation:

$$S(t) = S_0 - \gamma\left[\frac{X_0\exp(\mu_m t)}{1 - \frac{X_0}{X_m}\left(1 - \exp(\mu_m t)\right)} - X_0\right] - \eta\frac{X_m}{\mu_m}\ln\left[1 - \frac{X_0}{X_m}\left(1 - \exp(\mu_m t)\right)\right] \quad (5)$$

where X is the instantaneous biomass concentration, X_0 - initial biomass concentration, X_m - 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.

Therefore, logistic and Gompertz equations were applied to study cell growth, EPS production, and substrate consumption rate, the logistic incorporated modified Luedeking–Piret model was applied to study the EPS profile and substrate utilization, and the reparametrized Gompertz survival model was utilized to study the substrate consumption/growth profiles.

To evaluate the mathematical models used in the experiments, R-squared (R^2, R_{adj}^2), Mean-Square Errors (MSE), Root-Mean-Square Errors (RMSE) and Mean Absolute Errors (MAE) values were considered.

The modified Logistic Gompertz and Gompertz survival models were fitted to the experimental data using the Levenbergh^Marquardt algorithm, to minimize the objective function (PRESS WH et al, 1992; HUANG L., 2009), while for the logistic incorporated Ludeking–Piret model, the initial values were obtained by the linearization technique, presented by Mohammad et al. (1995).

Table 1. Statistical coefficients

Response	Model	RMSE	MSE	R^2
Biomass	Logistic	0.009	0.010	0.999
	Gompertz	0.009	0.010	0.999
	Logistic-Luedeking Pirret (LLP)	0.069	0.076	0.964
Crude polysaccharide content	Logistic	0.024	0.027	0.999
	Gompertz	0.025	0.028	0.999
	Logistic-Luedeking Pirret (LLP)	2.943	3.265	0.999
Substrate concentration	Logistic	0.034	0.038	0.998
	Gompertz	0.050	0.056	0.995
	Logistic-Luedeking Pirret (LLP)	0.643	0.714	0.261

Results

Dynamics of bacterial growth and EPS production

Statistical coefficients for all the applied kinetics models are presented in Table 1, along with the experimental data which indicate values higher than 0.99 for the R^2 coefficient, in the case of Logistic and Gompertz models.

To study the kinetics of the fermentation process using the *K. oxytoca* ICCF 419 strain, time profiles for biomass, substrate and crude product were first evaluated separately, and then, results were compiled as shown in the Fig. 1 to give a better view about the optimal model for the further use.

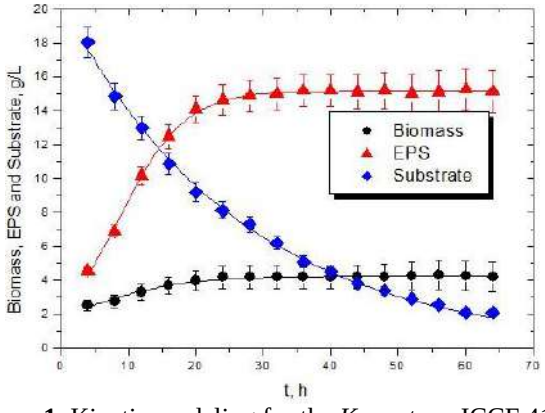

Figure 1. Kinetic modeling for the *K. oxytoca* ICCF 419 – Logistic model.

Fig. 1 presents concentration profiles for the Logistic model and illustrates the relation between biomass, substrate, and the EPS biosynthesis. The following values were recorded: a maximum for dry biomass at 4.2 g/L, a constant increase in crude polysaccharide content, with a maximum of 15 g/L and a rapid decrease in substrate concentration, with a minimum value after 64 hrs fermentation.

Investigation of fermentation parameters effects using RSM

Based on a previous screening study, different concentrations of medium components such as lactose, corn extract, KH_2PO_4 and citric acid were selected for the optimization experiment by using a CCRD design, with 4 variables and 3 levels.

Accordingly, three-dimensional graphs were generated for pair-wise combination of the four factors and in Fig. 2, 3 and 4 are presented some of the interactions that positively influenced the EPS production, biomass growth and substrate consumption, such as: KH_2PO_4 and citric acid or lactose, corn extract and lactose, and corn extract and KH_2PO_4.

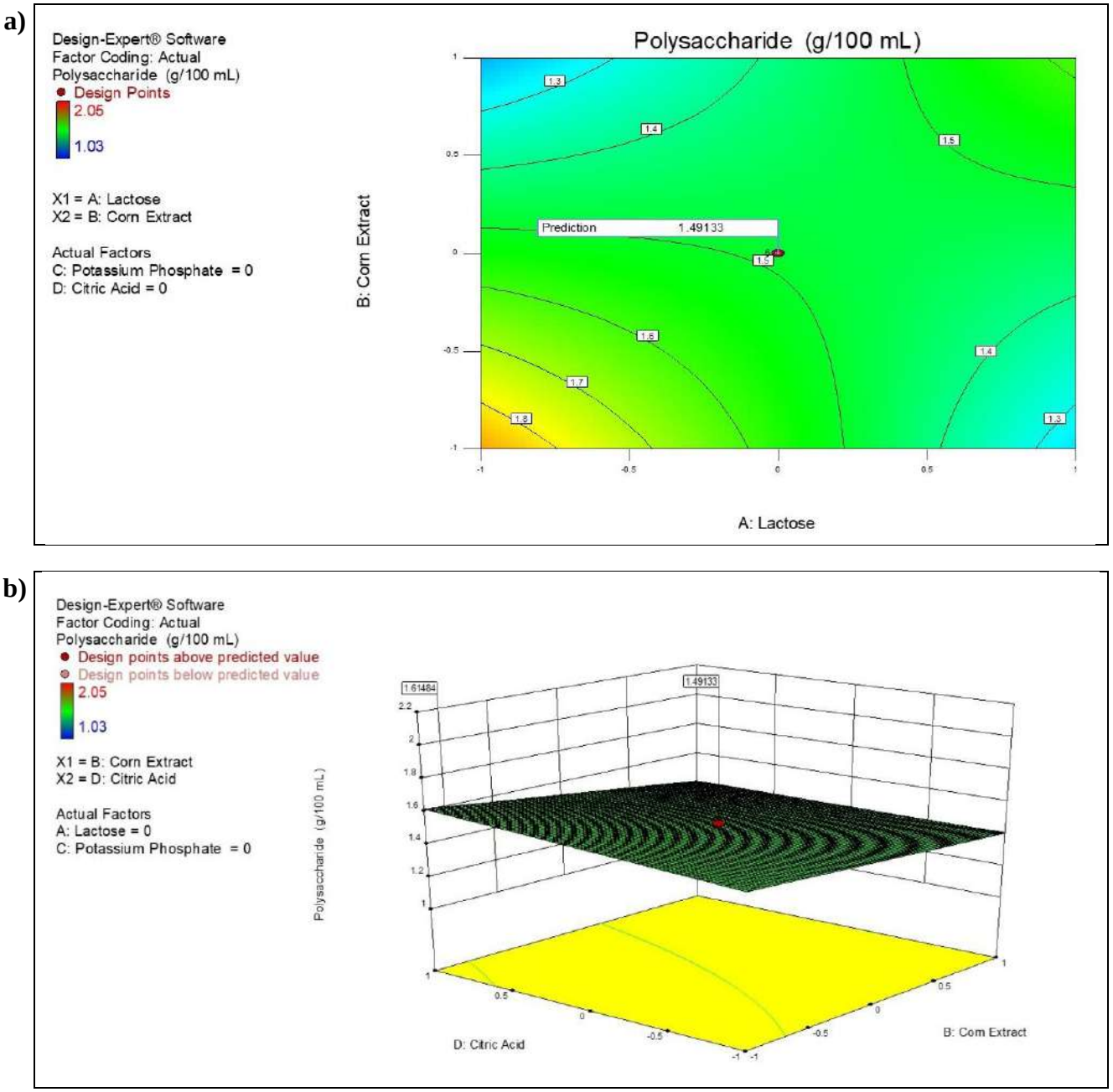

Figure 2. RSM profile of the EPS production: **a)** the CD factors interaction (between KH_2PO_4 and citric acid) and **b)** the AC factors interaction (between lactose and KH_2PO_4).

Experimental data indicate that after 64 hrs of fermentation, the highest concentration of crude EPS (20.5 g/L) was determined for run 20 (%w/v: lactose 3.0, corn extract 1.15, KH_2PO_4 0.1 and citric acid 0.3). High concentrations of EPS, such as 19.0, 18.75 and 17.5 g/L were also determined in case of runs 26 and 4, 23 and 1, respectively. Culture conditions were (% w/v): lactose 3.0 or 8.0, corn extract 1.15 or 3.0, KH_2PO_4 0.3, 0.2 or 0.1 and citric acid 0.1 or 0.2. The lowest production values were measured for runs 13, 3 and 8, where EPS content was determined as 10.3, 10.95, and 11.2 g/L, with initial culture conditions (% w/v): lactose 8.0 or 3.0, corn extract 1.15 or 3.0, KH_2PO_4 0.1 and citric acid 0.1 or 0.3.

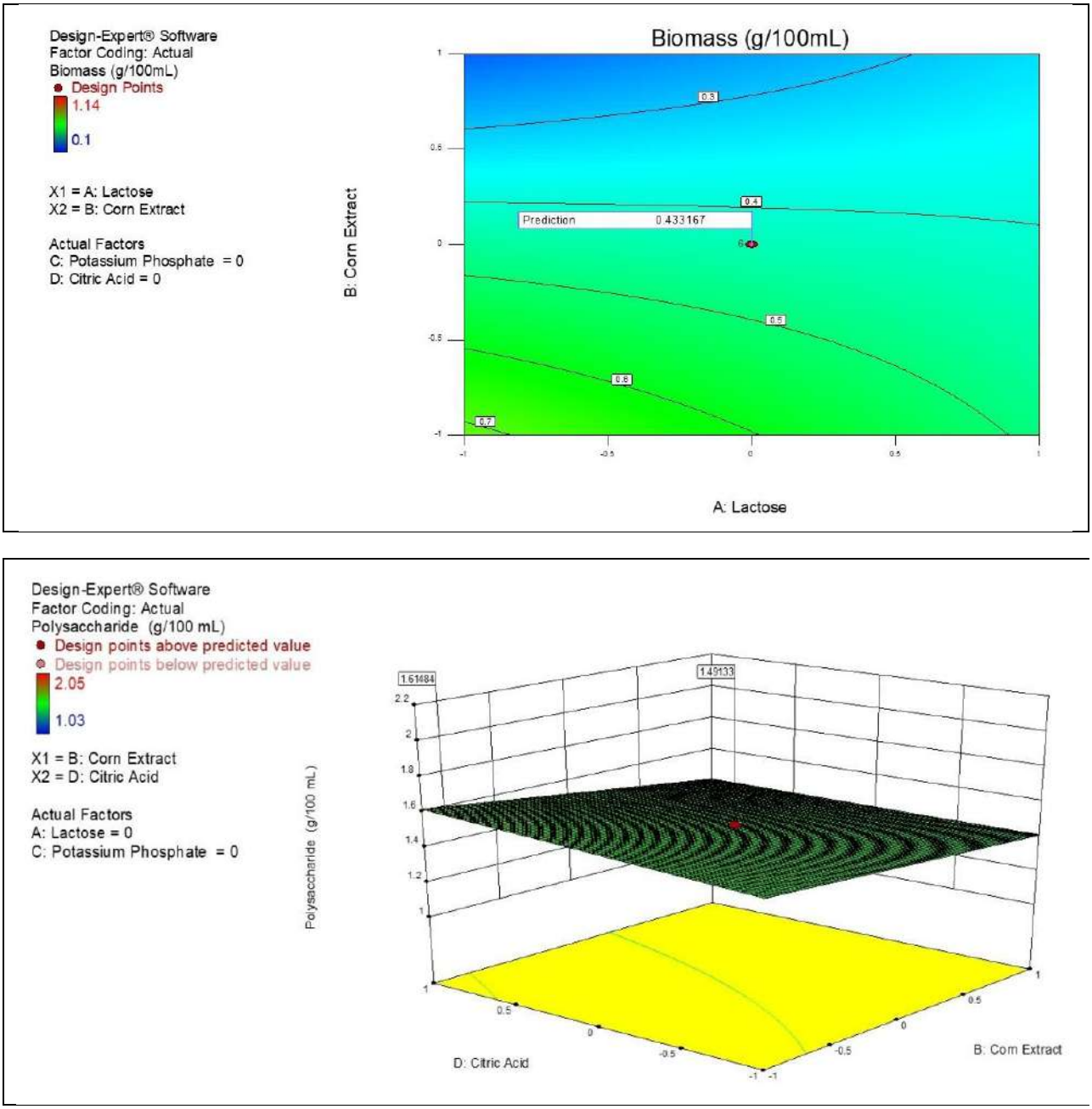

Figure 3. RSM profile of the biomass growth: **a)** the CD factors interaction (between KH_2PO_4 and citric acid) and **b)** the AB factors interaction (between lactose and corn extract).

The highest values for dry biomass were determined as 11.4 and 10.4 g/L, corresponding to runs 23 and 20, with initial fermentation conditions (% w/v): lactose 3.0, corn extract 1.15, citric acid 0.3 and KH_2PO_4 0.3 or 0.1. The lowest values were determined as between 1.0 and 1.95 g/L, caused by higher concentration of lactose and corn extract.

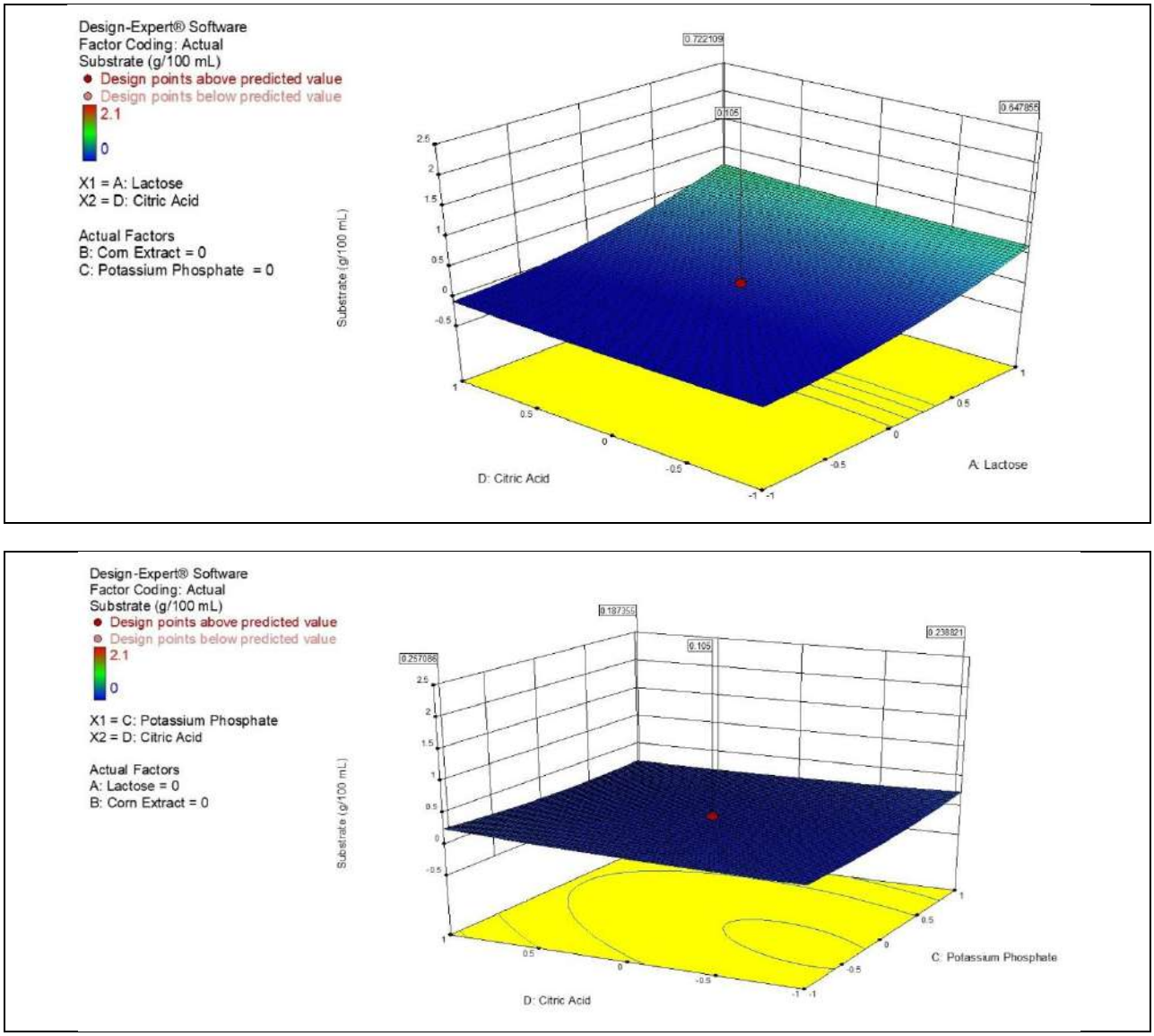

Figure 4. RSM profile of the substrate consumption: **a)** the BC factors interaction (between corn extract and KH_2PO_4) and **b)** the AB factors interaction (between lactose and corn extract).

Lactose, used as a carbon source for cell growth, but also as a substrate for the EPS production, was totally consumed (0 g/L in the fermentation broth) in case of run 23, for which was determined an EPS content of 18.75 g/L, with initial culture conditions (%w/v): lactose 3.0, corn extract 1.15, K_2PO_4 0.2 and citric acid 0.3.

A validation experiment was conducted and the following values were obtained: 17.41 g/L of crude exopolysaccharide, 2.53 g/L dry biomass and 0.13 g/L lactose remained in the fermentation broth (nearly totally substrate consumption).

Following the RSM modeling and data interpretation, bioprocess parameters were estimated considering the fermentation medium composed of (%w/v): lactose 3.0, corn extract 1.34, KH_2PO_4 0.1 and citric acid 0.1, with a C/N ratio of 23.45. The predicted values were: 17.7 g/L for the crude EPS content, 2.6 g/L for dry biomass and 0.163 g/L substrate in the fermentation broth. Under the optimization conditions, experimental data was very close to the predicted values of EPS content: 17.41 g/L of crude EPS, 2.53 g/L dry biomass and 0.13 g/L lactose in the fermentation broth.

Discussions

K. oxytoca is a Gram-negative and rod-shaped micro-organism, ubiquitous in the environment, normally found in water, vegetation and soil, but also in the bowel of man and animals, and which is known to be an opportunistic pathogen, an endophytic nitrogen-fixing bacterium, and an EPS producer. It is also a major component of the microflora characteristic to stressed environments, and it is frequently isolated from various plant roots in the bio-prospecting studies, in order to study different metabolic properties interesting for the bioremediation field (i.e. potential to remove phenol, cadmium, mercury, endosulfan,

nitroglycerin, acrylonitrile etc) (BRISSE et al, 2006; GALLO et al, 2012; CHEN et al, 2013; SINGH et al, 2016).

The present study aimed to investigate the effects of some bioprocess parameters, on an EPS producing *K. oxytoca* strain (ICCF 419), when lactose was selected as the main carbon source, and as a precursor for the polymer synthesis.

Most of the microbes have not the capacity to degrade lactose, because they do not produce the lactose-hydrolyzing enzymes, but *K. oxytoca* ICCF 419 strain demonstrated its ability to utilize lactose as a carbon source, which is not a commonly substrate used in the fermentation media, and it synthesized a promising yield of crude exopolysaccharide. Other studies reported production of polysaccharides by using lactose-utilizing microorganisms (i.e. *Pseudomonas elodea*, *Bacillus subtilis, Streptococcus thermophilus*, lactic acid bacteria etc. (CAGRI-MEHMETOGLU et al, 2012; RABHA et al, 2012; ZEIDAN et al, 2017).

Kinetics studies

Comparing the results presented in Table 1, the Logistic and Gompertz models are similar and suitable to use for the entire time-domain data analysis, than the LLP model, which has a linear allure and allows only a linear growth domain analysis (up to 44 hrs of fermentation).

In the case of cell formation, it was noticed a rapid growth in the first 24 hrs, followed by a slight and constant growth in the stationary phase, with a maximum determined at 4.33 g dry biomass/L after 56 hrs bioprocess. Logistic and Gompertz models are considered suitable to study the biomass growth, with a correlation coefficient (R^2) of 0.999.

Medium composition and the relation between carbon and nitrogen sources are significant factors that influence EPS biosynthesis, while substrate has a key role in polysaccharide production. Time profiles for this parameter were evaluated with Logistic and Gompertz models, with correlation coefficients (R^2) of 0.998 and 0.995.

An immediate decrease of lactose in fermentation broth was observed when *Klebsiella* started to exponentially multiplicate, the substrate being almost completely consumed between 8 and 64 hrs of bioprocess and reaching a minimum value of 2 g/L.

The strain started to produce EPS during the exponential phase, then a maximum yield of crude polysaccharide of 15 g/L was determined after 56 hrs of fermentation; after that, it followed the decline phase, during which the content of EPS in the fermentation broth started to decrease, due to its possible hydrolysis (MOGHANNEM et al, 2017). As in the case of biomass profile, correlation coefficient (R^2) for the kinetic models was 0.999.

Therefore, employing a Logistic model gives the closest interpretation of the experimental data in case of *K. oxytoca* ICCF 419, with a correlation coefficient (R^2) of 0.998.

Optimization studies

Higher EPS contents (17.5-20.5 g/L), and the most common values for EPS production (14-17 g/L) determined by using CCRD experiment were obtained when the C/N was calculated as between 27.32-27.93, and 30-55, respectively. These findings are promising, by comparison with other results, obtained with a *K. oxytoca* strain isolated from milk, which was grown on lactose 5%w/v, and after 72 hrs of bioprocess, produced 6.1 g EPS/L (DLAMINI et al 1997). The lowest values were obtained when the corresponding C/N ratios were calculated as 10.47 and 72.87, respectively. The relation between carbon and nitrogen source is strengthening the idea that a C/N ratio value of 27-30 is recommended for a maximum yield of polysaccharide.

Lower concentrations of carbon and nitrogen, as well as phosphate (a minimum of 0.2% w/v) are favorably influencing cell growth; the maximum value for dry biomass was observed when the C/N was approximately 27. Concentration of KH_2PO_4 had no significant effect on the EPS production, while a high concentration of biomass was not desirable. Therefore, biomass growth was influenced by corn extract, KH_2PO_4 and citric acid, and by the interactions between corn extract and lactose or citric acid. Lactose itself and its interaction with KH_2PO_4 had no significant influences. The highest biomass value was determined as 11.4 g/L, but it was not recommended when maximizing the EPS production.

Following the statistical analysis, it was found that the interactions between AB factors (lactose and corn extract), as well as BC factors (corn extract and potassium phosphate) have a positive influence on the substrate consumption, and in relation with the EPS production. However, the individual contributions of citric acid or phosphate or the interaction between them does not significantly influence lactose consumption. During the experiment, it has been observed that lactose at an initial concentration of 3 or 5.5% (w/v) is completely metabolized by our strain, with the advantage of producing high EPS content.

Crude EPS content as a response in the 2FI - RSM model

The F-value of 70.92 shows that the 2FI model is significant; there is only a 0.01% chance that this value could occur, due to the noise, while a value of "Prob>F" less than 0.05 indicates that model terms are significant. The "Lack of Fit F-value" of 11.90 implies that the model fits and there is only a 0.64% chance that this could occur due to the noise. ANOVA showed that terms like A, B, C, AB, AC, BC, and CD were significant, which means that lactose, corn extract and KH_2PO_4 by themselves were significantly effective in the EPS biosynthesis (p-values<0.0001); lower p-values registered for interactions between lactose and corn extract, and lactose and KH_2PO_4, had some influence on the EPS content. However, the citric acid itself or the interaction between lactose and citric acid had no significant influences (p-values of 0.0088 and 0.91).

The model has high values for R^2, R^2(adj) and R^2 (pred) of 0.97, 0.96 and 0.90 and it indicates that experimental results were fitted accordingly. Also, the value of 32.54 corresponding to the "Adeq Precision" indicates adequate signal to use these model in further spatial designing.

Biomass growth as a response in the 2FI - RSM model

The F-value of 39.34 suggests that the 2FI model is significant; the "Lack of Fit F-value" of 1.38 implies that the model fits and there is only a 38.31% chance that this could occur due to the noise. ANOVA analysis showed that terms like B, C, D, AB, BD, and CD are significant, meaning that the corn extract, KH_2PO_4 and citric acid by themselves were significantly effective (p-values <0.0001), while interactions between corn extract and lactose or citric acid had also influenced the biomass production. However, lactose itself and its interaction with KH_2PO_4 had no significant influences (p-values of 0.09 and 0.48).

High values for R^2, R^2(adj) and R^2(pred) of 0.95, 0.93 and 0.87 indicate that experimental results were fitted accordingly. The "Pred R-Squared" value of 0.87 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 24.56 corresponding to the "Adeq Precision" indicates adequate signal to use this model in further spatial designing.

The optimum values of biomass correlated with the EPS production were obtained when the C/N value was calculated as approx. 27, while interaction between lactose and corn extract had a significant influence on cell growth. As could be observed in the experimental results, higher concentrations (%w/v) of these nutrients (lactose – 5.5 or 8.0 and corn extract – 3.0) lead to lower biomass values, but nitrogen had a bigger influence than carbon if we compare run 29 and 27. In the first case, biomass was 1.35 g/L and in the second one, it was 8 g/L. The culture conditions (%w/v) were: lactose 3.0 and 5.5 and corn extract 3.0 and 1.15. KH_2PO_4 and citric acid in concentrations higher than 0.3 %w/v influenced the biomass and lead to decreased values of it: 1.15 g/L (run10) and 1.95 g/L (run 12).

Substrate consumption as a response in the 2FI - RSM model

The F-value of 29.98 indicates that the quadratic model is significant; the "Lack of Fit F-value" of 12.44 implies that the model fits and there is only a 0.62% chance that this could occur due to the noise. ANOVA analysis showed that terms like A, B, AB, BC, A^2, B^2 and C^2 are significant, meaning that lactose and corn extract by themselves were significantly effective (p-values <0.0001), including their interaction; lactose and citric acid interaction had no influence on substrate consumption.

The values of 0.97, 0.93 and 0.81 corresponding to R^2, R^2(adj) and R^2(pred), indicate that experimental results were fitted accordingly. The "Pred R-Squared" value of 0.81 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 29.98 corresponding to the "Adeq Precision" indicates adequate signal to use these models in further spatial designing.

A significant influence on substrate consumption was determined by the AB factors interaction, between lactose and corn extract. Therefore, to have a total substrate consumption, but taking into account the EPS production, optimal composition of fermentation medium was considered (%w/v): lactose 3.0, corn extract 1.15, and KH_2PO_4 and citric acid in concentrations higher than 0.2, if we correlate also with the values determined for the crude EPS and dry biomass (e.g. in case of run 23: 18.75 and 11.4 g/L, respectively).

Conclusions

Optimizing the fermentation is a key step of a microbial biotechnological process development, aiming to ensure the reliability, cost-efficiency and product quality. In this study, the effects of different bioprocess parameters on EPS production were investigated, by using the *K. oxytoca* ICCF 419 strain. Optimization and mathematical modeling were studied using RSM – CCRD design, and kinetics models such as Logistic and Gompertz.

The Logistic model was considered the most suitable to interpret experimental data, with an R^2 value > 0.99. Results indicated a crude EPS yield of 15 g/L and a maximum biomass concentration of 4.2 g/L after 56 hrs of bioprocess.

CCRD design was applied to study EPS formation in relation with microbial growth and substrate consumption, using 4 variables and 3 levels. EPS production was significantly influenced by lactose, corn extract and KH_2PO_4, while citric acid had no influence. Maximum experimental yield of crude EPS was determined as 20.5 g/L.

ANOVA analysis showed that interactions between the carbon and nitrogen sources were the most significant on the EPS production, with a R^2 value of 0.97.

Scaling-up the polysaccharide production using the *K. oxytoca* ICCF 419 strain, starting from the process parameters established following this study, is the next challenge related to the obtaining of a new biomaterial with potential pharmaceutical applications.

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References

1. AHMAD A, ALKHARFY KM, WANI TA, RAISH M. Application of Box-Behnken design for ultrasonic-assisted extraction of polysaccharides from *Paeonia emodi*, *International Journal of Biological Macromolecules*, 2015, 72: 990-997. https://doi.org/10.1016/j.ijbiomac.2014.10.011
2. ALIREZA JA. Evaluation suitability of different models for *Aspergillus niger* growth and glucose oxidase production in submerged fermentation, *J. Biotechnol*., 2008, 136, S106–S107. DOI: 10.1016/j.jbiotec.2008.07.244
3. ARIYANTI D, HADIYANTO H. Ethanol Production from Whey by *Kluyveromyces marxianus* in Batch

Fermentation System: Kinetics Parameters Estimation, Bulletin of Chemical Reaction Engineering & Catalysis, 2013, 7(3): 179-184. http://dx.doi.org/10.9767/bcrec.7.3.4044.179-184
4. BARANYI J, ROBERTS TA. A dynamic approach to predicting bacterial growth in food, *Int. J. Food Microbiol.*, 1994, 23(3-4): 277-294. https://doi.org/10.1016/0168-1605(94)90157-0
5. BAŞ D, BOYACI HI. Modeling and optimization I: Usability of response surface methodology, *Journal of Food Engineering*, 2007, 78(3): 836-845. https://doi.org/ 10.1016/j.jfoodeng.2005.11.024
6. BOA JM, LEDUY A. Pullulan from peat hydrolysate fermentation kinetics, *Biotechnology and Bioengineering*, 1987, 28(4): 361-366.
7. BRISSE S, GRIMONT F, GRIMONT PAD. The genus *Klebsiella*, Prokaryotes, 2006, 6: 159-196. DOI: 10.1007/0-387-30746-x_8
8. BUENO SM, GARCIA-CRUZ CH. Optimization of polysaccharides production by bacteria isolated from soil, *Brazilian Journal of Microbiology*, 2006, 37: 296-301.
9. CAGRI-MEHMETOGLU A, KUSAKLI S, VENTER M. Production of polysaccharide and surfactin by *Bacillus subtilis* ATCC 6633 using rehydrated whey powder as the fermentation medium, *Journal of Dairy Science*, 2012, 95(7): 3643-3649. DOI: https://doi.org/10.3168/jds.2012-5385
10. CHAO A, MA S, CHANG F, XUE W. Efficient production of pullulan by *Aureobasidium pullulans* grown on mixtures of potato starch hydrolysate and sucrose, *Brazilian Journal of Microbiology*, 2017, 48: 180-185. http://dx.doi.org/10.1016/j.bjm.2016.11.001
11. CHEN M, LIN L, ZHANG Y, SUN L, AN Q. Genome sequence of *Klebsiella oxytoca* SA2, an endophytic nitrogen-fixing bacterium isolated from the pioneer grass *Psammochloa villosa*. *Genome Announc*., 2013, 1(4): e00601-13. doi:10.1128/genomeA.00601-13
12. CHENG KK, LIN RH, LIU HJ, LIU DH. Kinetic analysis of anaerobic fermentation of 1,3-propanediol by *Klebsiella pneumoniae*, *The Chinese Journal of Process Engineering*, 2005, 5(4): 425-429.
13. CHOWDHURY BR, CHAKRABORTY R, CHAUDHURI UR. Validity of modified Gompertz and Logistic models in predicting cell growth of *Pediococcus acidilactici* H during the production of bacteriocin pediocin AcH, *Journal of Food Engineering*, 2007, 80(4): 1171-1175. https://doi.org/10.1016/j.jfoodeng.2006.08.019
14. COBAN BC, DEMIRCI A. Enhancement and modeling of microparticle-added *Rhizopusoryzae* lactic acid production, *Bioprocess Biosyst Eng*., 2016, 39: 323-330.
15. DAM EG, BROEK AM, BOERIU CG. Polysaccharides in human health care, *Natural Product Communications*, 2017, 12(6): 821-830.
16. DLAMINI AM, PEIRIS PS. Biopolymer production by a Klebsiella oxytoca isolate using whey as fermentation substarete, *Biotechnology Letters*, 1997, 19(2): 127-130.
17. DODIĆ JM, VUČUROVIĆ DG, DODIĆ SN, GRAHOVAC JA, POPOV SD, NEDELJKOVIĆ NM. Kinetic modelling of batch ethanol production from sugar beet raw juice, *Appl. Energy*, 2012, 99: 192-197.
18. European Pharmacopoeia 9.0, The 9th Edition, vol. 1, 2017.
19. FAN Y, WANG Y, QIAN PY, GU JD. Optimization of phthalic acid batch bio-degradation and the use of modified Richards model for modelling degradation, *Int. Biodeterior. Biodegrad.*, 2004, 53(1): 57-63. DOI: 10.1016/j.ibiod.2003.10.001
20. FERCHICHI M, CRABBE E, GIL GH, HINTZ W, ALMADIDY A. Influence of initial pH on hydrogen production from cheese whey, *J. Biotechnol.*, 2005, 120(4): 402-409. DOI: 10.1016/j.biotec.2005.05.017
21. GALLO G, BALDI F, RENZONE G, GALLO M, CORDARO A, SCALONI A, PUGLIA AM. Adaptative biochemical pathways and regulatory networks in *Klebsiella oxytoca* BAS-10 producing a biotechnologically relevant exopolysaccharide during Fe(III)-citrate fermentation, *Microbial Cell Factories*, 2012, 11:152. http://www.microbialcellfactories.com/content/11/1/152
22. GANDURI RK, PODHA S. Unstructured modeling of *Aureobasidium pullulans* fermentation for pullulan production. A mathematical approach, *International Journal of Engineering Research & Technology (IJERT)*, 2014, 3(10): 1076-1079.
23. GORANOV B, SHOPSKA V, DENKOVA R, KOSTOV G. Kinetics of batch fermentation in the cultivation of a probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* B1, *Acta Universitatis Cibiniensis Series E: Food Technology*, 2015, XIX(1): 61-72. 10.1515/aucft-2015-0006
24. GERMEC M, KARHAN M, KATHERINE L, BIALKA KL, DEMIRCI A, TURHAN I. Mathematical modeling of lactic acid fermentation in bioreactor with carob extract, *Biocatalysis Agricult. Biotechnol.*, 2018, 14: 254-263. https://doi.org/10.1016/j.bcab.2018.03.018
25. GERMEC M, CHENG KC, KARHAN M, DEMIRCI A, TURHAN I. Application of mathematical models to ethanol fermentation in biofilm reactor with carob extract, *Biomass Conversion and Biorefinery*, 2019. https://doi.org/10.1007/s13399-019-00425-1
26. GOMPERTZ B. On the nature of the function expressive of the law of human mortality, and on a new mode of determining the value of life contingencies. *Philos. Trans. R. Soc. Lond.*, 1825, 115: 513-583.
27. GUO J, LIU J, YANG Y, ZHOU Y, JIANG S, CHEN C. Fermentation and kinetics characteristics of a biofocculant from potato starch wastewater and its application, *Scientific Reports*, 2018, 8: 3631, DOI: 10.1038/ s41598-018-21796-x
28. HUANG L. Thermal inactivation of *Listeria monocytogenes* in ground beef under isothermal and dynamic temperature conditions, *Journal of Food Engineering*, 2009, 90: 380-387.

29. KHANNA N, KOTAY SM, GILBERT JJ, DAS D. Improvement of biohydrogen production by *Enterobacter cloacae* IIT-BT 08 under regulated pH. *J. Biotechnol.*, 2011, 152(1): 9-15. DOI: 10.1016/j.jbiotec.2010.12.014
30. KLIMEK J, OLLIS DF. Extracellular microbial polysaccharides: kinetics of *Pseudomonas* sp., *Azotobacter vinelandii*, and *Aureobasidium pullulans* batch fermentations, *Biotechnology and Bioengineering*, 1980, 22(11): 2321-2342.
31. KUMAR AS, MODY K, JHA B. Bacterial exopolysaccharides – a perception, *Journal of Basic Microbiology*, 2007, 47:103-117.
32. LIU J, XING J, CHANG T, MA Z, LIU H. Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSE using statistical experimental methods, *Process Biochemistry*, 2005, 40: 2757-2762. doi 10.1016/j.procbio.2004.12.025
33. MAHAJAN PM, GOKHALE SV, LELE SS. Production of nattokinase using *Bacillus natto* NRRL 3666: media optimization, scale up, and kinetic modeling, *Food SciBiotechnol*., 2010, 19(6): 1593-1603. doi: 10.1007/s10068-010-0226-4
34. MANAGAMURI U, VIJAYALAKSHMI M, GANDURI RK, BABU RS, PODA S. Optimization of culture conditions by Response Surface Methodology and Unstructured kinetic modeling for L-Asparaginase production by *Pseudonocardia endophytica* VUK-10, *Journal of Applied Pharmaceutical Science*, 2017, 7(1): 42-50. DOI: 10.7324/JAPS.2017.70106
35. MARQUES RMAR. Modeling and optimization of extracellular polysaccharide production by Enterobacter A47, PhD thesis, 2016, http://hdl.handle.net/10362/16539
36. MITRA R, DUTTA D. Growth profiling, kinetics and substrate utilization of low-cost dairy waste forproduction of β-cryptoxanthin by *Kocuria marina* DAGII, *R. Soc. open sci.*, 2018,5: 172318. http://dx.doi.org/10.1098/rsos.172318
37. MOGHANNEM SAM, FARAG MMS, SHEHAB AM, AZAB MS. Exopolysaccharide production from *Bacillus velezensis* KY471306 using statistical experimental design, Brazilian Journal of Microbiology, 2018, 49: 452-462. https://doi.org/10.1016/j.bjm. 2017.05.012
38. MOHAMMAD FHA, BADR-ELDIN SM, EL-TAYEB OM, ABD EL-RAHMAN OA. Polysaccharide production by *Aureobasidium pullulans* III. The influence of initial sucrose concentration on batch kinetics, *Biomass and Bioenergy*, 1995, 8(2): 121-129.
39. MONTGOMERY DC. Design and Analysis of Experiments, 10th Edition, *John Wiley & Sons, Inc*., 2019, ISBN: 978-1-119-49244-3
40. MORGAN PH, MERCER LP, FLODIN NW. General model for nutritional responses of higher organisms, *Proc. Natl. Acad. Sci.*, 1975, 72(11): 4327-4331.
41. MOSCOVICI M, IONESCU C, CARAIANI T, CĂȘĂRICĂ A, MARINESCU MC, ZĂHĂRESCU V, GHERA D, GHEORGHIU E, STAN A, SOARE M, MARDALE RM. Curdlan-type polysaccharide using a strain of *Agrobacterium rhizogenes*, *Romanian Biotechnology Letters*, 2009, 14(4): 4530-4537
42. PEARL R, REED LJ. On the rate of growth of the population of the United States since 1790 and its mathematical representation, *Proc. Natl. Acad. Sci.*, 1920, 6(6): 275-288.
43. PHUKOETPHIM N, SALAKKAM A, LAOPAIBOON P, LAOPAIBOON L. Kinetic models for batch ethanol production from sweet sorghum juice under normal and high gravity fermentations: logistic and modified Gompertz models, *J. Biotechnol*., 2017, 243: 69-75. https://doi.org/10.1016/j.jbiotec.2016.12.012
44. PRESS WH, TEUKOLSKY SA, VETTERING WT, FLANNERY BP. Numerical recipes in C: The art of scientific computing, *Cambridge, UK, Cambridge University Press*, 1992.

Rabha B, Nadra RS, Ahmed B, Effect of some fermentation substrates and growth temperature on exopolysaccharide production by *Streptococcus thermophilus* BN1, *International Journal of Bioscience, Biochemistry and Bioinformatics*, 2012, 2(1): 44-47.

45. RAJENDRAN A, THANGAVELU V. Application of Central Composite Design and Artificial Neural Network for the Optimization of Fermentation Conditions for Lipase Production by *Rhizopus arrhizus* MTCC 2233. *J. Bioprocess Biotechniq*, 2012, 2:118. doi: 10.4172/2155-9821.1000118
46. RICHARDS F. A flexible growth function for empirical use, *J. Exp. Bot.*, 1959, 10 (2): 290-301.
47. SANCHEZ OJ, MONTOYA S, VARGAS LM. Polysaccharide Production by Submerged Fermentation, Ramawat K, Merillon JM (eds) Polysaccharides. Springer, Cham., 2015, 451-473. DOI: 10.1007/978-3-319-16298-0_39
48. SCHOORL N, REGENBOGEN A. Massanalytische Zuckerbestimmung Zeitschr, *F. Anal. Chem.*, 1917, 56:191
49. SEKUROVA ON, SCHNEIDER O, ZOTCHEV SB. Novel bioactive natural products from bacteria via bioprospecting, genome mining and metabolic engineering, *Microbial Biotechnology*, 2019, 12 (5): 828-844. doi:10.1111/1751-7915.13398
50. SHARMA V, MISHRA HN. Unstructured kinetic modeling of growth and lactic acid production by *Lactobacillus plantarum* NCDC 414 during fermentation of vegetable juices, *LWT – Food Science and Technology*, 2014, 59: 1123-1128. http://dx.doi.org/10.1016/j.lwt.2014.05.039
51. SINGH L, CARIAPPA MP, KAUR M. *Klebsiella oxytoca*: An emerging pathogen?, Medical Journal Armed Forces India, 2016, 72: 59-61. http://dx.doi.org/10.1016/j.mjafi.2016.05.002
52. SINGH V, HAQUE S, NIWAS R, SRIVASTAVA A, PASUPULETI M, TRIPATHI CKM. Strategies for fermentation medium optimization: an in-depth review, *Frontiers in Microbiology*, 2017, doi: 10.3389/fmicb.2016.02087
53. SINGHASUWAN S, CHOORIT W, SIRISANSANEEYAKUL S, KOKKAEW N, CHISTI Y. Carbon-to-

nitrogen ratio affects the biomass composition and the fatty acid profile of heterotrophically grown *Chlorella* sp. TISTR 8990 for biodiesel production, *J. Biotechnol.*, 2015, 216, 169-177. DOI: 10.1016/j.jbiotec.2015.10.003
54. SIRAJUNNISA AR, VIJAYAGOPAL V, SIVAPRAKASH B, VIRUTHAGIRI T, SURENDHIRAN D. Optimization, Kinetics and Antioxidant activity of Exopolysaccharide produced from Rhizosphere isolate, *Pseudomonas fluorescens* CrN6, *Carbohydrate Polymers*, 2016, 135: 35-43.
55. SOARE (VLADU) MG, TOMULESCU C, PETRESCU M, LUPESCU I, MOSCOVICI M, POPA O, BĂBEANU N. Antimicrobial activity of newly isolated *Bacillus* sp. And *Pseudomonas* sp. strains and their potential use as biocontrol agents, *Scientific Bulletin. Series F. Biotechnologies. XXI, ISSN Online 2285-1372, ISSN-L 2285-1364*, 2017
56. STANNARD C, WILLIAMS A, GIBBS P. Temperature/growth relationships for psychrotrophic food-spoilage bacteria, *Food Microbiol.*, 1985, 2(2):115-122. https://doi.org/10.1016/S0740-0020(85)80004-6
57. TARRAH A, NOAL V, TREU L, GIARETTA S, DUARTE VS, CORICH V, GIACOMINI A. Comparison of growth kinetics at different temperatures of *Streptococcus macedonicus* and *Streptococcus thermophilus* strains of dairy origin, *J. Dairy Sci.*, 2018, 101: 7812-7816. https://doi.org/10.3168/jds.2018-14731
58. TELEKEN JT, ROBAZZA WS, GOMEZ GA. Mathematical modeling of microbial growth in milk, *Cienc Tecnol Aliment Campinas*, 2010, 31(4): 891-896.
59. THOMSON N, OLLIS DF. Extracellular microbial polysaccharides II. Evolution of broth power-law parameter for xanthan and pullulan batch fermentation, *Biotechnology and Bioengineering*, 1980, 22(4): 859-873.
60. VAMANU E. Polyphenolic nutraceuticals to combat oxidative stress through microbiota modulation, *Frontiers in Pharmacology*, 2019, 10:492. doi: 10.3389/fphar.2019.00492
61. VÁZQUEZ JA, GONZÁLEZ MP, MURADO M. Peptones from autohydrolysed fish viscera for nisin and pediocin production, *J. Biotechnol.*, 2004, 112(3): 299-311. DOI: 10.1016/j.jbiotec.2004.04.011
62. VENTORINO V, NICOLAUS B, DONATO P, PAGLIANO G, POLI A, ROBERTIELLO A, IAVARONE V, PEPE O. Bioprospecting of exopolysaccharide-producing bacteria from diferent natural ecosystems for biopolymer synthesis from vinasse, *Chem. Biol. Technol. Agric.*, 2019, 6:18. https://doi.org/10.1186/s40538-019-0154-3
63. VU B, CHEN M, CRAWFORD RJ, IVANOVA EP. Bacterial Extracellular Polysaccharides Involved in Biofilm Formation, *Molecules*, 2009, 14: 2535-2554. doi:10.3390/molecules14072535
64. WANG X, XU P, YUAN Y, LIU C, ZHANG D, YANG Z, YANG C, MA C. Modeling for gellan gum production by *Sphingomonas paucimobilis* ATXX 31461 in a simplified medium, *Applied and environmental microbiology*, 2006, 72(5): 3367-3374. DOI: 10.1128/AEM.72.5.3367-3374.2006
65. WEIBULL W. A statistical distribution function of wide applicability, *J. Appl. Mech.*, 1951, 18(3): 293-297
66. YADAV AN, KOUR D, RANA KL, YADAV N, SINGH B, CHAUHAN VS, RASTEGARI AA, HESHAM AL, GUPTA VK. New and Future Developments in Microbial Biotechnology and Bioengineering. Microbial Secondary Metabolites Biochemistry and Applications, *Elsevier*, Amsterdam, Netherlands, 2019, 333-344. https://doi.org/10.1016/B978-0-444-63504-4.00020-7
67. YU L, TANG Q, ZHANG Y, CHEN R, LIU X, QIAO W, LI W, RUAN H, SONG X. A novel Fe(III) dependent bioflocculant from *Klebsiella oxytoca* GS-4-08: culture conditions optimization and flocculation mechanism, *Scientific Reports*, 2016, 6: 34980. DOI: 10.1038/srep34980
68. ZAKERI A, PAZOUKI M, VOSSOUGI M. Kinetics investigation of cell growth, xanthan production and sugar cane molasses consumption by *Xanthomonas campestris*, *Iranian Journal of Chemical Engineering*, 2015, 12(4): 84-92
69. ZEIDAN AA, POULSEN VK, JANZEN T, BULDO P, DERKX MFP, ØREGAARD G, NEVES AR. Polysaccharide production by lactic acid bacteria: from genes to industrial applications, FEMS Microbiology Reviews, 2017, 41: S168–S200. doi: 10.1093/femsre/fux017
70. ZHONG C, XU A, WANG B, YANG X, HONG W, YANG B, CHEN C, LIU H, ZHOU J. Production of a value added compound from the H-acid waste water – Bioflocculants by *Klebsiella pneumonia*, Colloids and Surfaces B: Biointerfaces, 2014, 122: 583-590. http://dx.doi.org/10.1016/j.colsurfb.2014.07.036
71. ZWIETERING MH, JONGENBURGER I, ROMBOUTS FM, VAN'T RIET K. Modeling of the bacterial growth curve, *Applied Environmental Microbiology*, 1990. 56(6): 1875-1881