International Microbiology (2018) 21:129–142
https://doi.org/10.1007/s10123-018-0010-5

ORIGINAL ARTICLE

Expression of extracellular polysaccharides and proteins by clinical isolates of *Pseudomonas aeruginosa* in response to environmental conditions

Walid A. Lotfy[1] · Ramy G. Atalla[2] · Wael A. Sabra[2] · Ehab R. El-Helow[2]

Received: 4 March 2018 / Revised: 13 May 2018 / Accepted: 14 May 2018 / Published online: 11 June 2018

Abstract
The opportunistic pathogen *Pseudomonas aeruginosa* causes chronic respiratory infections in patients with cystic fibrosis (CF). Persistence of this bacterium is attributed to its ability to form biofilms which rely on an extracellular polymeric substance matrix. Extracellular polysaccharides (EPS) and secreted proteins are key matrix components of *P. aeruginosa* biofilms. Recently, nebulized magnesium sulfate has been reported as a significant bronchodilator for asthmatic patients including CF. However, the impact of magnesium sulfate on the virulence effect of *P. aeruginosa* is lacking. In this report, we investigated the influence of magnesium sulfate and other environmental factors on the synthesis of alginate and secretion of proteins by a mucoid and a non-mucoid strain of *P. aeruginosa*, respectively. By applying the Plackett-Burman and Box-Behnken experimental designs, we found that phosphates (6.0 g/l), ammonium sulfate (4.0 g/l), and trace elements (0.6 mg/l) markedly supported alginate production by the mucoid strain. However, ferrous sulfate (0.3 mg/l), magnesium sulfate (0.02 g/l), and phosphates (6.0 g/l) reinforced the secretion of proteins by the non-mucoid strain.

Keywords *Pseudomonas aeruginosa* · Polysaccharides · Alginate · Proteins

Introduction

Pseudomonas aeruginosa causes a variety of infections to the urinary tract, respiratory tract, skin and soft tissue, bones and joints, and gastrointestinal tract (Tielen et al. 2013; van 't Wout et al. 2015). *P. aeruginosa* infection is a serious problem in patients hospitalized with cancer, cystic fibrosis (CF), and burns with case fatality rate of 50% (Boyd and Chakrabarty 1995). In particular, patients with CF are susceptible to chronic *P. aeruginosa* infections of the airways, causing heightened inflammation, bronchial asthma, tissue destruction, and pulmonary dysfunction which can lead to death (Bonfield et al. 1999; Wang et al. 2015; Yang et al. 2011, 2008). Initially, patients are colonized with a classic *P. aeruginosa* morphotype, and subsequently, phase variation or mutation occurs resulting in adapted mucoid strains that predominate during the evolution of the disease (Purevdorj-Gage et al. 2005; Ramsey and Wozniak 2005; Römling et al. 1997; Schmidt et al. 1996; Yang et al. 2011, 2008). Recently, some studies suggested nebulized magnesium sulfate as a clinically significant bronchodilator treatment option in patients resistant to standard therapy of acute asthma (Sarhan et al. 2016). However, the effect of magnesium sulfate on the virulence of *P. aeruginosa* is lacking.

Virulence of *P. aeruginosa* is multifactorial, involving both secreted and cell-associated bacterial products. Secreted products include elastase, alkaline protease, protease IV, exotoxins, rhamnolipids, and siderophores while cell-associated bacterial products involve alginate production and O-antigen (Alionte et al. 2001; Baumann et al. 1993; Bever and Iglewski 1988; Bothwell et al. 2003; Döring and Høiby 1983; Finck-Barbançon et al. 1997; Hauser et al. 1998; Hinsa et al. 2003; Hobden 2002; Tielen et al. 2013, 2010; Xiao et al. 2006; Yahr et al. 1996; Yahr and Wolfgang 2006). *P. aeruginosa* utilizes two major mechanisms to evade the host defense system. The first mechanism is the production of extracellular products,

✉ Walid A. Lotfy
lotfywalid@yahoo.com

[1] Microbiology Department, Faculty of Dentistry, Pharos University in Alexandria, Alexandria, Egypt

[2] Department of Botany and Microbiology, Faculty of Science, Alexandria University, Alexandria, Egypt

such as proteases, toxins, and lipases (Tielen et al. 2010). Alkaline protease and elastase inhibit the function of the cells of the immune system (phagocytes, NK-cells, T cells), inactivate several cytokines (IL-1, IL-2, IFN-r, TNF), cleave immunoglobulins, and inactivate complement (Høiby et al. 2011; Horvat and Parmely 1988; Klausen et al. 2003; Matz and Girskov 2007; McIver et al. 1995; Otterlei et al. 1991). Moreover, *P. aeruginosa* uses a type III secretion system (TTSS) as a molecular syringe to deliver bacterial toxins directly into the cytosol of eukaryotic cells and to inhibit host immune response (Franklin et al. 2011; Phillips et al. 2003). The second mechanism is the alginate-dependent biofilm mode of growth in chronic infections (Kharazmi 1991). Mucoid strains overproduce the EPS alginate and are associated with CF (Franklin et al. 2011; Martin et al. 1993). Alginate has a crucial role in the process of pathogenicity; it protects bacterial cells from phagocytosis (Ma et al. 2012; Meluleni et al. 1995; Schlictman et al. 1994). Alginate affects leukocyte functions and plays an immunomodulatory role via suppression of lymphocyte transformation (Mathee et al. 1999). Furthermore, alginate is believed to prevent penetration of antibiotics into the infecting cells and to aid bacterial adherence to epithelial cells of the respiratory tract (Schlictman et al. 1994). Sessile bacteria can be up to 1000 times more resistant to antibiotics than their planktonic counterparts (Gilbert et al. 1997; Høiby 1994; Yang et al. 2011; 2008).

Serotyping of *P. aeruginosa* is based on the O-specific antigen. *P. aeruginosa* strain PAO1, a non-mucoid wild-type strain, belongs to the serotype O5 (Wang et al. 2015). *P. aeruginosa* FRD1, a sputum isolate from a cystic fibrosis patient, is an alginate-producing strain that possesses a distinctive mucoid colony morphology. Expression of virulence factors by *P. aeruginosa* is controlled by signal molecule-dependent cell-cell communication systems in response to the surrounding environment (Erickson et al. 2002; Singh et al. 2015). The aim of this work is to explore the effect of magnesium sulfate and other environmental variables on the production of alginate and extracellular proteins by *P. aeruginosa* FRD1 and PAO1 clinical isolates.

The one-dimensional search with successive changes on variables is still employed, even though it is well accepted that it is practically impossible for the one-dimensional search to accomplish an appropriate optimum combination in a finite number of experiments. Single-variable investigation methods are not only tedious, but can also lead to misinterpretation of results, especially taking into account that the interaction between different factors is overlooked. Statistical methods, through fractional factorial design (FFD) and response surface methodology (RSM), offer simultaneous study of many factors as well as the interactive effects of these factors together (Lotfy et al. 2006). To the best of our knowledge, concrete experimental studies of magnesium sulfate and other environmental factors affecting the performance of *P. aeruginosa* by statistically designed experiments are not employed.

Materials and methods

Microorganisms

Two strains of the species *P. aeruginosa* were used in this study namely, FRD1 and PAO1. The mucoid FRD1 strain, a sputum isolate from a cystic fibrosis patient, was kindly obtained from Prof. Bernd H. A. Rehm, Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand. The non-mucoid PAO1 strain (NCCB 2452) was donated from the culture collection NCCB, Netherlands. Stock cultures were maintained on nutrient broth supplemented with 30–40% (*v*/*v*) glycerol and kept at − 18 °C. Short-term viable cultures were maintained on nutrient agar plates at 4 °C. Cultures used in the experimental studies were not older than 5 weeks.

Culture media and solutions

For maintaining stock cultures, nutrient broth containing (g/l) beef extract (3.0) and peptone (5.0) was used. The basal culture medium used in the Plackett-Burman experiment contained (g/l) glucose, 20.0; $(NH_4)_2SO_4$, 0.6; K_2HPO_4, 2.2; KH_2PO_4, 0.8; NaCl, 0.2; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.05; yeast extract, 0.25; casamino acids, 0.25; $FeSO_4.7H_2O$, 0.6×10^{-3}; and trace elements (mg/l): $ZnSO_4.7H_2O$, 0.2; $CuSO_4.5H_2O$, 0.2; $MnSO_4.H_2O$, 0.2; and $CoCl_2.6H_2O$, 0.2 (Mian et al. 1978). Under some specified experimental conditions, yeast extract (0.5 g/l), casamino acids (0.5 g/l), and/or hydrogen peroxide (0.2 ml/l) were added to the medium.

All media were sterilized by autoclaving at 121 °C for 20 min. Solutions of K_2HPO_4, KH_2PO_4, $MgSO_4$, $CaCl_2$, and trace elements were autoclaved separately and added to the rest of the sterilized medium after cooling to room temperature. Ferrous sulfate solution was sterilized by filtration using a Millipore cellulose nitrate filter with a 0.2-μm pore size.

Bacterial growth

Shake flask experiments were carried out in 250-ml baffled Erlenmeyer flasks containing 50 ml medium and kept on a rotary shaker at 200 rpm at 30 °C. The pH was initially adjusted to 7.3 which decreased during the fermentation time due to alginate production. The inoculum was introduced as 10% (*v*/*v*) of the culture volume. For each trial in the experimental design matrices, growth parameters such as medium components and culture volume were adjusted as indicated in the "Results" section.

Determination of proteins and alginate

After 48-h incubation on a rotary shaker at 200 rpm at 30 °C, an aliquot of 10 ml culture broth was centrifuged at 5000 rpm at 20 °C for 30 min to precipitate the cells. Total protein was determined according to Lowry et al. (1951) to reflect the concentration of secreted virulence protein. Alginate dry weight was determined gravimetrically by the method described by Clementi et al. (1999). Briefly, the supernatant was cooled and alginate was then precipitated by the addition of three volumes of ice cold acetone which was then recovered by centrifugation at 5000 rpm for 30 min. The precipitate was dissolved in distilled water, precipitated again, centrifuged, and then finally dried at 80 °C for 24 h. For each determination, at least two samples were used.

Experimental designs

To investigate the production of EPS and exo-proteins as responses to environmental variations, two sequential multifactorial experimental designs were applied. The Plackett-Burman design which is a FFD (Lotfy et al. 2017; Plackett and Burman 1946; Yu et al. 1997) was used to reflect the relative importance of various environmental factors on the production of alginate and extracellular proteins by the strains FRD1 and PAO1, respectively, in liquid cultures. In this experiment, 11 self-governing variables were screened in 12 combinations organized according to the Plackett-Burman design matrix described in the "Results" section. These independent variables included culture volume, the components of the basal medium, in addition to yeast extract, casamino acids, and hydrogen peroxide. Phosphates were mixed and treated as a single variable. Similarly, trace elements were prepared as a stock solution to be examined as one factor. Each variable was examined at two levels, a high (+1) and a low (−1) level. All trials were performed in triplicates and the averages of alginate and extracellular proteins production observations were treated as the responses. The main effect of each variable on the response was calculated according to the following equation:

$$\text{Main} \quad \text{effect} = \frac{2[\sum R(H) - \sum R(L)]}{N}$$

where R(H) is the response parameter of an assembly in the screening design that contains the higher quantity of a given component, R(L) is the response parameter of an assembly in the screening design that contains the lower quantity of a given component, and N is the number of assemblies. The factor that had no effect would give a value of zero if no interactions existed. A main effect figure with a positive sign indicates that the high level of this variable is nearer to optimum and a negative sign indicates that the low level of this variable is nearer to optimum. Using Statistica 10 software, statistical t values and p values were calculated for determination of variable significance.

The Box-Behnken design which is a RSM (Box and Behnken 1960; Lotfy et al. 2006) was applied. In this experiment, the most significant variables resolved from the Plackett-Burman design for each strain were treated as independent variables. Each factor was examined at three different levels, low (−), high (+), and basal (0). Sixteen combinations and their observations were fitted to the following second-order polynomial mode:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

where, Y is the dependent variable (alginate or extracellular proteins in mucoid and non-mucoid strains, respectively); X_1, X_2, and X_3 are the independent variables; b_0 is the regression coefficient at center point; b_1, b_2, and b_3 are linear coefficients; b_{12}, b_{13}, and b_{23} are second-order interaction coefficients; and b_{11}, b_{22}, and b_{33} are quadratic coefficients. Based on the obtained equation, the values of the coefficients and levels that maximize dependent variables were predicted using Statistica 10 software. The coefficient of determination, R^2, and a plot showing the distribution of experimental versus predicted values were used to examine the quality of the model.

Results

Elucidation of the factors affecting the expression of EPS and extracellular proteins

The capability of the non-mucoid *P. aeruginosa* strain to produce virulence factors could be modulated by modifying environmental and culture factors, such as aeration and nutrient amount, or even the extra addition of hydrogen peroxide, a condition that prevailed in vitro (Mathee et al. 1999). In present work, the Plackett-Burman design was applied to screen for the most important environmental factors responsible for controlling EPS expression and the release of the proteinaceous virulence factors by the mucoid *P. aeruginosa* strain and protein production by the non-mucoid *P. aeruginosa* strain. The factors chosen for the statistical study were 11 and included the nutritional components together with aeration of the culture and the presence of hydrogen peroxide. The design was applied with 12 different fermentation conditions. The chosen levels of the culture components and the observed results are given in Table 1. With respect to EPS and excreted proteins, the calculated main effect values for each examined variable and statistical analysis results are shown in Table 2. The calculated statistical parameters revealed that the

Table 1 The Plackett-Burman design applied on the synthesis of EPS by FRD1 and production of exo-proteins by FRD1 and PAO1

Trial	Independent variable											FRD1		PAO1
	P (g/l)	S (g/l)	M (g/l)	C (g/l)	A (g/l)	F (mg/l)	T (mg/l)	V (ml)	H (ml)	Y (g/l)	CA (g/l)	EPS (g/l)	Proteins (g/l)	Proteins (g/l)
1	3.00	0.02	1.00	0.20	2.00	0.00	0.00	75.00	0.80	0.00	0.50	1.500	2.468	9.466
2	0.30	1.00	1.00	0.20	0.20	0.00	0.00	25.00	0.00	0.50	0.50	0.700	2.903	3.604
3	3.00	1.00	1.00	0.01	0.20	0.00	0.80	75.00	0.80	0.50	0.00	0.900	3.299	5.687
4	3.00	1.00	0.02	0.01	0.20	1.20	0.00	25.00	0.80	0.00	0.50	1.500	3.461	8.046
5	3.00	0.02	0.02	0.01	2.00	0.00	0.80	25.00	0.00	0.50	0.50	2.100	2.468	7.027
6	0.30	0.02	0.02	0.20	0.20	1.20	0.80	75.00	0.80	0.50	0.50	1.800	0.900	4.718
7	0.30	0.02	1.00	0.01	2.00	1.20	0.00	25.00	0.80	0.50	0.00	1.000	0.427	0.000
8	0.30	1.00	0.02	0.20	2.00	0.00	0.80	25.00	0.80	0.00	0.00	1.900	2.789	4.049
9	3.00	0.02	1.00	0.20	0.20	1.20	0.80	25.00	0.00	0.00	0.00	1.600	1.563	3.240
10	0.30	1.00	1.00	0.01	2.00	1.20	0.80	75.00	0.00	0.00	0.50	2.900	2.636	2.417
11	3.00	1.00	0.02	0.20	2.00	1.20	0.00	75.00	0.00	0.50	0.00	1.200	3.177	4.260
12	0.30	0.02	0.02	0.01	0.20	0.00	0.00	75.00	0.00	0.00	0.00	0.800	0.255	0.204

P, phosphates; *S*, sodium chloride; *M*, magnesium sulfate; *C*, calcium chloride; *A*, ammonium sulfate; *F*, ferrous sulfate; *T*, trace elements; *V*, culture volume; *H*, hydrogen peroxide; *Y*, yeast extract; *CA*, casamino acid

examined variables did not significantly affect the production of extracellular proteins by FRD1 (data not shown). However, the statistical analysis of the response results and the calculated main effects (Table 2) indicated the presence of marked variations with respect to the production of EPS by FRD1 and expression of exo-proteins by PAO1.

In general, variables with positive main effect signs are predicted to result in high response results at their examined high settings. The main effect results represented in Fig. 1a and Table 2 demonstrate that increasing the level of ammonium sulfate, trace elements, phosphates, or aeration intensity is advantageous for the EPS overall production (g/l) by the mucoid FRD1 strain. On the contrary, the high level of NaCl and the presence of hydrogen peroxide negatively affected alginate production by the same strain. On the basis of the calculated *t* values, the results of this experiment suggest also that ammonium sulfate, trace elements, and phosphate were the most substantial variables that significantly affect alginate production by the FRD1 strain.

Table 2 and Fig. 1b show the magnitude of effect for each of the 11 different variables on overall production of proteins (g/l) by the PAO1 strain under shake flask culture conditions.

Table 2 Statistical analysis of the Plackett-Burman experimental results

Variable	EPS production by FRD1 strain				Proteins production by PAO1 strain			
	Main effect	*t* value	*p* value	Significance levels (%)	Main effect	*t* value	*p* value	Significance levels (%)
P (g/l)	0.517	1.48	0.0848*	90	2.990	2.036	0.0345*	95
S (g/l)	− 0.417	− 1.15	0.1384	85	− 0.371	− 0.213	0.4178	55
M (g/l)	− 0.117	− 0.31	0.3814	60	1.852	1.125	0.1434	85
C (g/l)	− 0.050	− 0.131	0.44918	55	− 0.148	0.09	0.4650	50
A (g/l)	0.750	2.49	0.0159*	98	0.277	0.159	0.4384	55
F (mg/l)	− 0.350	− 0.95	0.182253	80	− 1.243	− 0.730	0.2411	75
T (mg/l)	0.550	1.61	0.069238*	90	0.269	0.154	0.4403	55
V (ml)	0.083	0.22	0.4151	55	1.010	0.588	0.2848	70
H (ml)	− 0.117	− 0.31	0.3814	60	− 0.665	− 0.384	0.3545	60
Y (g/l)	0.050	0.131	0.4491	55	0.585	0.337	0.3715	60
CA (g/l)	− 0.050	− 0.131	0.4491	55	3.806	3.01	0.0065*	99

*Significant (p value ≤ 0.1)

P, phosphates; *S*, sodium chloride; *M*, magnesium sulfate; *C*, calcium chloride; *A*, ammonium sulfate; *F*, ferrous sulfate; *T*, trace elements; *V*, culture volume; *H*, hydrogen peroxide; *Y*, yeast extract; *CA*, casamino acid

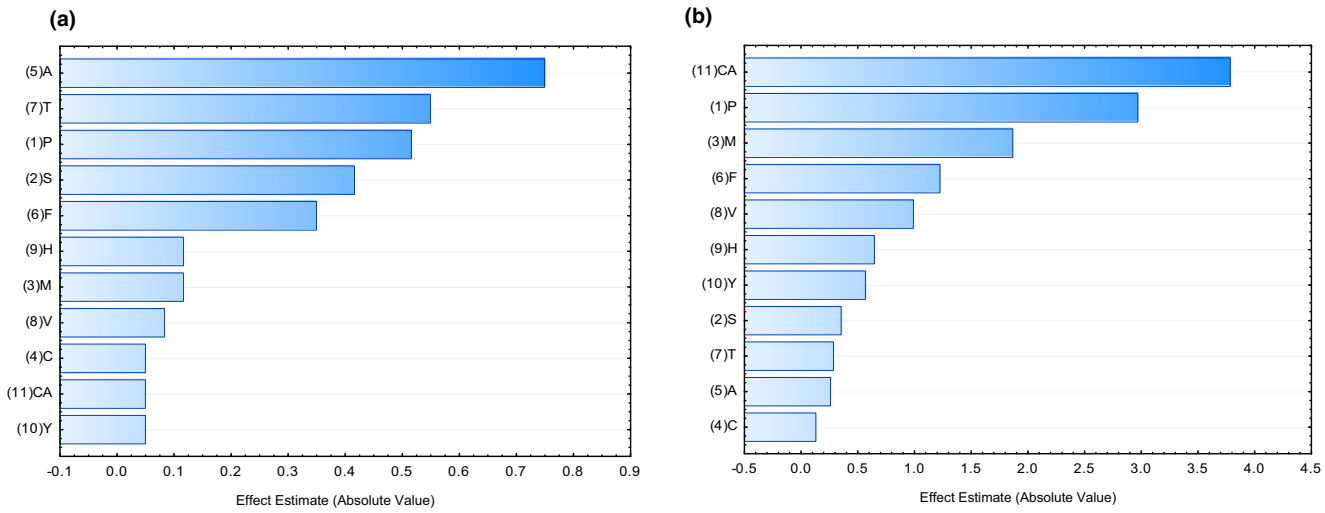

Fig. 1 Pareto plot showing the effect of each of the 11 different variables on (**a**) overall EPS production by FRD1 strain and (**b**) overall protein production by PAO1 strain. *Abbreviations*: *P*, phosphates; *S*, sodium chloride; *M*, magnesium sulfate; *C*, calcium chloride; *A*, ammonium sulfate; *F*, ferrous sulfate; *T*, trace elements; *V*, culture volume; *H*, hydrogen peroxide; *Y*, yeast extract; *CA*, casamino acid

Because of the proteinaceous nature of casamino acid and yeast extract, their main effects were not considered in this part of the work. The results suggest that high levels of phosphates, magnesium sulfate, ammonium sulfate, aeration, and the addition of trace elements to the minimal medium were advantageous for production of extracellular proteins (g/l). On the other hand, secretion of proteins was negatively affected by the presence of high levels of $FeSO_4$ and NaCl in the medium. According to the statistical analysis performed (Table 2), phosphates, $MgSO_4$, and $FeSO_4$ were treated as significant variables with respect to extracellular protein production by the PAO1 strain.

The main objective of the applied screening factorial experiment was to get a general picture of how the response is affected by changes in different environmental variables. Thus, a validation experiment was carried out in triplicates to evaluate the accuracy of the Plackett-Burman experiment. The predicted near optimum levels of independent variables were examined and compared to their contrary and their basal settings. As shown in Fig. 2, the near optimized medium formulation resulted in a 1.4-fold increase of alginate production by FRD1 strain when compared to the basal culture condition. On the other hand, total extracellular protein production showed a fourfold increase under the predicted near optimum condition compared to the basal levels (Fig. 2).

Application of the Box-Behnken statistical design

Based on the results of the Plackett-Burman experiment, the levels of the significant variables affecting the production of extracellular biopolymers and proteins were further investigated using the Box-Behnken design. For the FRD1 strain, the interactions of ammonium sulfate with the concentration of phosphates and the presence of trace elements were examined. Whereas the production of extracellular proteins by the PAO1 strain was further studied using concentrations of the most

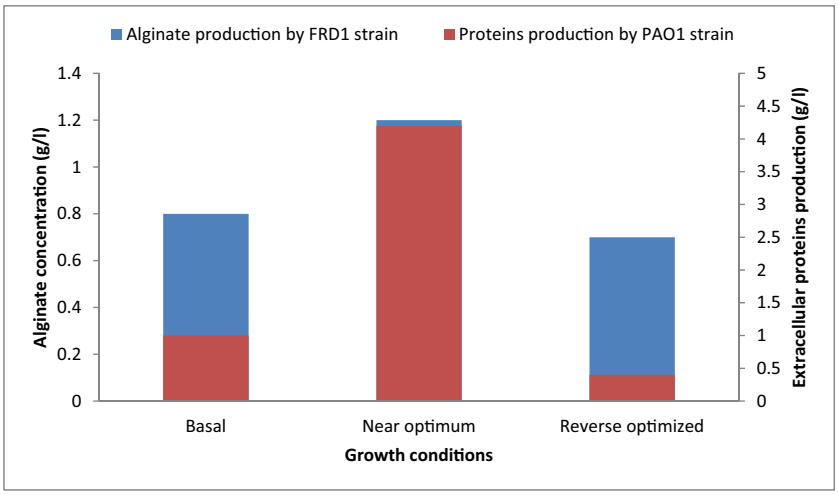

Fig. 2 Alginate and protein production by FRD1 and PAO1 strains, respectively, grown on basal, near optimum, and reverse optimized conditions

effective key factors, including phosphates, magnesium sulfate, and ferrous sulfate as independent variables. In both cases, each key factor was examined at three different levels in 12 trials as described in Tables 3 and 4, while the other factors were fixed at their basal levels. Trials 13–16 consist of the basal medium of this experiment and represent the control. The basal condition for alginate production by FRD1 strain was as follows: glucose, 20 g/l; phosphates, 3 g/l; sodium chloride, 0.02 g/l; magnesium sulfate, 0.02 g/l; calcium chloride, 0.01 g/l; ammonium sulfate, 2 g/l; ferrous sulfate, 0.3 mg/l; trace elements, 0.6 mg/l; culture volume, 75 ml; hydrogen peroxide, 0.2 ml; yeast extract, 0.5 g/l; and casamino acid, 0.1 g/l. The basal condition for protein production by the PAO1 strain was the same except for the concentration of magnesium sulfate, 1 g/l and casamino acid, 0.5 g/l.

The data shown in Table 3 represent different combination levels and the corresponding biopolymer production results observed after 2 days of incubation. According to the obtained results, the interactions of the three examined variables are presented in the form of three-dimensional surface plots in Fig. 3a–c. From these figures, it is clear that ammonium sulfate had the most considerable effect on alginate production. On the contrary, trace elements and concentration of phosphates within the examined ranges and under the present experimental conditions were very much less effective variables. Furthermore, the tested levels of phosphates and ammonium sulfate did not show an optimum peak in the graph, suggesting that further increases in their concentrations may result in more biopolymer yield.

Table 3 Concentrations of the key variables examined in the Box-Behnken experiment and observed alginate production responses of the FRD1 strain

Trials	Ammonium sulfate (g/l)	Trace elements (mg/l)	Phosphates (g/l)	Alginate (g/l)
1	0.20	0.60	0.30	1.00
2	4.00	0.60	0.30	0.80
3	0.20	0.60	6.00	1.00
4	4.00	0.60	6.00	2.60
5	2.00	0.00	0.30	0.80
6	2.00	1.20	0.30	1.00
7	2.00	0.00	6.00	1.40
8	2.00	1.20	6.00	1.40
9	0.20	0.00	3.00	1.00
10	0.20	1.20	3.00	0.80
11	4.00	0.00	3.00	1.80
12	4.00	1.20	3.00	1.40
13	2.00	0.60	3.00	1.40
14	2.00	0.60	3.00	1.20
15	2.00	0.60	3.00	1.40
16	2.00	0.60	3.00	1.60

Table 4 Concentrations of the key variables examined in the Box-Behnken experiment and observed protein production responses of the PAO1 strain

Trials	Phosphates (g/l)	Magnesium sulfate (g/l)	Ferrous sulfate (mg/l)	Proteins (g/l)
1	0.30	0.02	0.30	3.84
2	0.30	2.00	0.30	2.47
3	6.00	0.02	0.30	8.02
4	6.00	2.00	0.30	8.65
5	0.30	1.00	0.60	1.57
6	0.30	1.00	0.00	1.82
7	6.00	1.00	0.60	7.96
8	6.00	1.00	0.00	8.77
9	3.00	0.02	0.60	6.07
10	3.00	0.02	0.00	8.83
11	3.00	2.00	0.60	5.70
12	3.00	2.00	0.00	7.07
13	3.00	1.00	0.30	7.62
14	3.00	1.00	0.30	6.94
15	3.00	1.00	0.30	7.58
16	3.00	1.00	0.30	7.18

The statistical analysis and the values of coefficients were estimated by using Statistica 10 software (Table 5). For predicting the optimal point, the following second-order polynomial function was fitted to alginate concentration results of the applied Box-Behnken experiment:

$$Y = 1.26 + 0.73X_1 - 0.17X_2 + 0.78X_3 - 0.1X_1X_2 + 0.9X_1X_3 - 0.1X_2X_3 - 0.01X_1^2 + 0.17X_2^2 + 0.09X_3^2$$

where Y is the alginate production in g/l and X_1, X_2, and X_3 are the concentrations of ammonium sulfate (g/l), trace elements (mg/l), and phosphate (g/l), respectively.

The quality of the polynomial model equation was expressed by the coefficient of determination, R^2, which was 0.97. The closer the value of R^2 to 1.0, the better the correlation between actual and predicted values. Moreover, a parity plot showing the distribution of experimental versus predicted values by the mathematical model of alginate concentration (Fig. 4) reflects a linear relationship with $r = 0.98$ indicating a reasonable fitting model. According to this equation, the predicted optimum condition for alginate production contains ammonium sulfate, trace elements, and phosphates with concentrations of 4 g/l, 0.6 mg/l, and 6 g/l, respectively.

A confirmatory experiment was subsequently performed under the best-predicted nutritional conditions for alginate production in the flask culture. The pre-optimized Plackett-Burman culture medium was used as a control against a predicted Box-

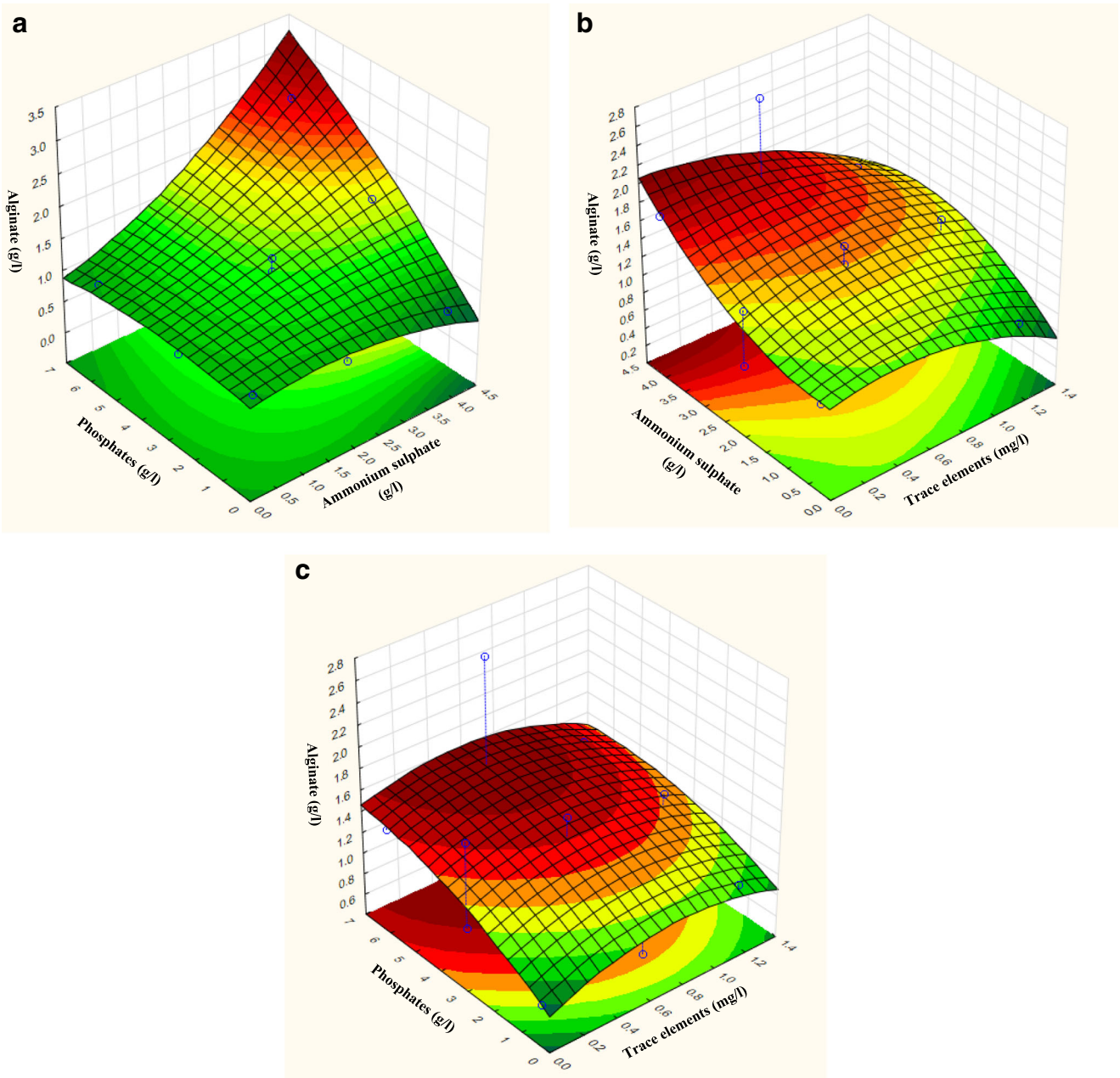

Fig. 3 Three-dimensional surface plots showing the interaction between K_2H/KH_2PO_4 and NH_4SO_4 (**a**), NH_4SO_4 and trace elements (**b**), and K_2H/KH_2PO_4 and trace elements (**c**) with respect to alginate production by FRD1 strain based on the Box-Behnken experimental results

Behnken optimized medium (Fig. 5) and alginate concentrations of 2.2 and 5.1 g/l, respectively, were observed.

The data shown in Table 4 represent different combinations of independent variables and corresponding concentrations of the exo-proteins virulence factors resulted within 2 days of incubation. The interactions of the three examined variables are shown in Fig. 6a–c. From these figures, it can be concluded that phosphate mixture is the most important variable that affected the production of extracellular proteins.

The results of this Box-Behnken application were fitted to a second-order polynomial model. The coefficient of determination R^2, which was found to be 0.996, indicating that 99.6% of the variability in the response can be explained by the model. This revealed that the equation is a suitable model to describe the response of the experiment. As shown in Fig. 7, the parity plot of the experimental versus predicted values indicated a good fitting model ($r = 0.984$). The following second-order polynomial function was fitted to protein

Table 5 Parameter coefficients for Box-Behnken design experiments

Effect	EPS production by FRD1 strain				Proteins production by PAO1 strain			
	Parameter coefficient	Standard error	Computed *t* value	*p* value	Parameter coefficient	Standard error	Computed *t* value	*p* value
Intercept	1.264451	0.047184	26.79828	0.000114*	5.94232	0.094503	62.87984	0.000009*
X_1	0.731579	0.121851	6.00389	0.009256*	5.68003	0.243891	23.28925	0.000173*
X_1^2	− 0.011219	0.081763	− 0.13721	0.899554	1.89055	0.163723	11.54727	0.001395*
X_2	− 0.171930	0.121885	− 1.41060	0.253175	− 0.58412	0.243956	− 2.39438	0.096361
X_2^2	0.169114	0.081763	2.06835	0.130449	− 0.13514	0.163723	− 0.82539	0.469661
X_3	0.782456	0.121750	6.42675	0.007636*	− 1.04190	0.243966	− 4.27068	0.023565*
X_3^2	0.092798	0.081763	1.13496	0.338869	0.58173	0.163723	3.55316	0.038005*
X_1X_2	− 0.100000	0.163299	− 0.61237	0.583583	1.00000	0.327210	3.05614	0.055161
X_1X_3	0.900000	0.163299	5.51135	0.011762*	− 0.28000	0.327210	− 0.85572	0.455057
X_2X_3	− 0.100000	0.163299	− 0.61237	0.583583	0.69500	0.327210	2.12402	0.123711

*Significant (p value ≤ 0.05)

For EPS production, X_1, X_2, and X_3 are the concentrations of ammonium sulfate (g/l), trace elements (mg/l), and phosphates (g/l), respectively

For protein production, X_1, X_2, and X_3 are the concentrations of phosphates (g/l), magnesium sulfate (g/l), and ferrous sulfate (mg/l), respectively

production results of the applied Box-Behnken experiment:

$$Y = 5.94 + 5.68X_1 - 0.58X_2 - 1.04X_3 + 1.0X_1X_2 - 0.28X_1X_3 + 0.69X_2X_3 - 1.89X_1^2 - 0.13X_2^2 + 0.58X_3^2$$

where Y is the protein production in g/l and X_1, X_2, and X_3 are the concentrations of phosphates (g/l), magnesium sulfate (g/l), and ferrous sulfate (mg/l), respectively.

According to this equation, the predicted optimum condition for protein production contains phosphates, magnesium sulfate, and ferrous sulfate with concentrations of 6 g/l, 0.02 g/l, and 0.3 mg/l, respectively. Finally, for verification, the Box-Behnken optimized medium (Fig. 8) was practically examined for extracellular protein production by PAO1 using the pre-optimized Plackett-Burman culture medium as a control. Extracellular protein concentrations of 10.5 and 6.3 g/l were recorded under the optimized and control conditions, respectively confirming the experimental predictions.

Discussion

As an opportunistic human pathogen, *P. aeruginosa* can cause CF and infections of the middle ear, eyes, wounds, and urinary tract (Mesaros et al. 2007). A hallmark of *P. aeruginosa* is its ability to produce EPS and extracellular virulence proteins to

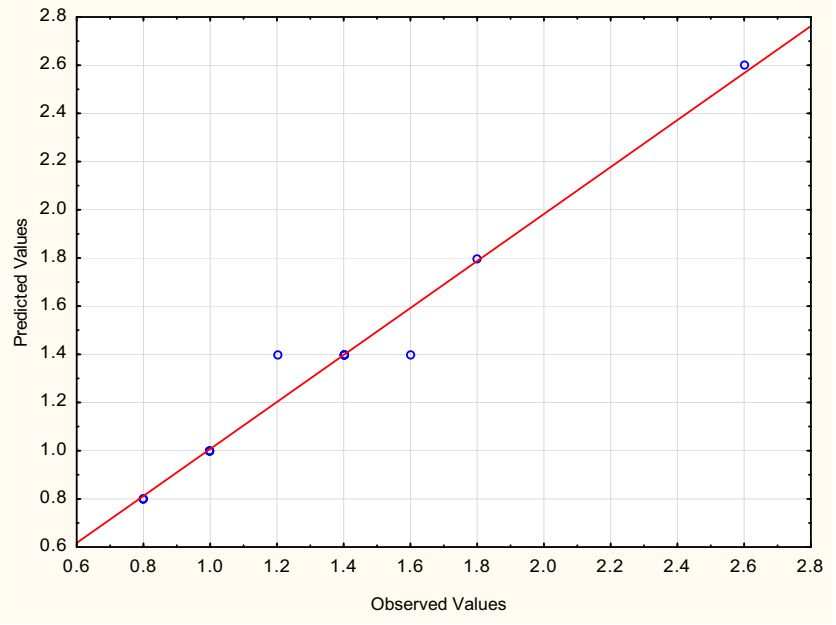

Fig. 4 Distribution of experimental values of alginate concentration versus predicted values ($r = 0.98$)

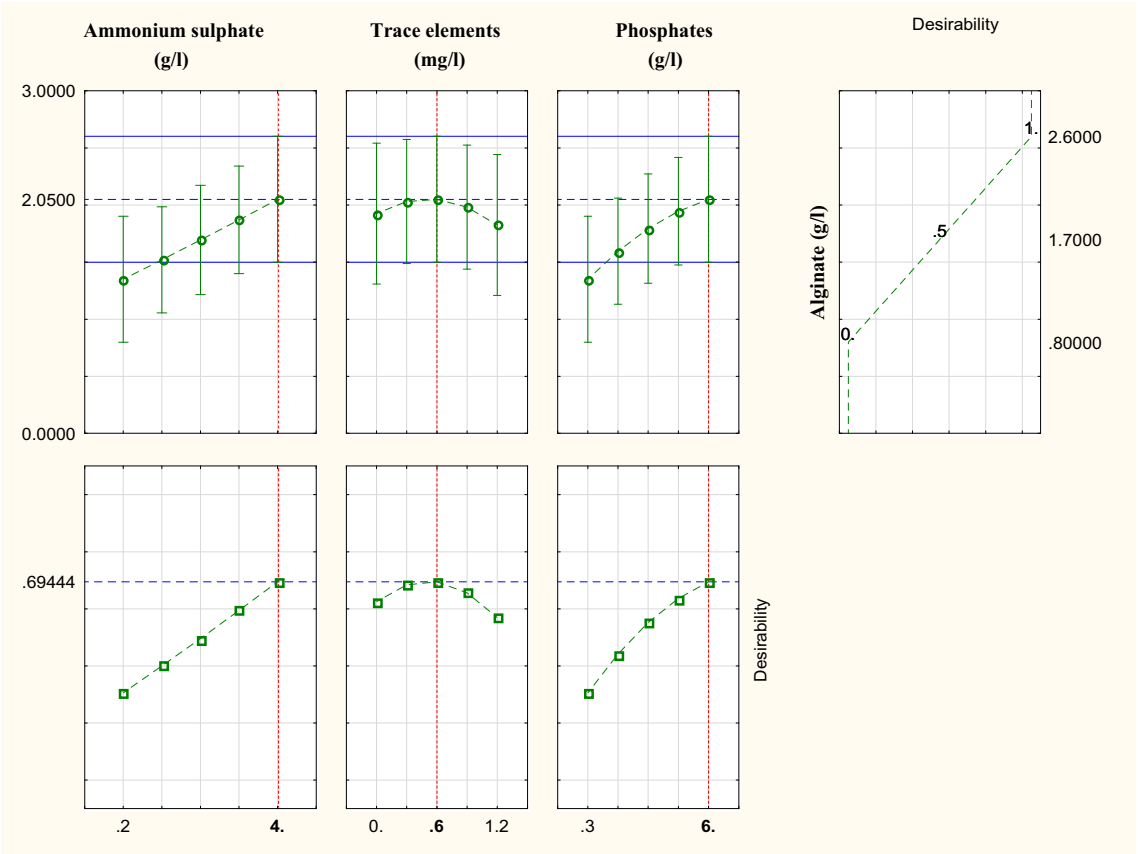

Fig. 5 Desirability charts of variables for maximum alginate production by FRD1 strain based on the Box-Behnken experimental results

establish biofilm-based infections that are difficult to eradicate. Toyofuku et al. (2012) demonstrated that approximately 30% of the identified matrix proteins of *P. aeruginosa* were outer membrane proteins, while some proteins were derived from secreted proteins and lysed cells (Toyofuku et al. 2012). Virulence of *P. aeruginosa* is dependent on a number of factors, involving both secreted proteins and alginate production (Alionte et al. 2001; Baumann et al. 1993; Bever and Iglewski 1988; Bothwell et al. 2003; Döring and Høiby 1983; Finck-Barbançon et al. 1997; Hauser et al. 1998; Hinsa et al. 2003; Hobden 2002; Tielen et al. 2013, 2010; Xiao et al. 2006; Yahr et al. 1996; Yahr and Wolfgang 2006). The main objective of the present study was to investigate various factors that control the production of alginate and extracellular proteins by *P. aeruginosa* clinical isolates.

Based on the results of the applied FFD and RSM, we should emphasize that the variables affected alginate formation by the mucoid strain were not exactly the same affecting extracellular protein production by the non-mucoid strain. It is, however, noted that the presence of excess phosphates in the medium increases alginate synthesis and secretion of proteins by the FRD1 and PAO1 strains, respectively. This observation is mainly attributed to the effect of phosphate functional groups on medium pH as well as metabolic activities including protein synthesis and cell wall composition. Phosphate is required for the synthesis of virulence proteins such as alkaline phosphatase and phospholipases by *P. aeruginosa* (Barker et al. 2004; Filloux et al. 1988). Similarly, inorganic polyphosphate has been reported to be essential for long-term survival and expression of virulence factors in *Shigella* and *Salmonella* spp. (Kim et al. 2002). Previous results indicated also that acidic metabolites such as free uronic acid and the biopolymer alginic acid are produced by mucoid and non-mucoid strains (Kim et al. 2002). Therefore, in addition to its metabolic roles, the effects of phosphates observed in this work may support the importance of the buffering capacity of the surrounding medium for pathogenic bacteria.

Moreover, it has been previously reported that inorganic polyphosphate (poly-P, a chain of tens or many hundreds of phosphate residues linked by high-energy phosphoanhydride bonds) and the enzyme synthesizing it have been found in all pathogenic bacteria (Kornberg et al. 1999). With respect to alginate synthesis in *P. aeruginosa*, the sugar subunits are activated with guanosine diphosphate (GDP) (Hay et al. 2009). Depending

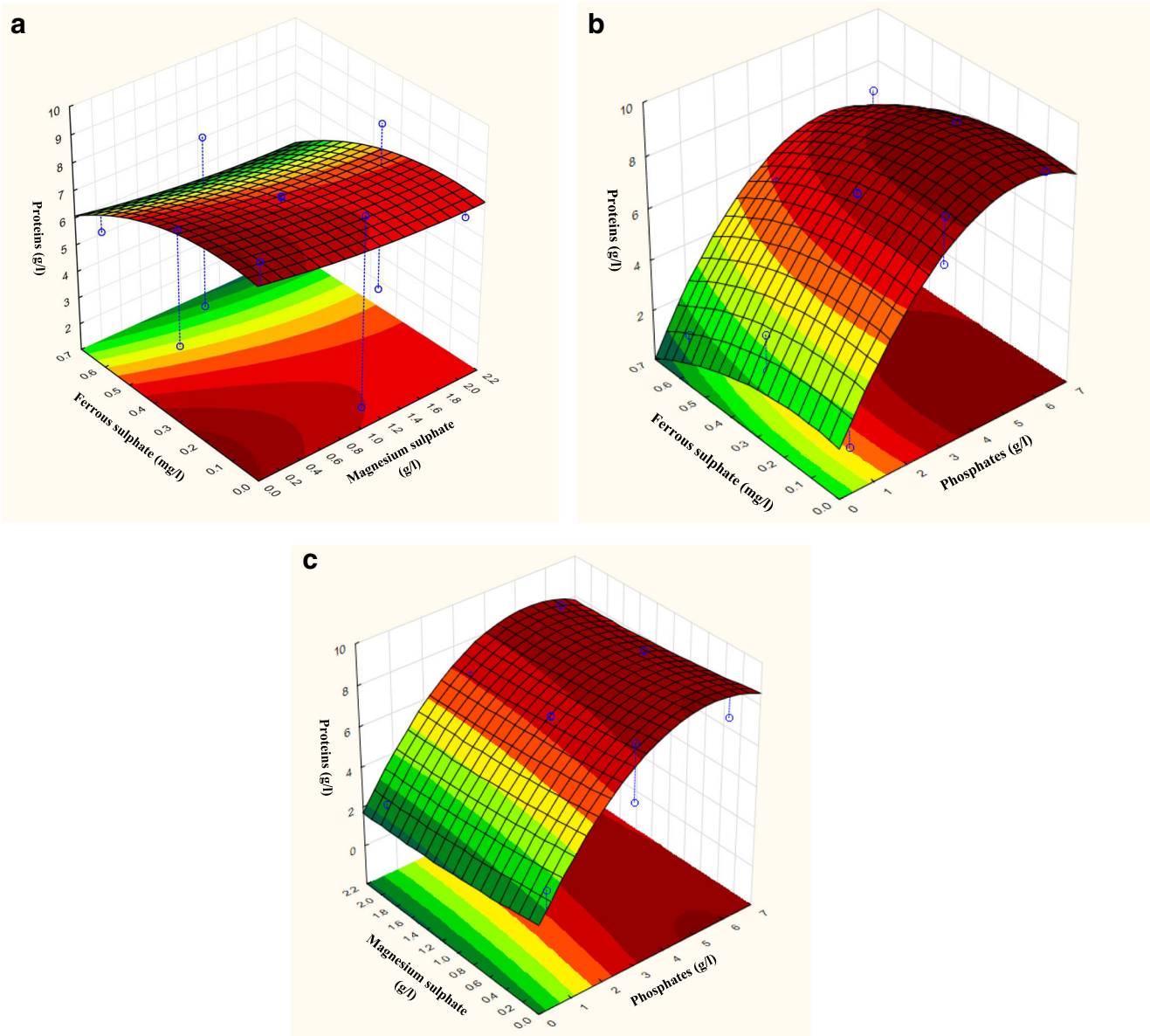

Fig. 6 Response surface plot showing the interaction of $FeSO_4$ with $MgSO_4$ levels (**a**), $FeSO_4$ with K_2H/KH_2PO_4 levels (**b**), and $MgSO_4$ with K_2H/KH_2PO_4 levels (**c**) with respect to proteins production by PAO1 strain based on the Box-Behnken experimental results

on the cell type and condition, poly-P performs varied functions (Fraley et al. 2007). For instance, mutants of PAO1 lacking poly-P kinase 1 and the enzyme responsible for most poly-P synthesis in *Escherichia coli* and other bacteria are defective in motility, quorum sensing, biofilm formation, and the secretion of virulence factors.

Our data suggest also ammonium sulfate and trace elements as substantial variables that significantly affect alginate production by the FRD1 strain. We found that the presence of a relatively high ammonium sulfate level (4.0 g/l) increased the ability of bacterial cells to produce alginate. This is in accordance with previous investigations concerned with the functional characterization of several enzymes and proteins involved in alginate synthesis (Filloux 2011; Keiski et al. 2010; Whitney et al. 2011). Our observations demonstrated also that the presence of trace elements in the surrounding environment supported alginate biosynthesis by the FRD1 strain. A possible explanation for this observation is the prominence of metals to activate enzymes involved in the production of alginate precursor (Regni et al. 2004, 2002).

The results of the applied FFD and RSM experiments demonstrated also that extracellular protein production by the PAO1 strain is significantly affected by the levels of $FeSO_4$ and $MgSO_4$ in the environment. Parallel to our findings, the importance of iron and magnesium availability as major limiting factors with respect to the growth of parasitic bacteria has been previously reported (Meyer 2000).

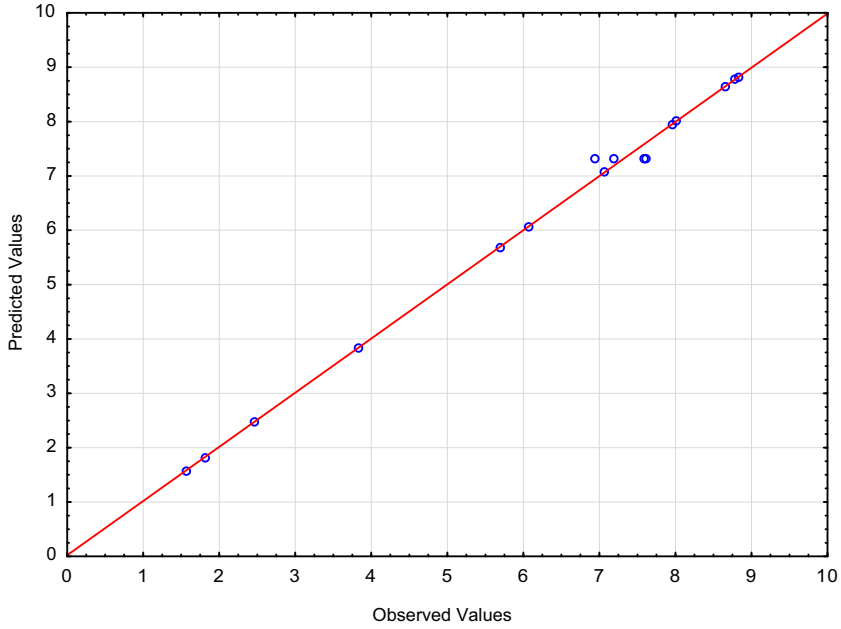

Fig. 7 Distribution of experimental values of protein concentration versus predicted values ($r = 0.984$)

The results showed that, under our experimental conditions, a concentration of 0.3 mg/l ferrous sulfate supports high protein secretion by the PAO1 strain. Earlier findings proved the presence of a complicated regulation network that involves iron signaling, quorum sensing, and chemotaxis that coordinate the macrocolony formation in non-mucoid *P. aeruginosa* biofilms (Harmsen et al. 2010; Yang et al. 2011). It has been also demonstrated that *P. aeruginosa* switch on the expression of iron-regulated outer membrane proteins that assist iron acquisition (Anwar et al. 1991).

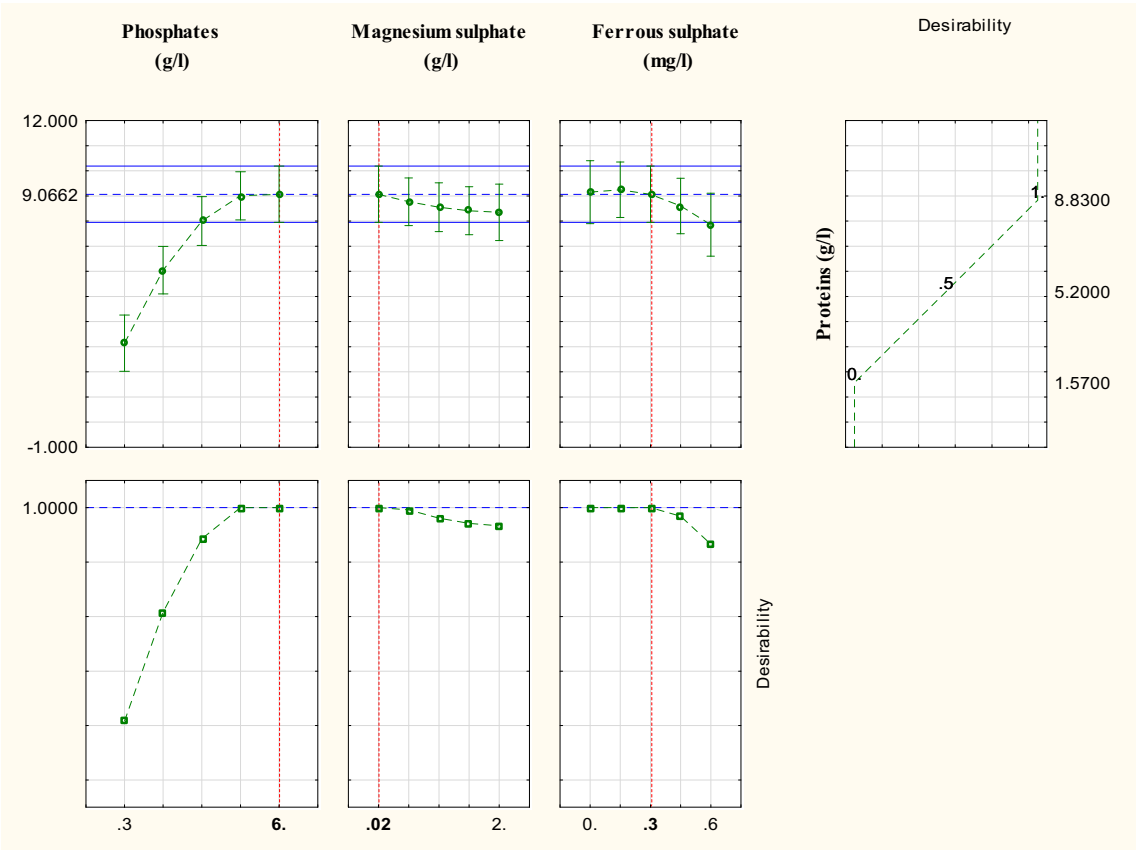

Fig. 8 Desirability charts of variables for maximum protein secretion by PAO1 strain based on the Box-Behnken experimental results

Recent investigations have reported that nebulized $MgSO_4$ has significant bronchodilator effect in acute asthma and leads to clinical improvement (Sarhan et al. 2016). However, these studies have focused on host defense rather than parasite virulence. On the other hand, the present work demonstrated that the concentration of magnesium sulfate in the surrounding environment affects biofilm formation as well as the expression of extracellular proteins by *P. aeruginosa*. The results of the applied FFD indicated that the presence of a relatively high concentration of magnesium sulfate (1 g/l) downregulated the biosynthesis of alginate by the FRD1 strain. Moreover, analyzing the results of the RSM experiment suggested a medium that contains magnesium sulfate in a concentration of 0.02 g/l as an optimized condition for the expression of extracellular proteins by the PAO1 strain. Analogous to this result is the previous finding showing that pathogenesis of PAO1 strain is highly associated with the production of T2SS-dependent exo-proteins such as metalloproteinase whose catalytic mechanisms rely on metals (Bleves et al. 2010; Braun et al. 1998; Voulhoux et al. 2001; Voulhoux et al. 2003). Moreover, the growth of *P. aeruginosa* in low magnesium resulted in altered subcellular compartmentalization of large enzyme complexes such as ribosomes (Guina et al. 2003).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Alionte L, Cannon B, White CD, Caballero A, O'Callaghan R, Hobden J (2001) *Pseudomonas aeruginosa* LasA protease and corneal infections. Curr Eye Res 22:266–271

Anwar H, Strap J, Costerton J (1991) Growth characteristics and expression of iron-regulated outer-membrane proteins of chemostat-grown biofilm cells of *Pseudomonas aeruginosa*. Can J Microbiol 37:737–743

Barker AP, Vasil AI, Filloux A, Ball G, Wilderman PJ, Vasil ML (2004) A novel extracellular phospholipase C of *Pseudomonas aeruginosa* is required for phospholipid chemotaxis. Mol Microbiol 53:1089–1098

Baumann U, Wu S, Flaherty KM, McKay DB (1993) Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel beta roll motif. EMBO J 12:3357

Bever RA, Iglewski BH (1988) Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. J Bacteriol 170:4309–4314

Bleves S, Viarre V, Salacha R, Michel GP, Filloux A, Voulhoux R (2010) Protein secretion systems in *Pseudomonas aeruginosa*: a wealth of pathogenic weapons. Int J Med Microbiol 300:534–543

Bonfield TL, Konstan MW, Berger M (1999) Altered respiratory epithelial cell cytokine production in cystic fibrosis. J Allergy Clin Immunol 104:72–78

Bothwell MR, Smith AL, Phillips T (2003) Recalcitrant otorrhea due to *Pseudomonas* biofilm. Otolaryngol Head Neck Surg 129:599–601

Box GE, Behnken DW (1960) Some new three level designs for the study of quantitative variables. Technometrics 2:455–475

Boyd A, Chakrabarty A (1995) *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. J Ind Microbiol Biotechnol 15:162–168

Braun P, de Groot A, Bitter W, Tommassen J (1998) Secretion of elastinolytic enzymes and their propeptides by *Pseudomonas aeruginosa*. J Bacteriol 180:3467–3469

Clementi F, Moresi M, Parente E (1999) Alginate from *Azotobacter vinelandii*. In: Bucke C (ed) Carbohydrate biotechnology protocols. Humana Press, Totowa, pp 23–42. https://doi.org/10.1007/978-1-59259-261-6_3

Döring G, Høiby N (1983) Longitudinal study of immune response to *Pseudomonas aeruginosa* antigens in cystic fibrosis. Infect Immun 42:197–201

Erickson DL, Endersby R, Kirkham A, Stuber K, Vollman DD, Rabin HR, Mitchell I, Storey DG (2002) *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. Infect Immun 70:1783–1790

Filloux A (2011) Protein secretion systems in *Pseudomonas aeruginosa*: an essay on diversity, evolution, and function. Front Microbiol 2:155

Filloux A, Bally M, Soscia C, Murgier M, Lazdunski A (1988) Phosphate regulation in *Pseudomonas aeruginosa*: cloning of the alkaline phosphatase gene and identification of phoB-and phoR-like genes. Mol Gen Genet MGG 212:510–513

Finck-Barbançon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleiszig SM, Wu C, Mende-Mueller L, Frank DW (1997) ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol Microbiol 25:547–557

Fraley CD, Rashid MH, Lee SS, Gottschalk R, Harrison J, Wood PJ, Brown MR, Kornberg A (2007) A polyphosphate kinase 1 (ppk1) mutant of *Pseudomonas aeruginosa* exhibits multiple ultrastructural and functional defects. Proc Natl Acad Sci 104:3526–3531

Franklin MJ, Nivens DE, Weadge JT, Howell PL (2011) Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. Front Microbiol 2:167

Gilbert P, Das J, Foley I (1997) Biofilm susceptibility to antimicrobials. Adv Dent Res 11:160–167

Guina T, Wu M, Miller SI, Purvine SO, Eugene CY, Eng J, Goodlett DR, Aebersold R, Ernst RK, Lee KA (2003) Proteomic analysis of *Pseudomonas aeruginosa* grown under magnesium limitation. J Am Soc Mass Spectrom 14:742–751

Harmsen M, Yang L, Pamp SJ, Tolker-Nielsen T (2010) An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. FEMS Immunol Med Microbiol 59:253–268

Hauser AR, Kang PJ, Engel JN (1998) PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol Microbiol 27:807–818

Hay ID, Remminghorst U, Rehm BH (2009) MucR, a novel membrane-associated regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. Appl Environ Microbiol 75:1110–1120

Hinsa SM, Espinosa-Urgel M, Ramos JL, O'toole GA (2003) Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. Mol Microbiol 49:905–918

Hobden JA (2002) *Pseudomonas aeruginosa* proteases and corneal virulence. DNA Cell Biol 21:391–396

Høiby N (1994) Diffuse panbronchiolitis and cystic fibrosis: East meets West. Thorax 49:531–532

Høiby N, Ciofu O, Johansen HK, Z-j S, Moser C, Jensen PØ, Molin S, Givskov M, Tolker-Nielsen T, Bjarnsholt T (2011) The clinical impact of bacterial biofilms. Int J Oral Sci 3:55–65

Horvat RT, Parmely MJ (1988) *Pseudomonas aeruginosa* alkaline protease degrades human gamma interferon and inhibits its bioactivity. Infect Immun 56:2925–2932

Keiski C-L, Harwich M, Jain S, Neculai AM, Yip P, Robinson H, Whitney JC, Riley L, Burrows LL, Ohman DE (2010) AlgK is a TPR-containing protein and the periplasmic component of a novel exopolysaccharide secretin. Structure 18:265–273

Kharazmi A (1991) Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa*. Immunol Lett 30:201–205

Kim K-S, Rao NN, Fraley CD, Kornberg A (2002) Inorganic polyphosphate is essential for long-term survival and virulence factors in *Shigella* and *Salmonella* spp. Proc Natl Acad Sci 99:7675–7680

Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S, Tolker-Nielsen T (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol Microbiol 48:1511–1524

Kornberg A, Rao NN, Ault-Riche D (1999) Inorganic polyphosphate: a molecule of many functions. Annu Rev Biochem 68:89–125

Lotfy WA, Abd-El-Karim NM, El-Sharouny EE, El-Helow ER (2017) Isolation and characterization of a haloalkaliphilic protease producer bacterium from Wadi Natrun in Egypt. Afr J Biotechnol 16:1210–1220

Lotfy WA, Ghanem KM, Ehab R (2006) Citric acid production by a novel *Aspergillus niger* isolate: II. Optimization of process parameters through statistical experimental designs. 11: 32–40

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Ma L, Wang S, Wang D, Parsek MR, Wozniak DJ (2012) The roles of biofilm matrix polysaccharide Psl in mucoid *Pseudomonas aeruginosa* biofilms. FEMS Immunol Med Microbiol 65:377–380

Martin D, Schurr M, Mudd M, Govan J, Holloway B, Deretic V (1993) Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc Natl Acad Sci 90:8377–8381

Mathee K, Ciofu O, Sternberg C, Lindum PW, Campbell JI, Jensen P, Johnsen AH, Givskov M, Ohman DE, Søren M (1999) Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiology 145:1349–1357

Matz C, Girskov M (2007) Biofilms as refuge against predation. The Biofilm Mode of Life: Mechanisms and Adaptations, Horizon Scientific Press, pp 195–213

McIver KS, Kessler E, Olson JC, Ohman DE (1995) The elastase propeptide functions as an intramolecular chaperone required for elastase activity and secretion in *Pseudomonas aeruginosa*. Mol Microbiol 18:877–889

Meluleni GJ, Grout M, Evans DJ, Pier GB (1995) Mucoid *Pseudomonas aeruginosa* growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients. J Immunol 155:2029–2038

Mesaros N, Nordmann P, Plésiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, Van Laethem Y, Jacobs F, Lebecque P, Malfroot A (2007) *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. Clin Microbiol Infect 13:560–578

Meyer J-M (2000) Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. Arch Microbiol 174:135–142

Mian F, Jarman T, Righelato R (1978) Biosynthesis of exopolysaccharide by *Pseudomonas aeruginosa*. J Bacteriol 134:418–422

Otterlei M, Østgaard K, Skjåk-Bræk G, Smidsrød O, Soon-Shiong P, Espevik T (1991) Induction of cytokine production from human monocytes stimulated with alginate. J Immunother 10:286–291

Phillips RM, Six DA, Dennis EA, Ghosh P (2003) In vivo phospholipase activity of the *Pseudomonas aeruginosa* cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. J Biol Chem 278:41326–41332

Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 33:305–325

Purevdorj-Gage B, Costerton W, Stoodley P (2005) Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. Microbiology 151:1569–1576

Ramsey DM, Wozniak DJ (2005) Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. Mol Microbiol 56:309–322

Regni C, Naught L, Tipton PA, Beamer LJ (2004) Structural basis of diverse substrate recognition by the enzyme PMM/PGM from *P. aeruginosa*. Structure 12:55–63

Regni C, Tipton PA, Beamer LJ (2002) Crystal structure of PMM/PGM: an enzyme in the biosynthetic pathway of *P. aeruginosa* virulence factors. Structure 10:269–279

Römling U, Schmidt KD, Tümmler B (1997) Large genome rearrangements discovered by the detailed analysis of 21 *Pseudomonas aeruginosa* clone C isolates found in environment and disease habitats. J Mol Biol 271:386–404

Sarhan HA, El-Garhy OH, Ali MA, Youssef NA (2016) The efficacy of nebulized magnesium sulfate alone and in combination with salbutamol in acute asthma. Drug Des Devel Ther 10:1927

Schlictman D, Kavanaugh-Black A, Shankar S, Chakrabarty A (1994) Energy metabolism and alginate biosynthesis in *Pseudomonas aeruginosa*: role of the tricarboxylic acid cycle. J Bacteriol 176: 6023–6029

Schmidt KD, Tümmler B, Römling U (1996) Comparative genome mapping of *Pseudomonas aeruginosa* PAO with *P. aeruginosa* C, which belongs to a major clone in cystic fibrosis patients and aquatic habitats. J Bacteriol 178:85–93

Singh G, Tamboli E, Acharya A, Kumarasamy C, Mala K, Raman P (2015) Bioactive proteins from Solanaceae as quorum sensing inhibitors against virulence in *Pseudomonas aeruginosa*. Med Hypotheses 84:539–542

Tielen P, Kuhn H, Rosenau F, Jaeger K-E, Flemming H-C, Wingender J (2013) Interaction between extracellular lipase LipA and the polysaccharide alginate of *Pseudomonas aeruginosa*. BMC Microbiol 13:159

Tielen P, Rosenau F, Wilhelm S, Jaeger K-E, Flemming H-C, Wingender J (2010) Extracellular enzymes affect biofilm formation of mucoid *Pseudomonas aeruginosa*. Microbiology 156:2239–2252

Toyofuku M, Roschitzki B, Riedel K, Eberl L (2012) Identification of proteins associated with the *Pseudomonas aeruginosa* biofilm extracellular matrix. J Proteome Res 11:4906–4915

van 't Wout EF, van Schadewijk A, van Boxtel R, Dalton LE, Clarke HJ, Tommassen J, Marciniak SJ, Hiemstra PS (2015) Virulence factors of *Pseudomonas aeruginosa* induce both the unfolded protein and integrated stress responses in airway epithelial cells. PLoS Pathog 11:e1004946

Voulhoux R, Ball G, Ize B, Vasil ML, Lazdunski A, Wu LF, Filloux A (2001) Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. EMBO J 20:6735–6741

Voulhoux R, Bos MP, Geurtsen J, Mols M, Tommassen J (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. Science 299:262–265

Wang S, Hao Y, Lam JS, Vlahakis JZ, Szarek WA, Vinnikova A, Veselovsky VV, Brockhausen I (2015) Biosynthesis of the common polysaccharide antigen of *Pseudomonas aeruginosa* PAO1: characterization and role of GDP-d-rhamnose: GlcNAc/GalNAc-diphosphate-lipid α1, 3-d-rhamnosyltransferase WbpZ. J Bacteriol 197: 2012–2019

Whitney JC, Hay ID, Li C, Eckford PD, Robinson H, Amaya MF, Wood LF, Ohman DE, Bear CE, Rehm BH (2011) Structural basis for alginate secretion across the bacterial outer membrane. Proc Natl Acad Sci 108:13083–13088

Xiao G, Déziel E, He J, Lépine F, Lesic B, Castonguay MH, Milot S, Tampakaki AP, Stachel SE, Rahme LG (2006) MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. Mol Microbiol 62: 1689–1699

Yahr TL, Goranson J, Frank DW (1996) Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. Mol Microbiol 22: 991–1003

Yahr TL, Wolfgang MC (2006) Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. Mol Microbiol 62:631–640

Yang L, Hu Y, Liu Y, Zhang J, Ulstrup J, Molin S (2011) Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. Environ Microbiol 13:1705–1717

Yang X, Kuk J, Moffat K (2008) Crystal structure of *Pseudomonas aeruginosa* bacteriophytochrome: photoconversion and signal transduction. Proc Natl Acad Sci 105:14715–14720

Yu X, Hallett S, Sheppard J, Watson A (1997) Application of the Plackett-Burman experimental design to evaluate nutritional requirements for the production of *Colletotrichum coccodes* spores. Appl Microbiol Biotechnol 47:301–305