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Research Article
Influence of Environmental Factors on Extracellular Fructan and Oligosaccharide Production by *Gluconobacter nephelii*

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Abstract

Background and Objective: Several species of the Acetic Acid Bacteria (AAB) such as *Acetobacter* and *Gluconobacter* can produce Extracellular Fructans (EF) comprising of fructose polymers and fructooligosaccharides (FOS). Although, only few strains of *Bacillus*, *Zymomonas* and *Erwinia* species have proven to be efficient producers of EF for multiform practical use. In previous study a novel strain of AAB *Gluconobacter nephelii* P1464 was tested in this regard and the productivity of fructose polymers was found to be comparable or even higher than reported for other producers. The purpose of this study was to specify the key factors and their interactions that contribute to the formation of EF by *G. nephelii* P1464. **Materials and Methods:** Response Surface Methodology (RSM) was applied to optimize the conditions of poly and oligosaccharide production by *G. nephelii* P1464. Based on the Plackett-Burman and the Box-Behnken designs agitation rate, sucrose and yeast extract (YE) concentrations were confirmed as most important factors that contribute to the maximum production of EF by *G. nephelii* P1464. **Results:** The three variable regression model for fructan synthesis in shake flask cultures showed the global maximum 31.66 g L^{-1} at the initial sucrose concentration 208.63 g L^{-1}, the initial YE concentration 11.13 g L^{-1} and the agitation rate 298 rpm. At the fixed level of YE concentration 9 g L^{-1} the maximum concentration of FOS predicted by a two-variable regression model reached 16.76 g L^{-1} at the initial sucrose concentration 327.9 g L^{-1} and agitation rate 197 rpm. Positive interaction was detected between the agitation rate and the YE or sucrose concentrations for the fructan and oligosaccharide synthesis, respectively. **Conclusion:** Obtained results suggest, that the combination of Plackett-Burman and Box-Behnken designs should be an effective and reliable tool to select the relevant factors and determine their interactions and optimal levels, thus contributing to the maximum production of fructose polymers and FOS by acetic acid bacteria. This is the first report on the use of RSM to optimize cultivation conditions for EF production by AAB. Obtained results demonstrate, that the novel AAB strain *G. nephelii* P1464 could be used as a competitive and promising producer to obtain extracellular fructose polymers as well as FOS.

Key words: *Gluconobacter nephelii*, cultivation conditions, optimization, extracellular fructans, oligosaccharides

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Extracellular fructans of bacterial origin, generally known as levans, constitute a significant part among other microbial exopolysaccharides (EPS)[1-3]. These homopolysaccharides are D-fructose polymers mainly formed by β-(2,6) glycosidic links and characterized by a much higher degree of polymerisation as compared to fructans of plant origin, known as inulins[4].

Bacterial levan, as other microbial EPS, still attracts the growing attention and research effort because it has an extensive technological potential in several fields[2,3,5].

Bacterial fructans are applicable as functional food ingredients[1,2,6,7], which beneficially affect the processes in the human body, through the stimulation of favourable in test in almicroflora, especially bifidobacteria[4,7,8]. Such polysaccharides are commonly defined as prebiotics[4,6]. Levan can be used in medicine as a plasma substitute, source of fructose, anti-inflammation, detoxifying agent, drug activity promoter and a broad-spectrum immunomodulator having radio protecor properties and anti-tumoractivity[1,7,9].

Besides bacterial levan can be used as a thickener, emulsifier, encapsulating agent, as a carrier of color and flavor in food as well as pharmaceutical and cosmetic industries[2,3,7,9]. Recently it also has gained a promising application in nanotechnology, particularly for novel nano-drug delivery systems[9]. Although, despite the obvious functional and technological advantages the commercial use of microbial fructans, remains still limited, mainly due to their relatively high production costs. Extensive attempts to optimize the conditions for levan synthesis are still not resulted insufficiently high productivity and yield to achieve a cost-effective process and there are grounds to believe that the use of more efficient producing strains can be a decisive factor in this respect[5,10]. Levan can be synthesized by bacteria that belong to various genera, including *Zymomonas, Streptococcus*, *Xanthomonas*, *Bacillus*, *Erwinia* and *Pseudomonas*[1,8,11]. Acetic acid bacteria (AAB) such as several species of the genera *Acetobacter* and *Gluconobacter* also can produce extra cellular fructans[3,4,8,11,12]. However, only afew strains of *Bacillus, Zymomonas* and *Erwinia* species have proven to be effective enough to obtain sufficient amounts of levan for practical uses[1,8,11]. It should be noted, that in case of AAB the FOS production has been observed only for some strains of *Gluconoacetobacter diazotropicus*[13] and there are no such reports in relation to *Gluconobacter* spp.

In the previous studies a novel strain[14] of AAB *Gluconobacter nephelii* P1464 was tested in this respect and preliminary results showed the fructan productivity and yield as comparable or even higher than reported for other producers[3]. Although, the one factor at a time (OFAT) approach[15] has been used for these initial studies, which is unable to identify the interaction of factors as well to perform a full optimization of culture conditions. Besides, the potential of *G. nephelii* for the FOS production then also was not examined.

The purpose of this study was to specify the key factors and their interactions that contribute to the formation of extracellular fructans by *G. nephelii* P1464 using the Plackett-Burman and Box-Behnken factorial design.

MATERIALS AND METHODS

Strain and culture conditions: *Gluconobacter nephelii* P1464 and A10 strains were obtained from Microbial Strain Collection of Latvia and LU MBI culture collection (Riga, Latvia), respectively. Cultures was grown aerobically at 30°C for 48 h in basic Hestrin and Schramm (HS) medium, containing (g L^{-1}): D-glucose 20, peptone 5, yeast extract 5, Na_2HPO_4+12H_2O 7.3, citric acid 1.15, $MgSO_4$ 0.5, pH adjusted[16] to 5.6, when appropriate media was solidified with 20 g L^{-1} agar.

Preinocula were initially prepared from cultures cultivated for 72 h on agarized HS media containing glucose (20 g L^{-1}), as a carbon source. Inoculating strain was cultivated in 200 mL flasks (50 mL of medium in each) on a rotary shaker (I 26 New Brunswick Scientific, USA) at 100 rpm. For cultivation in shake flasks aliquots of the corresponding cultures (1%) were transferred to 300 mL flasks containing 100 mL of the HS medium with varied sucrose and yeast extract concentration (50-220 and 3-10 g L^{-1}, respectively) and incubated at varied agitation (100-240 rpm) frequencies.

Fructan acquisition: After 48 h of cultivation in shake flasks, culture samples were centrifuged for 10 min at 8000×g) and 2.5 volumes of chilled (4°C) ethanol were added to one volume of culture supernatants and the mixtures were stored for 24 h at 4°C. Precipitates were collected by centrifugation for 10 min at 10000×g, washed twice with deionized water. Collected pellets were dried at 60°C. The EPS (fructans) were determined by measuring the dry weight, total reducing sugars and fructose content of the precipitates. The detection threshold of fructan was 5 g L^{-1}. Full scale fructan preparations were obtained except that repeated (3 times) fructan precipitation/dissolving cycles and deproteinization of fructan solutions with 50 mmol L^{-1} $CaCl_2$ (final concentration 5 mmol L^{-1}) were performed. The fructan preparations were freeze-dried and stored in a dessicator.

Analytical determinations

Determination of biomass: Cell growth was monitored spectrophotometrically (Libra S22, UK) at 600 nm, according the calibration curve:

$$\text{Biomass dry weight (g L}^{-1}) = 0.19 \times OD_{600}\ \text{nm}$$

Determination of reducing sugars, mono and disaccharides: Reducing sugars were determined by dinitrosalicylic acid (DNS) method[17].

The EPS mono and disaccharides were determined by Megazyme Sucrose/Fructose/D-Glucose Assay Kit [http://secure.megazyme.com/Sucrose-Fructose-D-Glucose-Assay-Kit].

Fructan quantification: The quantity of fructan was estimated using the gravimetric method[11]. The content of fructan was analyzed as D-fructose and obtained quantity of D-fructose was divided by the factor 1.11 to calculate the amount of fructan[11].

Determination of the total oligosaccharide concentration: Determination of the total oligosaccharide (tri-and tetrasaccharides) concentration was performed using HPLC-RID. Runs were performed on an agilent zorbax carbohydrate analysis column (4.6×150 mm, 5 μm). The temperature of the column and RID was 35°C. The isocratic solvent mixture was 30% H_2O and 70% ACN. The 1-kestose, 1.1-kestotetraose and raffinose (Megazyme) were used as external oligosaccharide standards. The calibration curves for each standard were made in the concentration range of 0.5-10 mg mL^{-1} using chromatographic peak areas as the dependent variable. The total amount of oligosaccharides in the sample solution was expressed as the sum of the peak areas corresponding to the region of tri and tetrasaccharides in the chromatogramm.

Factorial design, data processing and analysis: The 12-run Plackett-Burman two level factorial design[18] was employed to screen out the key factors affecting the fructan formation and growth of bacteria. The 15-run Box-Behnken three level design[18] was carried out to subsequently evaluate second-order effects and interactions between the most important factors.

In order to fit the response function as a linear or quadratic polynomial model experimental data were treated by the multiple regression analysis using the software statgraphics centurion (Manugistics, Inc., Mar., US) and IBM SPSS Statistics 20 (SPSS Inc., Ill., US). The Fisher's F-test for analysis of variance (ANOVA) was performed to assess the statistical significance of models and the student's t-test was employed to check the significance of regression coefficients. The Leave One Out Cross Validation (LOOCV) procedure was employed to validate developed regression models[19]. The linear plots of the actual experimental data against those predicted by the multiple regression models were used throughout the study to assess the goodness-of-fit for observed relationships according to adjusted R^2 values.

The online computational tool Wolfram Alpha (Wolfram Research, Inc., Ill., US, http://www.wolframalpha.com/) was used in order to locate the optimum of second-order response surfaces. Analytical measurements were performed at least at duplicate and data is given as the Mean±SE. The $p<0.05$ were considered to be statistically significant for all the parametric and nonparametric tests.

RESULTS

Screening of significant variables by Plackett-Burman design: There has been extensive evidence that the quantities and the composition of bacterial exopolysaccharides (EPS) are strain dependent and affected by varied environmental conditions[2,3,20]. Also, this initial studies indicated[14] that formation of extracellular fructans by the Acetic Acid Bacteria (AAB) *Gluconobacter nephelii* depends on cultivation conditions such as concentration of sucrose and yeast extract in the medium and agitation rate, as well as it varies between different strains. To clarify and quantitatively assess the relative importance of these and some other conditions cultivation experiments were carried out in accordance with the Plackett-Burman two level factorial design. The design matrix for 12 trials together with response data representing the observed concentrations of extracellular fructans and bacterial cells in the cultivation medium are shown in Table 1. The multiple regression analysis revealed that the highly significant ($0.001<p<0.0016$) linear polynomials can be fitted to the both sets of response data, although there are some differences regarding the significance and relative importance of the factors under study as follows from the directly comparable regression coefficients of coded (Table 1) independent variables. The results of such analysis are summarized in Pareto charts (Fig. 1) and they show that only part of factors could contribute significantly to the formation of fructans, besides differently from their effects on cell growth. Thus, in both cases (Fig. 1a, b), the concentration of sucrose in the cultivation medium appeared

Table 1: Plackett-Burman design matrix and response data representing the concentrations of *Gluconobacter nephelii* extracellular fructans and bacterial cells in the cultivation medium

	Variables							
Run	X_1	X_2	X_3	X_4	X_5	X_6	Biomass±SE (g L^{-1})	Fructans±SE (g L^{-1})
1	+	+	+	+	+	+	0.254±0.011	45.93±0.29
2	–	+	+	+	+	+	0.322±0.013	15.29±1.08
3	–	–	+	–	+	+	0.291±0.016	12.65±0.69
4	+	–	–	+	–	+	0.241±0.002	49.48±0.98
5	–	+	–	–	+	–	0.309±0.011	15.64±1.97
6	–	–	+	–	–	+	0.276±0.014	19.12±0.98
7	–	–	–	+	–	–	0.290±0.001	17.71±0.01
8	+	–	–	–	+	–	0.285±0.020	21.21±0.39
9	+	+	–	–	–	+	0.265±0.006	28.31±0.20
10	+	+	+	–	–	–	0.308±0.012	14.32±1.28
11	–	+	+	+	–	–	0.303±0.012	12.65±1.87
12	+	–	+	–	+	–	0.253±0.015	24.00±0.20

	Real levels	
	-1	+1
X_1 sucrose concentration (g L^{-1})	50	150
X_2 yeast extract (g L^{-1})	2.5	7.5
X_3 peptone (g L^{-1})	2.5	7.5
X_4 agitation (rpm)	100	180
X_5 initial pH	4.8	6.2
X_6 *G. nephelii* strain	A10	P1464

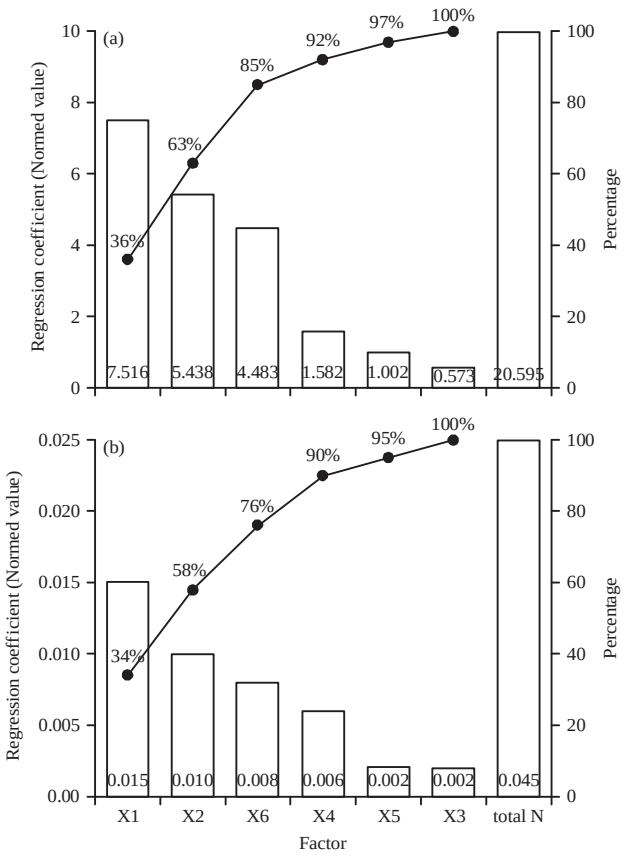

Fig. 1(a-b): Pareto chart: The impact of experimental factors affecting the formation of (a) Fructans and (b) Biomass by *Gluconobacter nephelii*

as the most important factor ($0.00001<p<0.005$), while the next influential proved to be the agitation rate ($p<0.005$) and the concentration of yeast extract ($p<0.01$) for the fructan formation (Fig. 1a) and cell growth (Fig. 1b), respectively.

Also the different strains of *G. nephelii* which were in use in this study emerged as a significant factor ($0.001<p<0.031$) in both cases whereas, the values of initial pH ($0.50<p<0.69$) as well as the peptone concentration ($0.27<p<0.54$) in the cultivation medium did not appear as statistically significant factors at the levels tested (Fig. 1, Table 1). Besides, like in initial studies[14], *G. nephelii* P1464 exhibited higher concentration of extracellular fructans.

At the same time the possible quadratic effects and interactions between selected independent variables remain unclarified because only linear, so-called main effects can be assessed by the Plackett-Burman two level screening design. Such second-order effects could be essential for the further optimization of cultivation conditions to attain a maximum production of extracellular fructans.

Optimization by Box-Behnken design and statistical analysis: The further cultivation experiments were performed according to the Box-Behnken three level factorial design which is considered as an efficient way for fitting quadratic models[21]. Based on the previous data obtained by the Plackett-Burman design (Fig. 1), sucrose concentration, agitate on rate and yeast extract concentration were defined as the variables of interest which needs to be further optimized. The design matrix for 15 trials, including three center points, together with response data representing the observed concentrations of extracellular fructose polymers and oligosaccharides in the cultivation medium of *G. nephelii* P1464 are shown in Table 2. Subsequent application of multivariate regression revealed that the second-order polynomials of high statistical significance ($p<0.00001$) can be fitted to the both sets of data (Table 3). These functions, therefore, can be considered as statistically robust multiple quadratic regression models linking the concentrations of extracellular fructans and oligosaccharides with the nutritional (sucrose and yeast extract concentrations) and operational (agitation rate) parameters of *G. nephelii* P1464 shake-flask cultivation.

The matching quality of the data obtained by multiple regression models was evaluated by the linear plots (Fig. 2) of the actual concentrations of extracellular fructans and oligosaccharides against those predicted by proposed models. The highly significant adjusted R-square values also indicate that the models (Table 3) adequately represent the actual relationships between the concentration of extracellular

saccharides and cultivation conditions and only a relatively small proportion (3.96-12.79%) of the total variance remains unexplained. The validation of models using the LOOCV procedure although, resulted in the certain reduction of the R^2 values (Table 3), but still remained within the limits of high ($p<0.00001$) statistical significance. The analysis of variance (ANOVA) for the regression models are summarized in the Table 4. Both models represent all three variables under study and contain not only linear and quadratic, but also the

Table 2: Box-Behnken design matrix and response data representing the concentrations of *G. nephelii* P1464 extracellular fructose polymers and oligosaccharides in the cultivation medium

	Variables				
Run	X_1	X_2	X_3	Fructan±SE (g L^{-1})	Oligosaccharide±SE (g L^{-1})
1	–	–	0	8.35±0.26	1.23±0.02
2	+	–	0	21.81±0.59	1.35±0.14
3	–	+	0	8.89±0.58	2.38±0.24
4	+	+	0	22.71±0.69	14.18±0.16
5	–	0	–	7.55±0.07	1.59±0.36
6	+	0	–	21.73±1.31	7.89±0.20
7	–	0	+	6.58±0.00	2.18±0.21
8	+	0	+	27.09±0.15	11.28±0.11
9	0	–	–	10.49±0.15	4.73±0.01
10	0	+	–	13.22±0.66	6.30±0.21
11	0	–	+	14.15±0.37	5.99±0.04
12	0	+	+	31.98±0.22	11.55±0.56
13	0	0	0	21.16±0.07	8.67±0.04
14	0	0	0	24.72±0.15	8.06±0.46
15	0	0	0	22.94±0.04	8.37±0.25

	Real levels		
	-1	0	+1
X_1 sucrose concentration (g L^{-1})	50	135	220
X_2 yeast extract (g L^{-1})	3	6	9
X_3 agitation (rpm)	100	170	240

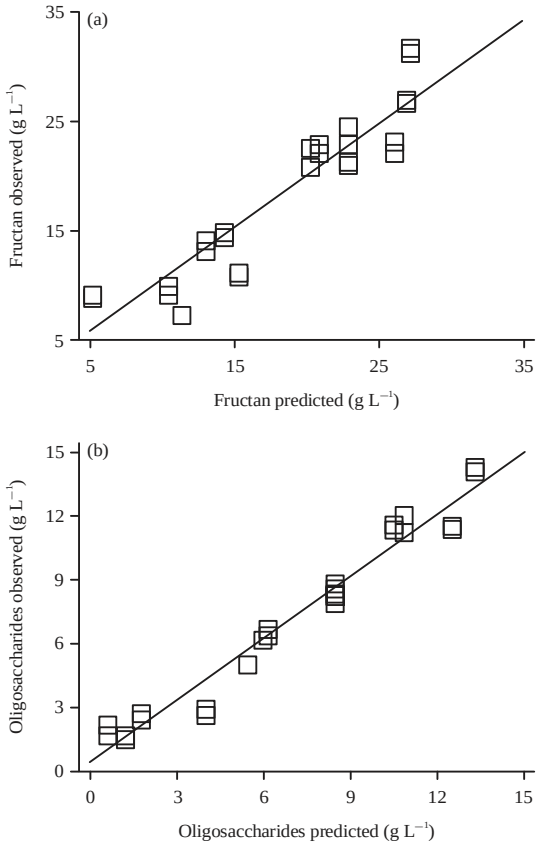

Fig. 2(a-b): Linear plots of the actual concentrations of *G. nephelii* P1464 (a) Extracellular fructans and (b) Oligosaccharides against those predicted by the regression models[a]. [a]Model elements together with the statistical indices are represented in Table 2 and 3

Table 3: Elements and the statistical indices for multiple regression models which link the concentrations of extracellular fructans and oligosaccharides of *G. nephelii* P1464 and varied cultivation conditions

Regression model	Dependent variable[a]	Parameters	Regression coefficient	SE	t-value	p-value	R^2%[a]	$R^2_{adjusted}$%[a]	R^2%[b]	$R^2_{adjusted}$%[b]
I	Fructan	Constant	22.9387	1.3141	17.455	0.0000	87.66	83.73	77.90	77.11
		X_1	7.8345	0.8047	9.735	0.0000				
		X_2	2.6541	0.8047	3.298	0.0033				
		X_3	3.3500	0.8047	4.163	0.0004				
		X_1^2	-4.5224	1.1846	-3.818	0.0009				
		X_2^2	-2.7959	1.1846	-2.360	0.0275				
		XX_3^2	-2.6804	1.1846	-2.263	0.0339				
		X_2X_3	3.7754	1.1381	3.317	0.0031				
II	Oligosaccharides	Constant	8.4542	0.3040	27.814	0.0000	96.18	94.96	92.57	92.30
		X_1	4.6656	0.2237	20.856	0.0000				
		X_2	1.4019	0.2237	6.267	0.0000				
		X_3	1.2962	0.2237	5.794	0.0000				
		X_1^2	-1.2412	0.3283	-3.780	0.0010				
		XX_3^2	-1.4124	0.3283	-4.302	0.0003				
		X_1X_3	0.6988	0.3164	2.209	0.0379				
		X_2X_3	1.0263	0.3164	3.244	0.0037				

[a]Model elements are represented in Table 1, [b]Obtained by the LOOCV[19] of models

Table 4: Variance analysis of multiple regression models[a] linking the concentrations of extracellular fructans and oligosaccharides of *G. nephelii* P1464 and varied cultivation conditions

	Variables							
Model	Dependent	Independent	Variance source	Sum of squares	df	Mean square	F-ratio	p-value
I	Fructan	$X_1, X_2, X_3, X_1^2, X_2^2, XX_3^2, X_2X_3$	Model	1618.67	7	231.238	22.32	0.0000
			Residual	227.959	22	10.3618		
			Total	1846.62	29			
II	Oligosaccharides	$X_1, X_2, X_3, X_1^2, X_3^2, X_1X_3, X_2X_3$	Model	443.476	7	63.3537	79.12	0.0000
			Residual	17.6156	22	0.80071		
			Total	461.092	29			

[a]Model elements together with the statistical indices are represented in Table 1 and 2, df: Degree of freedom

interaction terms although there are certain differences (Table 3). Thus, for the model describing the fructan formation (model I, Table 3) all three quadratic terms are statistically significant together with only one significant interaction between the yeast extract concentration and agitation intensity. By contrast, in the case of oligosaccharides (model II, Table 3) only the linear effect of yeast extract concentration appears together with the additional interaction between the sucrose concentration and agitation rate. Such specificities, in turn, have an impact on the further searches for optimum in these models. Both functions have three variables, so each of them relates to the 3D hypersurface located in the 4D hyperspace, hence, out of the direct geometrical interpretation that allows, however, the searches by an analytical way. Thus, the regression model, which contains 3 quadratic terms (model I, Table 3), proved the global maximum where the predicted value of response reaches 31.6631 g L^{-1} fructans at the factor levels (coded values) $X_1 = 0.8662$, $X_2 = 1.7093$ and $X_3 = 1.8287$. In terms of factor real values (Table 2) this means that that the maximum fructan concentration in the cultivation medium can be achieved at the initial sucrose concentration (X_1) 208.63 g L^{-1}, the initial yeast extract concentration (X_2) 11.13 g L^{-1} and the agitation rate (X_3) 298 rpm. By contrast, the other function describing the formation of oligosaccharides (model II, Table 3) does not exhibit the globalmaximum in respect of all three variables. Although, it is possible in both cases to find the optimum of regression model for two variables if the third variable is set at a constant level and as a consequence, to visualize the corresponding response surface. Such an analysis would be even necessary taking into account the observed interactions between the factors which could substantially affect the response variable. Really, if the initial concentrations of yeast extract (X_2) for this model (model II, Table 3) are set at a constant level the regression models with two independent variables can be obtained, describing quadratic response surfaces as the elliptic paraboloids (Fig. 3). In case the yeast extract concentration is kept constant at the upper level (Fig. 3a) the predicted value

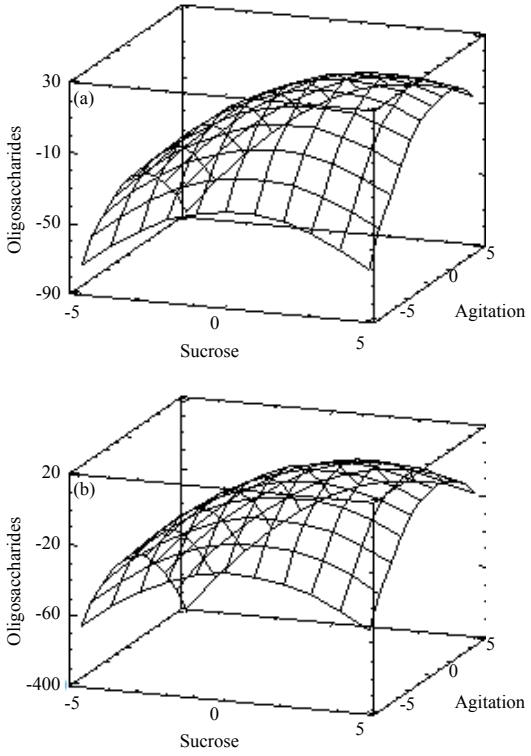

Fig. 3(a-b): Response surface plot showing changes of *G. nephelii* P1464 oligosaccharides as a dependent variable upon to concentration of sucrose and agitation rate as independent variables (X_1 and X_3, respectively) at fixed yeast extract concentration (X_2) at the (a) Upper and (b) Lower level. The global maximum is 16.7557 g L^{-1} at the factor levels (coded values) $X_1 = 2.269$ and $X_3 = 1.383$ at the upper level of yeast extract concentration. The model[a] equations: (a) Oligosaccharides (g L^{-1}) = $9.8561+4.6656*X_1+2.3225*X_3-1.2412*X_1^2-1.4124*X_3^2+0.6988*X_1X_3$, $R^2 = 96.180\%$, $p<0.0000$ and (b) Oligosaccharides (g L^{-1}) = $7.0524+4.6656*X_1+0.2700*X_3-1.2412*X_1^2-1.4124*X_3^2+0.6988*X_1X_3$; $R^2 = 96.180\%$; $p<0.00001$. [a]Model elements together with the statistical indices are represented in Table 2 and 3

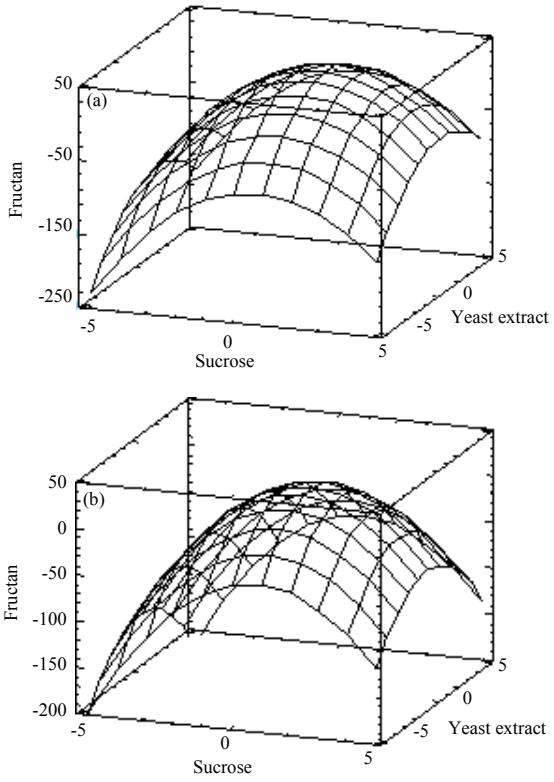

Fig. 4(a-b): Response surface plot showing changes of *G. nephelii* P1464 fructans as a dependent variable upon to concentration of sucrose and yeast extract as independent variables (X_1 and X_2, respectively) at fixed at the upper (a) and lower (b) level of agitation rate (X_3). The global maximum is 30.6977 g L^{-1} (a) At the factor levels (coded values) $X_1 = 0.866$ and $X_2 = 1.150$ at the upper level of agitation rate. The model[a] equation: (a) Fructans (g L^{-1}) = $23.6083+7.8345*X_1+6.4295*X_2-4.5224*X_1^2-2.7959*X_2^2$, $R^2 = 87.655\%$, $p<0.00001$ and (b) Fructans (g L^{-1}) = $16.9083+7.8345*X_1-1.1213*X_2-4.5224*X_1^2-2.7959*X_2^2$, $R^2 = 87.655\%$, $p<0.00001$. [a]Model elements together with the statistical indices are represented in Table 2 and 3

of response reaches 16.756 g L^{-1} oligosaccharides at the factor levels (coded values) $X_1 = 2.269$ and $X_3 = 1.383$. In terms of factor real values (Table 2) this indicates that the maximum oligosaccharide concentration in the cultivation medium could be achieved at the initial sucrose concentration (X_1) 327.9 g L^{-1} and the agitation rate (X_3) 197 rpm.

Keeping the yeast extract concentration at the lower level ($X_2 = -1$) results in decreasing oligosaccharide concentration by almost 30% together with a relatively smaller reduction of the sucrose concentration and a slight increase of agitation rate (5.7 and 7.6%, respectively) due to a significant interaction between factors in the regression model (Fig. 3b).

The formation of extracellular fructans by *G. nephelii* P1464 also can be evaluated and visualized in a similar way (Fig. 4). For instance, the maximum concentration of fructans in the cultivation medium at the upper level ($X_3 = +1$) of agitation rate could achieve 30.698 g L^{-1} (Fig. 4a) which is very slightly (3.05%) below the global maximum as mentioned above for the regression model with three variables (model I, Table 3). Besides, it can be achieved at a relatively lower (9.45 g L^{-1}) yeast extract concentration (Fig. 4a). The matching quality of the data obtained by the modified two-variable quadratic regression models were confirmed by the highly significant linear relationships of the actual concentrations of extracellular fructans and oligosaccharides against those predicted by models.

It is worth noting that the biomass formation and fructan yield (Yp/s) also can be described with similar three-variable regression models ($p<0.00001$) although with relatively lower adjusted R-squared values (64.11 and 60.46%, respectively). Since the biomass model includes quadratic terms of all three variables the global maximum (0.54 g L^{-1}) can be found at the sucrose concentration 212.72 g L^{-1} and the medium levels (Table 2, factor real values) of the yeast extract concentration 6 g L^{-1} and agitation rate 170 rpm. Besides, the modified two-variable regression models and relevant response surfaces can be derived as described (Fig. 3, 4) for the biomass formation and fructans yield at the fixed upper level of sucrose concentration.

DISCUSSION

Extensive evidence has been obtained that the quantities and the composition of microbial exopolysaccharides are strain dependent and affected by varied nutritional and environmental conditions[20,22-24]. It is therefore necessary to find and specify the optimal cultivation conditions for each individual species. Besides, it should be emphasized[4,13] that the growth of acetic acid bacteria on sucrose as a carbon source and the polysaccharide production from the *Gluconobacter* strains are still insufficiently studied. In order to solve such objectives, the response surface methodology and methods of factorial design have found wide applications, although within relatively limited bacterial species[10,11,25] among which acetic acid bacteria remain unrepresented. The combination of Plackett-Burman and Box-Behnken factorial designs[21] was used in this study to identify the most important factors and assess the effects of their interactions on the formation of fructans and oligosaccharides by *G. nephelii* on the sucrose-containing media.

The obtained results suggest that formation of extracellular fructans and oligosaccharides by *G. nephelii* P1464 depends on the concentration of sucrose and yeast extract in the cultivation medium as well as the agitation rate. These data are consistent with the observations about the most important factors affecting the formation of bacterial fructans and oligosaccharides[22,24-26].

It has been shown that the sucrose concentration has a particular impact in this respect[10,27,28], although its increase does not always contribute the synthesis of fructans. Thus, for *Acetobacter xylinum*, unlike *G. nephelii*, the elevated sucrose concentration (above 70 g L^{-1}) caused a decline of fructan content in the medium[3]. This could be explained by a lower overall resistance of *A. xilinum* to the osmotic stress and/or by the fact that its extracellular levansucrase (EC 2.4.11) is probably more susceptible to the substrate inhibition as compared to the *G. nephelii* strain under study. Also, the impact of increased yeast extract concentration as well of more intense aeration, which, in most cases, contribute to the formation of extracellular fructans are considered to be bacterial species-specific effects[10,22,25,28]. It is worth noting that the involvement of the second-order Box-Behnken factorial design allows more accurate estimation of the factor effects. Thus, the assessment of factors according the linear effects of Plackett-Burman design (Fig. 1, 2) that does not take into account the possible impact of interaction suggests that the agitation rate much more affects the fructan synthesis while the concentration of yeast extract has the greater impact on biomass formation. A similar effect of yeast extract has been reported for the fructan synthesis by *B. licheniformis*[29].

In contrast, cultivation experiments according the Box-Behnken design (Table 2, 3) clearly indicate that all three factors significantly affect the synthesis of fructans and oligosaccharides as well as the biomass formation. Moreover, for example during the cultivation of *G. nephelii* P1464, the sucrose and yeast extract concentrations exhibit different effects at different levels of agitation rate (Fig. 4).

CONCLUSION

Based on the Plackett-Burman and the Box-Behnken factorial designs the agitation rate, sucrose and yeast extract concentrations were highlighted and confirmed as most influential factors that contribute to the maximum production of both extracellular fructose polymers and fructo oligosaccharides by *G. nephelii* P1464 representing the original strain of very little studied species. The second-order polynomial models were developed, describing the production of these compounds at varying levels of influential factors and their global maxima detected thus indicating the optimal sets of culture conditions. Therefore, the use of Response Surface Methodology (RSM), including the combination of Plackett-Burman two-level design and Box-Behnken three-level design can be considered as a favourable strategy and a convenient tool to select the relevant factors and determine their optimal levels in order to maximize the formation of extracellular fructans and oligosaccharides by acetic acid bacteria *G. nephelii* P1464.

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