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Optimization of the culture medium using the Burman and Box-Behnken experimental designs for an enhanced production of alginate by *Azotobacter vinelandii*

ABD EL-RAHEEM R. EL-SHANSHOURY [1]*, EL-REFAIE KENAWY [2], AMRO A. AMARA [3,4], SAMIA F. MOHAMED [5], AFRAH F. SALAMA [6], SHIMAA KISHK [6]

[1] Bacteriology Research Unit, Microbiology Section, Botany Department, Faculty of Science, Tanta University, Tanta, Egypt

[2] Polymer Research Unit, Chemistry Department, Faculty of Science, Tanta University, Tanta, Egypt

[3] Protein Research Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technology Applications, Alexandria, Egypt

[4] Microbiology Division, Pharmaceutical Department, Faculty of Pharmacy at King Saud University (KSU), Riyadh, Kingdom of Saudi Arabia

[5] Pharmacology and Toxicology Unit, Department of Biochemistry, Animal Health Institute, Giza, Egypt

[6] Biochemistry Unit, Chemistry Department, Faculty of Science, Tanta University, Tanta, Egypt

Abstract

Alginates are regarded as high-value biomacromolecules. A pursuit for new bacterial species that can produce novel alginates for various applications in different industries including the pharmaceutical and food industries as well as the field of medicine is still needed. Variations in the molecular weight and sugar composition of alginates depend on the culture medium composition and different physical conditions provided during fermentation. The aim of this study was to obtain a maximum alginate production by the potent Egyptian bacterial strain *Azotobacter vinelandii*, out of the 30 tested *Azotobacter* isolates. The composition of the production medium and environmental growth conditions during fermentation were optimized to ensure consistency and to enhance alginate production. Nine variables were optimized using the Plackett-Burman randomization method. Eighteen trials were performed to verify the variables to obtain high alginate production levels. Temperature as well as sucrose and NaCl concentrations have been found to affect the alginate production by *A. vinelandii* significantly and were therefore chosen for further improvement of the medium using the Box-Behnken design. The Box-Behnken model was optimized mathematically using the Excel solver. The results were analyzed statistically by ANOVA multiple regression analysis and different statistical methods (coefficient, standard error, *t* statistic, and confidence interval). On using the Plackett-Burman and Box-Behnken designs, the biggest alginate production rates were found to be 3.8 and 4.94 g/l/day, respectively. Addition of 00.1 g/l $MgSO_4$, 0.01 g/l FSO4, and 0.001 g/l $NaMoO_4$ to the culture medium further enhanced the alginate production and yielded 5.4 g/l/day and 8.5 g/l/day. Thus, the obtained alginate was purified using Sephadex G-100 Gel chromatography and compared with a standard by IR correlation. The alginate was found to be composed of 50–70% carbohydrates and 60–80% uronic acid. The techniques used in this survey were found useful for improving the production of alginates.

Key words: alginate, *Azotobacter vinelandii,* Box-Behnken, optimization, Plackett-Burman, culture medium

Introduction

Alginate is a negatively charged polymer, present in viscous solutions in a soluble (in the absence of divalent cations) or in a gel-like (in the presence of divalent or trivalent cations) form. Alginates are exopolysaccharides composed of linear copolymers containing blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues (Auhim and Hassan, 2013). The blocks are arranged as homogeneous poly-G (GGGGGG) or poly-M (MMMMMM) sections or alternating M- and G-residues

* Corresponding author: Bacteriology Research Unit, Microbiology Section, Botany Department, Faculty of Science, Tanta University, Tanta 31527, Egypt; e-mail: abrabsh@gmail.com

(GMGMGM) in a block in a similar manner. Alginates differ in terms of M and G contents as well as compositions of each block, with a common molecular formula ($C_6H_8O_6$) and with a molar mass ranging between 10 and 600 kDa (Charles et al., 2012; Szekalska et al., 2016). The extracellular accumulation of alginates serves as a barrier for diffusion of oxygen or heavy metals and/or as a protection against other environmental threats (Segura et al., 2003). It plays an important role in biofilm formation (Thielen et al., 2005) and interacts with Ca^{2+} to form Ca^{+2} alginate complexes (Schmid et al., 2008). Although brown seaweeds are the main source for the commercial obtainment of alginates, bacterial biosynthesis constitutes an interesting alternative as it offers the production of alginates with more defined chemical structures and physical properties than those isolated from seaweeds. The valuable properties of bacterial alginates ensure an increasing interest from various industries (Sabra, 1999; Diaz-Barrera and Sot, 2010; Mejia et al., 2010). Alginates produced by bacteria are rich in mannuronic acid (Rehm and Valla, 1997; Sabra, 1999). Those produced by *A. vinelandii* have a high molecular mass of up to 1,350 kDa (Mejia et al., 2010; Diaz-Barrera et al., 2011), although a molecular mass of about 4000 kDa produced by a mutant strain of *A. vinelandii* has also been reported (Pena et al., 2002). Moreover, bacterial strains acetylate alginates, whereas alginates obtained from algae are not acetylated. Both, alginate acetylation and molecular mass affect alginate viscosity and other properties (Pena et al., 2006; Auhim and Hassan, 2013). Various bacteria, especially *Pseudomonas* and *Azotobacter* spp. can synthesize alginates; however, alginates from *Azotobacter* sp. are preferred for technical and medical applications. Strains of *A. chroococcum* can also produce alginates (Cote and Krull, 1988). *A. vinelandii* requires alginates for the encystment process; cysts in their mature form are surrounded by two discrete layers rich in alginates, which enable the sleeping cells to survive long periods of desiccation. Alginates from *Azotobacter* sp. contain polyguluronates, which form rigid gels in the presence of Ca^{2+} and are also important in the formation of cysts. Conversely, in the absence of polyguluronates, but in the presence of Ca^{2+}, relatively flexible gels are produced by *Pseudomonas aeruginosa* (Sherbroock-Cox et al., 1984).

Alginate is a biomaterial that has many applications. They are used in the production of ice creams, frozen custards, creams, and cake mixtures in the food industry and in beer manufacture to enhance the formation of foam (Neidleman, 1991). In biotechnological applications, alginates are used for cell immobilization, and in the production of ethanol by yeast and monoclonal antibodies by hybridoma cells (Crescenzi, 1995). Alginates increase the flocculate aggregation (Dekwer and Hempel, 1999) in water treatment processes (Grasdalen, 1983; Rehm and Valla, 1997; Sabra et al., 2001). They have found many applications in the biomedical and engineering sectors due to their favorable properties, including biocompatibility and ease of gelation. Alginate hydrogels have been particularly attractive in specific sectors such as biomedicals and pharmaceuticals, in particular; in developing controlled drug delivery systems, wound healing, tissue engineering, and the food industry (Remminghorst and Rehm, 2006; Galindo et al., 2007; Yao et al., 2009; Lee and Mooney, 2012; Rinaudo, 2014). Alginates are suitable for use as matrices for three-dimensional tissue cultures, adjuvants of antibiotics, and antiviral agents in cell transplantation in diabetes or neurodegenerative disease treatment (Szekalska et al., 2016).

Materials and methods

Microorganisms and basal medium

Soil samples, including cultivated and uncultivated ones, were collected from different localities in Tanta, Gharbia Governorate, Egypt. Thirty isolates belonging to the genus *Azotobacter* were isolated. The bacterial strains were purified by subculturing on a nitrogen-free medium, as described by Vancura and Mancura (1960). Its composition was as follows (per liter): 20 g sucrose; 0.65 g K_2HPO_4; 0.16 g KH_2PO_4; 0.2 g NaCl; 0.2 g $MgSO_4 \cdot 7\ H_2O$; 2 g CaCO3; 0.005 g $NaMoO_4 \cdot 2H_2O$; 0.005 g $Fe_2(SO_4)_3$; 0.005 g $NaBO_4$, and 15 g Bacto-agar, prepared at a pH of 7. Of 30 isolates, five highest N-fixers and alginate producers were selected and characterized. Among the five isolates chosen for further studies, isolate Az5, the highest alginate producing ability, identified by El-Shanshoury and coworkers (2013) as *A. vinelandii* was also used in this study.

Alginate production

Following the composition of the medium used for alginate production: 30 g/l sucrose; 2 g/l $NaNO_3$; 1 g/l

Table 1. Morphological characteristics and quantification of alginates produced by selected *Azotobacter* isolates

Isolate code	Morphology of colonies [1]		Alginate [2] [g/l]	Levels of alginate [3]
	color	elevation		
Az1	brown	raised	0.60	low
Az2	translucent	convex	1.45	high
Az3	translucent	raised	0.58	low
Az4	brown	raised	0.80	moderate
Az5	translucent	raised	1.50	high

[1] All isolates were isolated from soil, the surface of their colonies was smooth and the colony margin was entire; [2] Alginate produced by the selected Azotobacters in a nitrogen-free liquid medium; [3] Low producers (0.58–0.60 g/l), moderate producers (0.80 g/l), and high producers (1.45–1.50)

Table 2. Effect of different variables on alginate production rate by *A. vinelandii* utilizing the Plackett-Burman design

Experiment no.		X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	Alginate production rate [g/l/day]
+*a*	1	−1 (30)	1(7)	1(30)	1(2)	1(1)	1(0.5)	1(0.5)	1(0.05)	−1(200)	2.7
Plackett-Burman experiments	2	−1(30)	−1(5)	1(30)	−1(0.1)	1(1)	1(0.5)	1(0.5)	−1(0)	1(250)	0.3
	3	−1(30)	1(7)	−1(5)	1(2)	−1(0)	1(0.5)	1(0.5)	1(0.05)	−1(200)	0.6
	4	1(37)	1(7)	1(30)	1(2)	1(1)	−1(0)	1(0.5)	1(0.05)	1(250)	3.0
	5	1(37)	−1(5)	1(30)	−1(0.1)	1(1)	1(0.5)	−1(0)	1(0.05)	1(250)	2.4
	6	1(37)	−1(5)	−1(5)	1(2)	−1(0)	1(0.5)	1(0.5)	−1(0)	1(250)	0.7
	7	1(37)	−1(5)	−1(5)	1(2)	1(1)	−1(0)	1(0.5)	1(0.05)	−1(200)	1.0
	8	−1(30)	−1(5)	−1(5)	−1(0.1)	1(1)	1(0.5)	−1(0)	1(0.05)	1(250)	0.1
	9	1(37)	1(7)	−1(5)	−1(0.1)	−1(0)	1(0.5)	1(0.5)	−1(0)	1(250)	0.2
	10	1(37)	−1(5)	1(30)	−1(0.1)	−1(0)	-1(0)	1(0.5)	1(0.05)	−1(200)	1.9
	11	−1(30)	−1(5)	−1(5)	−1(0.1)	−1(0)	-1(0)	-1(0)	1(0.05)	1(250)	0.0
	12	−1(30)	1(7)	−1(5)	1(2)	−1(0)	−1(0)	−1(0)	−1(0)	1(250)	0.0
	13	−1(30)	1(7)	1(30)	−1(0.1)	1(1)	−1(0)	−1(0)	−1(0)	−1(200)	3.8
	14	1(37)	1(7)	1(30)	−1(0.1)	−1(0)	1(0.5)	−1(0)	−1(0)	−1(200)	1.3
	15	−1(30)	−1(5)	1(30)	1(2)	−1(0)	−1(0)	1(0.5)	−1(0)	−1(200)	2.4
	16	−1(30)	1(7)	−1(5)	1(2)	1(1)	−1(0)	−1(0)	1(0.05)	−1(200)	2.2
	17	1(37)	1(7)	1(30)	1(2)	1(1)	1(0.5)	−1(0)	−1(0)	1(250)	2.7
−*b*	18	1(37)	−1(5)	−1(5)	−1(0.1)	−1(0)	−1(0)	−1(0)	−1(0)	−1(200)	1.0

Where: X_1 – temperature, X_2 – pH, X_3 – sucrose, X_4 – $NaNO_3$, X_5 – KH_2PO_4, X_6 – NaCl, X_7 – $MgSO_4$, X_8 – $FeSO_4$, X_9 – shaking rate

KH_2PO_4; 0.5 g/l NaCl; 0.5 g/l $MgSO_4$; 0.05 g/l $FeSO_4$; 0.005 g/l $NaMoO_4$; 0.06 g/l $CaCl_2$, and 15 g/l Bacto-agar. Alginate production was performed in 250 ml Erlenmeyer flasks containing 100 ml alginate production medium. The medium composition and the environmental factors were altered during the running of both Plackett-Burman and Box-Behnken designs (Plackett and Box, 1946) as described in Tables 1 and 5.

Medium optimization for alginate production using the Plackett-Burman and Box-Behnken designs

The Plackett-Burman design (Plackett and Burman, 1946) was used for screening the effect of nine variables, including temperature [X_1], pH [X_2], sucrose [X_3], $NaNO_3$ [X_4], KH_2PO_4 [X_5], NaCl [X_6], $MgSO_4$ [X_7], $FeSO_4$ [X_8], and shaking rate [X_9]. Eighteen experiments were performed for the analysis of nine variables (Table 1). The results of the Plackett-Burman design were applied to the linear multiple regression analysis, using Microsoft Excel 2002, as described by Abdel-Fattah and Olama (2002). From the statistical analysis of the data collected in Table 1 and summarized in Table 2, the variables with confidence levels ≥ 90% were considered to be significantly affected by the alginate production rate. Variables with confidence levels between 70% and 90% were considered effective (Stowe and Mayer, 1966). The model created for the analysis of the Plackett-Burman experimental design using a multiple regression analysis was based on the First-order model, where $Y = \beta_0 + \sum \beta_i \cdot X_i \cdot Y$ was the predicted response, β_0 was the model constant, and X_i indicated the different variables used (Plackett and Burman, 1946). Three most influential variables out of nine were selected (sucrose [X_1], temperature [X_2] and NaCl [X_3]) for further optimization using the Box-Behnken experimental design. The randomization represented three levels: high (+1), medium (0), and low (−1). The variables were: [X_1] 30, 17.5, and 5 g/l; [X_2] 37, 33.5, and 30 °C; [X_3] 0.5, 0.25, and 0 g/l. The results obtained from running the Box-Behnken experiment (Table 5) were analyzed by a linear multiple regression analysis. The response surface and counterparts were predicted for each of the 2 responses using Microsoft Excel 2000 and Essential Exp., Version 2.205 software (Steppan et al.,1998). The designed models were applied using the coefficient results of each variable. Of the three variables randomized in the Box-Behnken experiment, the following equation was used:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where X_1, X_2, and X_3 were independent variables; β_1, β_2, and β_3 were linear coefficients; β_{12}, β_{13}, and β_{23} were cross product coefficients; and β_{11}, β_{22}, and β_{33} were the quadratic coefficients. Sucrose [X_1], temperature [X_2], and NaCl [X_3] were further optimized to calculate the best alginate production rate (Y) using the Microsoft Excel 2002 solver. The Y values determined by the Excel solver were experimentally verified using the calculated optimum values of sucrose [X_1], temperature [X_2], and NaCl [X_3]. The accuracy percentage of the model was calculated using the following formula: accuracy of the model = [$Y_{experiment} / Y_{calculated}$] × 100]. To prove the model's accuracy, the variables in the calculated Y were tested experimentally and the alginate production rate (Y experiment) was determined.

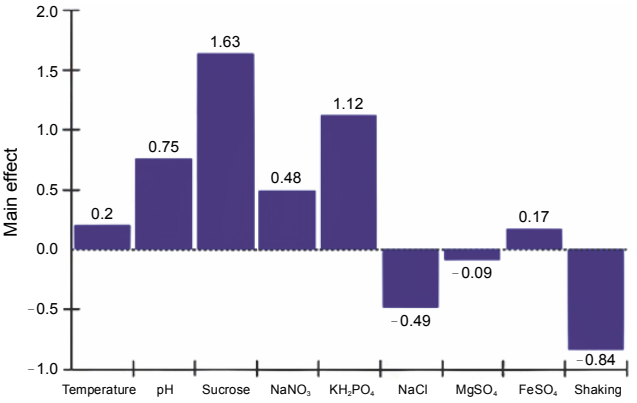

Fig. 1. Effects of environmental conditions and cultural constituents on the rate of alginate production by *A. vinelandii*

Partial purification and characterization of alginates

The growth culture of *A. vinelandii* was subjected to vortexing at 560 g for 3 min and the cells were separated from the supernatant by centrifugation at 1360 g. Proteins present in the supernatant were separated from alginates by adding 10% (w/v) trichloroacetic acid (TCA) (Van den Berg et al., 1995). The precipitated proteins were removed by centrifugation at 605 g for 30 min. After removing the protein fractions, a clear supernatant was collected and allowed to stand overnight at 4 °C. Next, the alginates were precipitated by adding 3 volumes of cold ethanol. Alginate precipitates were collected by centrifugation at 2840 g for 30 min and lyophilized. Alginates were purified using Sephadex G-100 column (1.5 × 45 cm) (Pharmacia Fine Chemicals) with a bed volume of approximately 73 ml. The column was equilibrated with BioUltra Phosphate buffer solution (pH 7.2) composed of 0.044 M Na_2HPO_4, 0.022 M NaH_2PO_4, and the elution was accomplished at a flow rate of 8 drops/min. Three-milliliter fractions were collected, then the total carbohydrate content, uronic acid level, and the contaminating proteins were determined. A carbazol assay was performed to determine the amount of uronic acid in the purified alginate according to the procedure

Table 3. Main effect of each variable on alginate production rate by *A. vinelandii* utilizing the Plackett-Burman design

Code	Variable (−1/+1)	Values		Unit	Main effect
		$(\sum +1)/n_{(+1)}$	$(\sum -1)/n_{(-1)}$		
A	Temperature (30/37)	1.555556	1.354444	°C	0.201111
B	pH (5/7)	1.831852	1.078148	pH	0.753704
C	Sucrose (5/30)	2.272222	0.637778	g/l	1.634444
D	$NaNO_3$ (0.1/2)	1.693333	1.216667	g/l	0.476667
E	KH_2PO_4 (0/1)	2.016667	0.893333	g/l	1.123333
F	NaCl (0/0.5)	1.211481	1.698519	g/l	−0.48704
G	$MgSO_4$ (0/0.5)	1.409259	1.500741	g/l	−0.09148
H	$FeSO_4$ (0/0.5)	1.54	1.37	g/l	0.17
I	Shaking rate (200/250)	1.036667	1.873333	rpm	−0.83667

Where: −1 (low) and +1 (high); main effect = $(\sum +1)/n_{(+1)} - (\sum -1)/n_{(-1)}$

Table 4. Linear multiple regression results of the Plackett-Burman design

Variables	Coefficient	SE	*t*-value	*P*-value	CL (%)	Rank
Intercept	0.146111	0.17572	8.31499	0.0000	100	
$FeSO_4$	0.0259903	0.204006	0.1274	0.9018	9.82	9
KH_2PO_4	0.334075	0.223789	1.49281	0.1738	82.62	2
$MgSO_4$	−0.166757	0.213748	−0.780157	0.4578	54.22	8
NaCl	−0.255913	0.199887	−1.28029	0.2363	76.37	4
$NaNO_3$	0.235113	0.220942	1.06414	0.3183	68.17	6
pH	0.182171	0.205343	0.887151	0.4009	59.91	7
Shaking rate	−0.281703	0.199093	−1.41493	0.1948	80.52	3
Sucrose	0.707217	0.211565	3.3428	0.0102	98.98	1
Temperature	0.202723	0.183497	1.10477	0.3014	70	5

Coefficient – coefficient of variance, SE – standard error, *t*-value – student *t*-test, *P*-value – corresponding level of significance, CL [%] – confidence level [%]

Table 5. ANOVA of the Plackett-Burman Model

Source	SS	Df	MSS	F	*P*-value
Model	19.7964	9	2.1996	3.96	0.0328
Residual	4.44637	8	0.555796		
Total	24.2428	17			

R^2 – 81.659; percent and standard error – 0.745518; Df – degree of freedom; SS – sum of squares; ASS – adjusted sum of squares; MSS – mean sum of squares; F – Fisher function; *P* – corresponding level of significance

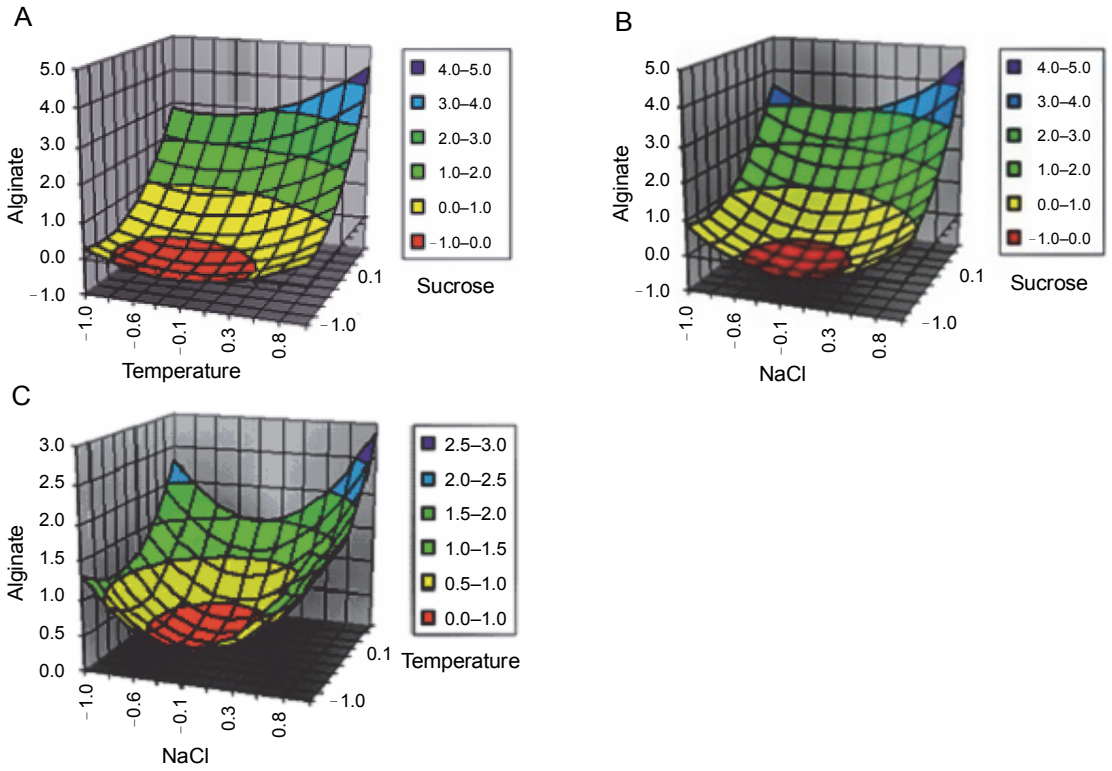

Fig. 2. A) Response surface plot showing the effects of sucrose and temperature, B) sucrose and NaCl, and C) temperature and NaCl on the rate of alginate production by *A. vinelandii*

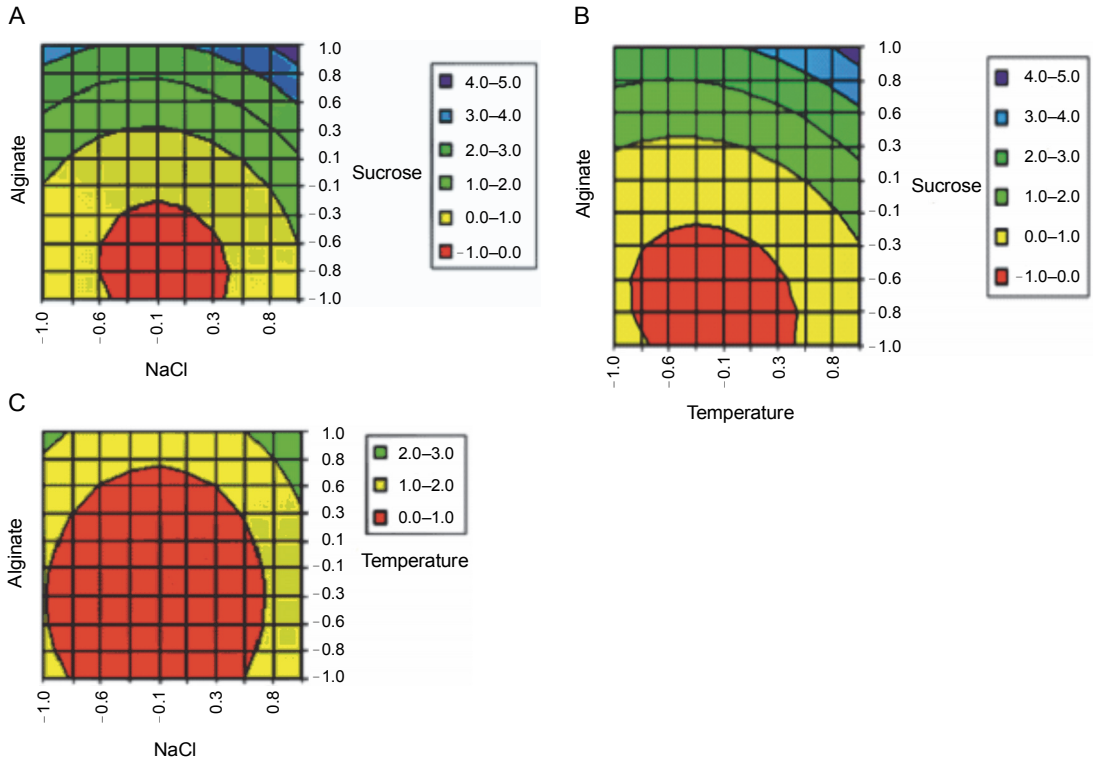

Fig. 3. A) Contour plot showing the effects of sucrose and temperature, B) sucrose and NaCl and C) temperature and NaCl on the rate of production of alginate by *A. vinelandii*

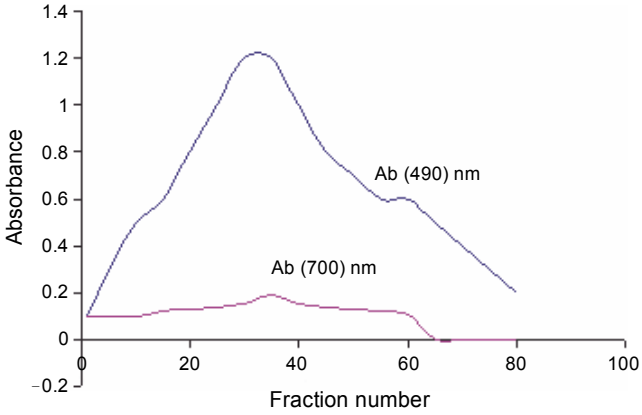

Fig. 4. Purification of alginate produced by *A. vinelandii* using gel chromatography; Ab (490) nm represents total carbohydrates and Ab (700) nm represents the protein content of the sample

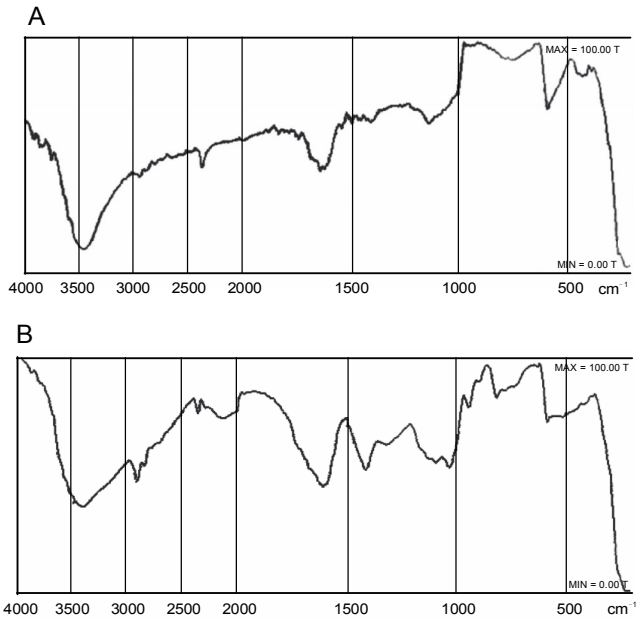

Fig. 5. A) IR analysis of purified alginate sample from *A. vinelandii* and B) standard algal alginate sample

by Bitter and Muir (1962). The protein content of the alginate samples was determined spectrophotometrically according to the method by Lowry and coworkers (1951). One milligram of pure dried alginate was used for an infrared (IR) analysis using a Perkin-Elmer 1430 spectrophotometer, following the method described by Sherbroochk-Cox and coworkers (1984). The IR spectrum of the isolated and purified alginates was compared with a known alginate sample.

Results and discussion

Alginates are biopolymers produced by brown algae and a few bacterial species (Sabra et al., 2001). *Azotobacter* sp. and *Pseudomonas aeruginosa* are able to produce significant amounts of alginates (Clementi, 1997; Remminghorst and Rehm, 2006). In contrast to *P. aeruginosa*, *A. vinelandii* is a nonpathogenic bacterium that has been used for the development of a biotechnological process to produce alginates (Clementi, 1997; Sabra et al., 2001; Amara et al., 2006; Hussain and Amara, 2006). In this study, in the initial experiments (presented in Table 1), of the 30 local Egyptian *Azotobacter* strains isolated and screened out, five isolates produced alginate with variable amounts. Az5 isolate (*A. vinelandii*), among other alginate producers, was selected and subjected for further studies to improve alginate production levels and its characteristics. Alginates have a wide range of applications and are used as thickeners, stabilizers, gelling agents, and emulsifiers in the food, as well as in textile and pharmaceutical industries. Recent biotechnological advances show a promising potential application of alginates in the making of medical drugs (Yao et al., 2009; Lee and Mooney, 2012; Rinaudo, 2014).

Strategies for the biotechnological production of high-quality and/or tailor-made bacterial alginates are being developed by evaluation the influences of different nutritional and operational conditions (Sabra et al., 2001; Trujillo-Roldan et al., 2004; Diaz-Barrera and Soto, 2010; Diaz-Barrera et al., 2011; Auhim and Hassan, 2013). A response surface methodology (RSM) is a well-known method applied in the optimization of medium constituents and other critical variables responsible for the production of biomolecules (Montgomery, 1997). An experimental design is a common practice in biotechnology (Kalil et al., 2000), whereas optimization by changing one factor at a time is a time-consuming process as reported by various researchers, for example, Lakshman and coworkers (2004). Experimental design is an organized approach that yields more reliable information per experiment than unplanned approaches (Nickel et al., 2005). A number of statistical experimental designs have been used for optimizing different fermentation variables (Xu et al., 2002; Rao et al., 2004). The Plackett–Burman design (Plackett and Box, 1946) is a well-established and widely used statistical technique for screening and selection of critical culture variables (Rao, 2004; Kaur and Satyanarayana, 2005). The Plackett-Burman experimental design method was implemented using +1 and −1 for each variable as shown in Table 2. Eighteen experiments were conducted and the results have been summarized

Table 6. Box-Behnken design for optimized production of alginate by *A. vinelandii*

Experiment No.	Sucrose X_1	Temperature X_2	NaCl X_3	Alginate [g/l/day]
1	−1(5)	−1(30)	0(0.25)	0.53
2	1(30)	−1(30)	0(0.25)	2.7
3	−1(5)	1(37)	0(0.25)	0.75
4	1(30)	1(37)	0(0.25)	4.19
5	−1(5)	0(33.5)	−1(0)	0.49
6	1(30)	0(33.5)	−1(0)	3.564
7	−1(5)	0(33.5)	1(0.5)	0.87
8	1(30)	0(33.5)	1(0.5)	4.94
9	0(17.5)	−1(30)	−1(0)	1.4
10	0(17.5)	−1(30)	1(0.5)	1.7
11	0(17.5)	1(37)	−1(0)	2.51
12	0(17.5)	1(37)	1(0.5)	2.78
13	0(17.5)	0(33.5)	0(0.25)	0.37
14	0(17.5)	0(33.5)	0(0.25)	0.07
15	0(17.5)	0(33.5)	0(0.25)	0.5

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{33}$; X_2 and X_3 are independent variables; β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{13}, and β_{23} are cross-product coefficients; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients

Table 7. Multiple regression results of the Box-Behnken design

Variables	Coefficient	SE	*t*-value	*P*-value	CL [%]	Rank
Intercept	0.313333	0.199338	1.57187	0.1768		
Sucrose	1.59425	0.122069	13.0602	0.0000	100	1
Sucrose* Sucrose	1.04883	0.179681	5.8372	0.0021	99.79	3
Sucrose* Temperature	0.3175	0.172632	1.83917	0.1253	87.47	6
Sucrose* NaCl	0.249	0.172632	1.44238	0.2088	79.12	7
Temperature	0.4875	0.122069	3.99364	0.0104	98.96	3
Temperature* Temperature	0.680333	0.179681	3.78634	0.0128	98.72	4
Temperature* NaCl	−0.0075	0.172632	−0.043445	0.9670	3.3	8
NaCl	0.29075	0.122069	2.38185	0.0630	93.7	5
NaCl* NaCl	1.10383	0.179681	6.14329	0.0017	99.83	2

Coefficient – coefficient of variance; SE – standard error; *t*-value – student *t*-test; *P*-value – corresponding level of significance, CL [%] – confidence level [%]

in Table 1 as an alginate production rate (g/l/day). The variations in the results showed the importance of utilizing an experimental design in nutritional condition optimization to map the points that affect the alginate production rates. The main effect of each variable was calculated and summarized in Figure 1 and Table 3. Positive values mean that such variables needed to be increased and the negative ones that they needed to be lowered. A temperature of 37°C, pH of 7, sucrose at 30 g/l, $NaNO_3$ at 2 g/l, and KH_2PO_4 at 1 g/l and 0.5 g/l

Table 8. ANOVA statistical analysis of variance of the Box-Behnken Model

	Df	SS	MSS	F	*P*-value
Model	9	32.54723	3.616359	30.33676	0.000772
Residual	5	0.596036	0.119207		
Total	14	33.14326			

R^2 – 98.2016; percent and standard error = 0.345264; Df – degree of freedom; SS – sum of squares; MSS – mean sum of squares; F – Fisher function; *P*-value – corresponding level of significance

Table 9. Final optimization of alginate production by *A. vinelandii* using Excel solver

Media content	Distilled water* [g/l]	Distilled water^ [g/l]
Sucrose	30.00	30.000
NaCl	0.50	0.500
$CaCl_2$	0.06	0.060
$NaNO_3$	0.10	0.100
KH_2PO_4	1.00	1.000
$MgSO_4$	0.00	0.010
$FeSO_4$	0.00	0.010
$NaMoO_4$	0.00	0.001
Alginate [g/l/day]	5.40	8.500
Model accuracy	88.84	140.000

* the experiment followed the Excel solver optimization for the *Y* value derived from the Box-Behnken method; ^ the experiment followed the Excel solver optimization for the *Y* value derived from Box-Behnken, but variables with 0 g/l amounts changed

positively affected the alginate production rate, while no addition of NaCl nor $MgSO_4$ and a shaking rate of 200 rpm showed negative effects (Fig. 1 and Table 3), which is in contrast to the results obtained by Diaz-Barrera and coworkers (2011), in continuous cultures. The nine variables analyzed using the linear multiple regression analysis method aimed at producing a fitted model. The coefficient level of the variance in the conducted model was calculated from the *P*-value (Table 2). The analysis of variance using the ANOVA method (Table 5) resulted in a *P*-value of 0.0328, significant at the 95% confidence level. The First-order model was alginate production rate (*Y*) = 1.46111 + 0.0259903 · $FeSO_4$ + 0.334075 · KH_2PO_4 – 0.166757 · $MgSO_4$ – 0.255913 · NaCl + 0.235113 · $NaNO_3$ + 0.182171 · pH – 0.281703 · Shaking rate + 0.707217 · Sucrose + 0.202723 · Temperature. The R^2 statistics indicated that the model fitted and explained 81.659% of the variability in the alginate production rate. The adjusted R^2 statistics, which is more suitable for comparing models with different numbers of independent variables, was 61.0253%. The estimates showed that the standard error of the residual was 0.745518. Based on *t* statistics, *P*-value, and the confidence level percentage from the Plackett-Burman design (Table 4 and Table 5) as well as the results from our previous study on poly-β-hydroxybutyrate (PHB) optimization, using the same strategy (El-Shanshoury et al., 2013), we conlcluded that the Plackett-Burman design is a promising tool to improve alginate production. KH_2PO_4 at 2 g/l and a shaking rate of 225 rpm should not be used because they promote the PHB production, which in turn interferes with the alginate production rate, which is in accordance with the results obtained by Pena and coworkers (1997), and Mejia and coworkers (2010). Because sucrose and NaCl concentrations, as well as temperature, significantly influenced alginate production,

they were selected for further optimization. Other variables did not significantly influence alginate production, hence they were used in the amounts as in the Plackett-Burman experiment number 13, which exhibited the maximum alginate production rate. Using the Box-Behnken method, 18 experiments were designed and different alginate production rates were determined (summarized in Table 4). An analysis of variance using the ANOVA test resulted in a *P*-value of 0.000772, which showed a highly significant relationship between the variables, at 99% confidence level (Table 8). The R^2 statistical analysis indicated that the fitted model explained 98.2016% of the variance. The multiple linear regression models of the Box-Behnken analysis experiments described the relationship between the alginate production rate and three independent variables: sucrose (X_1), temperature (X_2), and NaCl (X_3) as indicated in Table 6. The equation of the fitted model was: alginate production rate [*Y*] = 0.313333 + 1.59425 · sucrose + 1.04883 · sucrose · sucrose + 0.3175 · sucrose · temperatures + 0.249 · sucrose · NaCl + 0.4875 · temperatures + 0.680333 · temperatures · temperature – 0.0075 · temperature · NaCl + 0.29075 · NaCl + 1.10383 · NaCl · NaCl. The *P*-value of the independent variables was 0.9670, but calculated for temperature · NaCl was greater or equal to 0.10, which was not statistically significant at the 90% or higher confidence level. From the statistical analysis, the sucrose concentration has the most significant effect on the alginate production rate, where it showed the *P*-value equal to 0.0102 and the highest confidence level percentage, which is in contrast to the results obtained by Mejia and coworkers (2010) in a two-stage fermentation process for alginate production. This was clear from the analysis of the Box-Behnken design, where the confidence level percentage of sucrose equaled 100% of the P-value from the ANOVA test, which was $P = 0.000772$; the R^2 indicated that the model fitted explained 98.20% of the variance. The highest significant value as well as the highest confidence level percentage in both the Plackett-Burman and the Box-Behnken experiments were obtained from sucrose. These results are in agreement with most of the previous studies and prove the power of experimental design in mapping the effective points on the optimization.

The response surface and counter-plots of each of the two optimized variables, their interaction, and effect on alginate production have been shown in Figures 2 and 3. The highest level of the three variables, as obtained from the maximum point of the polynomial model, was further optimized using the solver function of Microsoft Excel tools and was found for sucrose [X_1] = 30 g/l, temperature [X_2] = 37 °C, and NaCl [X_3] = 0.5 g/l with a calculated prediction of the alginate production rate equal to 6.077833 [*Y*]. The flask experiment was conducted, and *Y* value was 5.4 g/l/day with model accuracy equal to 88.84%. The culture medium was supplemented with different minerals at different concentrations. By adding 0.01 g/l $MgSO_4$, 0.01 g/l FSO_4, and 0.001 g/l $NaMoO_4$ to the culture medium, the alginate production rate was found to improve up to 8.5 g/l/day (model accuracy 140%) as shown in Table 8. These nutrients, in addition to sucrose, were possibly responsible for the increase in the alginate production rate as suggested by Mejia and coworkers (2010). However, the overall results obtained in this study confirmed that the experimental design methodology used herein is a suitable method for the optimization of alginate production by *A. vinelandii*, amounting up to 8.5 g/l/day. However, using *Pholiota squarrosa*, after the optimization of medium components using a response surface methodology, an extracellular alginate production 0.876.32 g/l of cultivation medium has been reported by Wang and Lu (2004). Morover, it is possible to engineer *A. vinelandii* strains possessing increased levels of transcription from the *algD* operon, and thus increasing the levels of alginate production. Indeed, when this was combined with the disruption of the polyhydroxybutyrate pathway (thus allowing more carbon sources for alginate biosynthesis) up to 7 g/l of alginate was obtained (Pena et al., 2002; Calindo et al., 2007). Furthermore, a disruption of the Na+-translocating NADH: ubiquinone oxidoreductase complex in *A. vinelandii* lead to an alginate overproducing phenotype; this alginate also had a higher degree of acetylation and a lower G/M ratio, though the exact mechanism remains unclear (Nunez et al., 2009; Gaytanet al., 2012).

The isolated and purified alginate samples were analyzed for total carbohydrates and total protein contents and were found to be 490 and 700 nm, respectively. The fractions showed a low protein level (300 μg/ml) and high alginate contents (Fig. 4). The alginates were reprecipitated and lyophilized for further analysis. The purified alginate samples were compared with a known A0682 alginic acid sodium salt from brown algae using the IR spectrophotometer, and the peaks of the alginate

were nearly the same as those of the reference sample, which indicated a high purity of the alginate (Fig. 5).

Conclusions

The analysis of 30 *Azotobacter* strains isolated from Egyptian soils showed the best growth on a nitrogen-free medium. Five best nitrogen fixing isolates were investigated for their ability to produce alginates. The alginate production rates obtained in the 18 experiments conducted, proved the importance of the Plackett-Burman design in mapping the performance of each variable during the alginate production process. The ranking of the variables regarding their levels of significance showed that sucrose was the most significant variable followed by KH_2PO_4, shaking rate, NaCl, and temperature. Using the Plackett-Burman and Box-Behnken methods/designs, the Excel solver, and a statistical and logical selection of the variables, we were able to improve the rate of alginate production by *A. vinelandii*. Strategies used in this study are recommended in optimizing the production of bioproducts, especially those controlled by complicated enzymatic systems. An advancement in the regulation of alginate biosynthesis in bacteria and introducing mutation into genes encoding enzymes involved in alginate biosynthesis enable the production of alginates with tailor-made features and a wide range of applications. Understanding the mechanisms of epimerization, acetylation, and degradation, as well as their regulation in *A. vinelandii* is crucial for obtaining high-quality products required by new markets. Because many *A. vinelandii* strains are regarded as secure, the utilization of alginate delivered from an ecofriendly *Azotobacter* strain, as used in this study, could bring an added value to alginates used for technical and medical applications.

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