Full Paper

Kinetic studies on the expression of alginate and extracellular proteins by *Pseudomonas aeruginosa* FRD1 and PAO1

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Introduction

*Pseudomonas aeruginosa* is a Gram-negative opportunistic human pathogen. It can cause infections of the urinary tract, wounds, middle ear and eyes. *P. aeruginosa* is also the etiologic agent of chronic lung infections in patients with cystic fibrosis (CF) (Soheili et al., 2015; Tielen et al., 2013). Most of these infections are biofilm-associated (Lee et al., 2011; Ma et al., 2012; van’t Wout et al., 2015) in which the cells are attached to living or non-living surfaces, or to each other. Thereby, cells of *P. aeruginosa* are embedded in a self-produced extracellular polymeric matrix. Various quantities of polysaccharides, lipids, nucleic acids and proteins can construct the biofilm matrix of *P. aeruginosa* (Lee et al., 2011; Ma et al., 2012; Tielen et al., 2010, 2013; Yang et al., 2011).

Biofilm mucoidy is caused by the overproduction of alginate (Ramsey and Wozniak, 2005; Starkey et al., 2009; Yang et al., 2008) which is a high molecular weight extracellular polymeric substance (EPS). Alginate provides a highly hydrated matrix that protects *P. aeruginosa* from dehydration, antibiotics (Wang et al., 2015; Yang et al., 2011), disinfectants (Grobe et al., 2001) and phagocytosis (Leid et al., 2005; Pier et al., 2001). Furthermore, non-mucoid biofilms of *P. aeruginosa* may be protected in the CF lung by covering themselves within other biofilm mucoidy (Yang et al., 2011).

In addition to biofilm formation, many infectious bacterial strains secrete toxins and degradative enzymes that play critical roles in microbe-associated processes, including pathogenesis (Cezairliyan and Ausubel, 2017). *P. aeruginosa* is able to produce and secrete several proteins which are important for the nutrition of the bacterium, biofilm formation (Harrison et al., 2006; Tielen et al., 2010), or being virulence factors (Filloux, 2011; Tolker-Nielsen and Molin, 2004). Some proteins associated with the non-mucoid *P. aeruginosa* matrix are derived from...
secreted proteins and lysed cells, while the large majority of the matrix proteins originate from outer membrane vesicles (Toyofuku et al., 2012, 2014).

The dynamics of interaction between the host and the pathogen is affected by pathogen virulence and host defense (Carval and Ferriere, 2010). Nebulized MgSO4 has been suggested as a clinically significant bronchodilator treatment option in acute asthma, including CF patients (Sarhan et al., 2016). However, these studies have focused on host protection rather than the virulence of the pathogen. Moreover, only a single study has been reported so far that has evaluated the boosting influence of MgSO4 on the virulence effect of P. aeruginosa (Lotfy et al., 2018). On the other hand, new antibacterial strategies could be developed based on understanding the mechanisms by which P. aeruginosa synthesizes its virulence factors (Wang et al., 2015). To our knowledge, no study has been undertaken which relates the kinetic measurements of P. aeruginosa FRD1 and PAO1 to the growth and biosynthesis of virulence factors.

Recently, our preliminary studies demonstrated that the total levels of alginate and/or extracellular proteins produced in P. aeruginosa shake flask cultures are significantly affected by the concentrations of magnesium sulfate, ferrous sulfate, ammonium sulfate, trace elements and phosphates in the surrounding environment (Lotfy et al., 2018). Here, we are interested to investigate the regulatory effects of such environmental factors on the amounts of alginate and extracellular proteins with a special focus on: (1) monitoring the target products expressed per biomass unit rather than the total amounts produced in the culture; (2) performing the experiments under a controlled bioreactor condition to attain a consistent environment, when compared to shake flask cultures; and (3) investigating the kinetics of growth and synthesis of the target products as a function of the growth phase.

For this purpose, we examined various environmental factors that affect the specific product yield coefficients (expressed as g product/OD600) of alginate and extracellular proteins by a mucoid (FRD1) and a non-mucoid (PAO1) clinical isolates of P. aeruginosa, respectively. We also provide additional bases for understanding the kinetics of expressing alginate and extracellular proteins by the cells using a 5 L stirred tank bioreactor.

Materials and Methods

Microorganisms and growth media. Two different P. aeruginosa strains, designated as FRD1 and PAO1, were used in this study. The former (FRD1) is a mucoid strain isolated from a cystic fibrosis case and was kindly provided by Prof. Bernd H. A. Rehm, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand. The second strain (PAO1) is non-mucoid and was obtained from the culture collection NCCB, Netherlands.

The used media included nutrient broth containing (g/l) beef extract (3.0) and peptone (5.0) for maintaining stock cultures and medium C (Mian et al., 1978) with previously described modifications (Lotfy et al., 2018) to be used as the experimental basal medium. Media were sterilized by autoclaving at 121°C for 20 minutes. Solutions of K2HPO4, KH2PO4, MgSO4, CaCl2 and trace elements (ZnSO4·7H2O, CuSO4·5H2O, MnSO4·H2O, CoCl2·6H2O) 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 Millipore cellulose nitrate filter with a 0.2 μm pore size.

Stock cultures were maintained on nutrient broth 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.

Shake flask cultures. Shake flask cultures were carried out in 250 ml baffled Erlenmeyer flasks containing 50 ml medium, or other volumes mentioned in the results section. Cultures were kept on a rotary shaker at 200 rpm and 30°C. The pH was initially adjusted to 7.3 which decreased during the fermentation time due to alginate production. Liquid seed cultures were incubated for 24 h and the inoculum was introduced as 10% (v/v).

Bioreactor cultures. Figure 1 shows a schematic diagram of the bioreactor used in this study. Fermentations were carried out in a 5 L stirred tank bioreactor (Biostat B, B-Braun Biotechnolgia, Germany) with a working volume of 2.5–3.5 L. The bioreactor was equipped with temperature, pH, and agitation speed measure, and control units which were connected to a process computer (UBICON: Universal Bioprocess Control System, GFB, Braunschweig, Germany). The bioreactor has a stirrer shaft with three six-bladed disk impellers. Agitation was controlled in the range of 200–1,000 rpm. Temperature was measured by a Pt100 temperature sensor and controlled at 32°C. The thermostat system was an open, pressure free system that included an electric heater and a valve for cooling water supply. The pH was measured by autoclavable pH electrode (Ingold) and kept at 7.2 ± 0.2. This was achieved by the automatic addition of HCl (0.1M) or NaOH (0.1M) by an acid and base pump in the control unit of B-Braun. The electrode was calibrated before sterilization and checked afterwards during sampling.

Seed cultures of the same medium composition used in the bioreactor were prepared by shake flask cultures incubated for 24 h. A seed culture that served as the inoculum...
was introduced at a concentration of 10% (v/v) to start up each batch culture in the bioreactor.

**Growth monitoring.** The cell density was monitored in liquid culture samples by measuring the optical density (OD) at 600 n.m. using a spectrophotometer (model T70/Vis, PG Instruments Ltd). Samples were adequately diluted when the OD value exceeded 0.6.

**Protein and alginate determination.** Aliquots of 10 ml culture broth were centrifuged at 5,000 rpm at 20°C for 30 min to precipitate the cells. The total protein in each sample was determined according to Lowry et al. (1951) to reflect the concentration of secreted virulence protein. Alginate dry weight was determined gravimetrically according to the method described by Clementi et al. (1999). Briefly, the supernatant was cooled and alginate was then precipitated by the addition of 3-volumes of ice cold acetone which was then recovered by centrifugation at 5,000 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.** The Plackett-Burman experimental design, a fractional factorial design, (Lotfy et al., 2017; Plackett and Burman, 1946) was applied for studying the specific product yield coefficients (expressed as g product/OD_{600}) of alginate and extracellular proteins using a mucoid (FRD1) and a non-mucoid (PAO1) clinical isolate of *P. aeruginosa*, respectively. In this experiment, eleven variables were screened in twelve combinations organized according to the Plackett-Burman design matrix described in the Results section. For each variable, a high (+1) and a low (−1) level were tested. All trials were performed in triplicate and the averages of calculated specific yields (g/OD_{600}) of alginate and extracellular proteins were treated as the responses. The main effect of each variable on the response was calculated according to the following equation:

\[
\text{Main effect} = \frac{2 \left[ \Sigma R(H) - \Sigma R(L) \right]}{N},
\]

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 the determination of variable significance. A scale-free value between 0 and 1 was designated to each response by Statistica 10 software to generate a desirability chart. Subsequently, these values were combined to attain an overall desirability chart by taking the arithmetic mean.

**Calculation of the process parameters.** The specific growth rate \((\mu)\), defined as the increase in cell mass per unit time, was calculated as follows:

\[
\mu = \ln \left( \frac{m_f^2 - m_i^1}{t_2 - t_1} \right),
\]

where \( m \) is the optical density of the culture measured at 600 n.m. and \( t \) is the time in hours.

The product specific yield for alginate and extracellular proteins were calculated as follows:

\[
\text{Alginate specific yield} = Y_{\text{alginate}} = \frac{C_{\text{alginate}}}{X} \quad \text{(g/OD}_{600})
\]

Specific yield of extracellular proteins

\[
= Y_{\text{protein}} = \frac{C_{\text{protein}}}{X} \quad \text{(g/OD}_{600})
\]

where \( C \) is the concentration and \( X \) is the optical density of the culture measured at 600 n.m.

Specific alginate production rate \((q)\) was calculated with the help of Microsoft Excel 2010 software. First, both alginate and cell density were described polynomially as a function of time. For a small time interval \( t \), the specific alginate productivity, \( q_{\text{alginate}} \), was calculated according to the following equation:

\[
q_{\text{alginate}}(t) = \frac{dC_{\text{alginate}}(t)}{dt} \cdot \frac{1}{X(t)} \quad \text{(g/OD}_{600}/h).
\]

Similarly, the specific protein productivity \( q_{\text{protein}} \) was calculated by the following equation:

\[
q_{\text{protein}}(t) = \frac{dC_{\text{protein}}(t)}{dt} \cdot \frac{1}{X(t)} \quad \text{(g/OD}_{600}/h).
\]

**Results**

**Exploration of the environmental factors affecting the specific yields of EPS and extracellular proteins**

The Plackett-Burman design was applied to screen for the most important fermentation factors responsible for controlling the specific yields of EPS and extracellular proteins by the mucoid and non-mucoid *P. aeruginosa* strains, respectively. Factors chosen for the statistical study included the nutritional components together with aeration of the culture (examined by changing culture volume) and the addition of hydrogen peroxide. The chosen levels of the independent variables are given in Table 1. The applied design that included 12 different growth conditions in flask cultures and the observed data are shown in Table 1. The main effects and statistical analysis were calculated as specific yields (g/OD_{600}) of EPS and extracellular proteins by the strains FRD1 and PAO1, respectively, after an incubation period of 24 h (Table 2).
Based on the calculated main effect results, magnesium sulfate, trace elements and hydrogen peroxide were the most significant variables that affected EPS specific yield by the FRD1 cells under the experimental culture conditions. As shown graphically in Fig. 2, their presence at relatively high levels markedly increases the efficiency of the cells to produce alginate. For predicting the critical levels of these significant variables, within experimental constraints, desirability charts of variables were generated (Fig. 3). The levels of 0.99 g/l magnesium sulfate, 0.55 mg/l trace elements and 0.79 ml hydrogen peroxide found to be most favourable with a predicted EPS specific yield of 4.5 g/OD\textsubscript{600}. Similarly, but to a lesser extent, it can be concluded that high levels of calcium chloride followed by culture volume affected the alginate response positively. On the other hand, the relatively high levels of yeast extract, casamino acids, ferrous sulfate, phosphates followed by ammonium sulfate negatively influenced alginate specific yield by the same strain.

With respect to the response of the PAO1 strain to express extracellular proteins, variations in the levels of culture volume, casamino acids and ferrous sulfate resulted in significant effects (Table 2). The results presented in Fig. 2 reveal that the production of extracellular proteins is positively affected by the examined high levels of culture volume and casamino acids whereas it is negatively affected by the level of ferrous sulfate. The data illustrated in Fig. 4 demonstrate the profiles for the predicted values and desirability with respect to the specific yield of extracellular proteins (g/OD\textsubscript{600}). The critical levels of ferrous sulfate, culture volume and casamino acids were found to be 0.0 mg/l, 33.5 ml and 0.47 g/l, respectively, with a predicted specific yield of extracellular proteins of 6.248 (g/OD\textsubscript{600}).

According to the signs attributed to each of the calculated main effect values (Table 2) and Fig. 2, it can be predicted that, a near optimum condition for the specific yield of EPS by the strain FRD1 has high settings of magnesium sulfate, sodium chloride, calcium chloride, trace elements, medium volume and hydrogen peroxide, and low settings of phosphates, ammonium sulfate, ferrous sulfate, yeast extract and casamino acids. However, a near optimum growth condition for the specific yield of extracellular proteins by PAO1 has high levels of phosphates, magnesium sulfate, calcium chloride, ammonium sulfate, trace elements, medium volume and hydrogen peroxide whereas it is negatively affected by the level of ferrous sulfate. The data illustrated in Fig. 4 demonstrate the profiles for the predicted values and desirability with respect to the specific yield of extracellular proteins (g/OD\textsubscript{600}).

### Table 1.
The Plackett-Burman design applied on the EPS yield (g/OD\textsubscript{600}) and proteins yield (g/OD\textsubscript{600}) by FRD1 and PAO1 strain, respectively.

| Trial | Independent variable | EPS yield | Proteins yield |
|-------|----------------------|-----------|----------------|
|       | 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) | |
| 1     | 3.00    | 0.02   | 1.00    | 0.20   | 0.00   | 0.00   | 0.00   | 75.00  | 0.80   | 0.00   | 0.50   | 2.222   | 6.207   |
| 2     | 0.30    | 1.00   | 1.00    | 0.20   | 0.00   | 0.00   | 0.00   | 25.00  | 0.00   | 0.50   | 0.50   | 0.593   | 3.162   |
| 3     | 3.00    | 1.00   | 0.01    | 0.20   | 0.00   | 0.80   | 0.00   | 75.00  | 0.80   | 0.50   | 0.50   | 1.250   | 3.395   |
| 4     | 3.00    | 0.02   | 0.01    | 0.01   | 2.00   | 0.00   | 0.00   | 0.00   | 0.80   | 0.00   | 0.50   | 1.526   | 2.910   |
| 5     | 3.00    | 0.02   | 0.02    | 0.01   | 2.00   | 0.00   | 0.80   | 25.00  | 0.00   | 0.50   | 0.50   | 1.261   | 2.910   |
| 6     | 0.30    | 0.02   | 0.02    | 0.02   | 2.00   | 0.00   | 0.80   | 75.00  | 0.80   | 0.50   | 0.50   | 1.682   | 4.735   |
| 7     | 0.30    | 0.02   | 0.02    | 0.01   | 2.00   | 0.00   | 0.80   | 25.00  | 0.00   | 0.50   | 0.50   | 5.556   | 0.000   |
| 8     | 0.30    | 1.00   | 0.02    | 0.02   | 2.00   | 0.00   | 0.80   | 75.00  | 0.80   | 0.50   | 0.50   | 2.164   | 0.924   |
| 9     | 0.30    | 1.00   | 1.00    | 0.01   | 2.00   | 1.20   | 0.80   | 75.00  | 0.80   | 0.50   | 0.50   | 5.556   | 0.000   |
| 10    | 0.30    | 1.00   | 1.00    | 0.01   | 2.00   | 1.00   | 0.80   | 75.00  | 0.80   | 0.50   | 0.50   | 2.164   | 0.924   |
| 11    | 3.00    | 1.00   | 0.02    | 0.02   | 0.01   | 2.00   | 1.00   | 0.80   | 75.00  | 0.80   | 0.50   | 0.50   | 2.164   | 0.924   |
| 12    | 0.30    | 0.02   | 0.02    | 0.01   | 2.00   | 0.00   | 0.00   | 75.00  | 0.80   | 0.50   | 0.50   | 0.829   | 0.349   |

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.

### Table 2.
Statistical analysis of the Plackett-Burman experimental results for FRD1 and PAO1 strains.

| Variable | Main effect | EPS yield (g/OD\textsubscript{600}) by FRD1 strain | Proteins yield (g/OD\textsubscript{600}) by PAO1 strain |
|----------|-------------|-----------------------------------------------|-----------------------------------------------|
| P (g/l)  | -0.545      | 0.2292                                        | 0.1117                                        |
| S (g/l)  | 0.043       | 0.0172                                        | 0.4933                                        |
| M (g/l)  | 1.183       | 3.6286                                        | 0.0023*                                       |
| C (g/l)  | 0.702       | 0.3123                                        | 0.3806                                        |
| A (g/l)  | -0.069      | 0.0265                                        | 0.4897                                        |
| F (mg/l) | -0.742      | 0.3418                                        | 0.3697                                        |
| T (mg/l) | 1.142       | 3.5561                                        | 0.0026*                                       |
| V (ml)   | 0.001       | 0.4224                                        | 0.4142                                        |
| H (ml)   | 0.244       | 0.8015                                        | 0.3806                                        |
| Y (g/l)  | -0.850      | 0.5313                                        | 0.3663                                        |
| CA (g/l) | -0.801      | 0.3513                                        | 0.3663                                        |

*Significant (p-value ≤ 0.05).

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.
Pseudomonas aeruginosa growth kinetics

It is also clear that the maximum yield records observed under the pH-controlled bioreactor conditions are markedly elevated when compared with the maximum yields of EPS and extracellular proteins shown in Table 1 (5.556 and 6.207 g/OD600, respectively) confirming the near optimum predictions concluded from the applied preliminary factorial screening study.

Kinetic analysis of EPS and extracellular proteins as metabolic yields

Based on the data of FRD1 and PAO1 batch bioreactor cultures, the specific growth rate ($\mu$, h⁻¹) and specific EPS productivity ($q_{\text{alginate}}$, calculated as g/OD600/h), or specific
extracellular proteins productivity ($q_{\text{protein}}$, calculated as g/OD$_{600}$/h), were determined at different incubation time intervals. In Fig. 7, the values for specific growth rates together with $q_{\text{alginate}}$ were plotted against the cultivation time. As shown in this figure, the maximum specific growth rate ($\mu_{\text{max}} = 1.11$ h$^{-1}$) was recorded at 3 h of cultivation. The results indicate also that maximum specific EPS productivity results (24.77 g/OD$_{600}$/h) occurred during the exponential phase at 3 h. Within the exponential phase of growth, 1 h prior to the transition towards the stationary phase, it is likely that alginate specific productivity became close to zero.

Similarly, the results shown in Fig. 8 suggest that the majority of the extracellular proteins expressed by cells of the non-mucoid PAO1 strain are growth associated. The maximum specific productivity of extracellular proteins (12.5 g/OD$_{600}$/h) occurred during the exponential phase at 3 h. During the stationary phase, $q_{\text{protein}}$ was very low and ranged between 0.15 and 0.846 (g/OD$_{600}$/h).

**Discussion**

The host-pathogen interaction is commonly affected by the host defence as well as the virulence of the pathogen (Carval and Ferriere, 2010). Therefore, treatments that decrease pathogen fitness would assist the host to defend itself and reduce the infection prevalence. It has been reported that nebulized MgSO$_4$ has a significant bronchodilator effect in acute asthma including CF patients and leads to clinical improvement (Sarhan et al., 2016). However, these studies have focused on host defence as a response to the treatment regardless of pathogen virulence. On the other hand, we have recently demonstrated through preliminary studies that the levels of magnesium sulfate, ferrous sulfate, ammonium sulfate, trace elements and phosphates in the surrounding environment have significant effects on the formation of alginate and/or extracellular proteins by *P. aeruginosa* strains in shake flask cultures (Lotfy et al., 2018). Consequently, it can be predicted that dealing with such factors as environmental variables can introduce information that help to decrease the virulence efficiency of the pathogen by interference with the ability of bacterial cells to form biofilms and/or to express extracellular proteinaceous virulence factors.

*P. aeruginosa* is one of the most frequently observed bacterial pathogens in patients with chronic infections such as wounds and CF (Vital-Lopez et al., 2015). The persistence of *P. aeruginosa* in these infections is mainly based on its capability to form biofilms and to express extracel-
lular virulence proteins. Conventional antibiotic treatments against biofilms are generally ineffective (Vital-Lopez et al., 2015). However, understanding the relationship between bacterial growth and the secretion of alginate and proteins, in addition to the environmental factors that control their production, will make it possible to design specific treatment strategies against such biofilm-based infections. Therefore, developing treatments against *P. aeruginosa* requires a quantitative understanding of the relationship between bacterial growth and secretion of alginate as well as proteins in addition to the factors that significantly control their production. For this purpose, we examined various environmental factors that affect the specific product yield coefficients (expressed as g product/OD$_{600}$) of alginate and extracellular proteins by a mucoid (FRD1) and a non-mucoid (PAO1) clinical isolates of *P. aeruginosa*, respectively.

In general, mucoid strains of *P. aeruginosa* are less proteolytic than the non-mucoid strains (Wozniak et al., 2003). In fact, isolates that initially infect the lungs of CF patients have a non-mucoid phenotype typical to other environmental isolates, are highly motile and secrete high levels of protease exotoxins and siderophores (Gasser et al., 2015; Zaborina et al., 2000). The capability of the non-mucoid *P. aeruginosa* strain to produce virulence factors can be modulated by modifying environmental and cultural factors, such as aeration and nutrient amount, or even the extra addition of hydrogen peroxide, a condition that prevailed *in vitro* (Bjarnsholt et al., 2005). Our experimental results suggested that the significant variables affecting the specific yield of extracellular proteins by the non-mucoid strain were not exactly the same as those affecting alginate yield by the mucoid strain. It is, however, noted that an increase in magnesium sulfate, trace elements and hydrogen peroxide is commonly advantageous for the expression of extracellular alginate and proteins. In this context, and in an experiment with a mixture of mucoid and non-mucoid strains (1:1), it was previously reported that aeration favourably allows the growth of only mucoid phenotypes, and that non-mucoid strains are sensitive to oxidative stress (Sabra et al., 2002). Moreover, the hydrogen peroxide secreted by macrophages in the lungs was reported to initiate the conversion to mucoidy in non-mucoid strains (Bjarnsholt et al., 2005).

Interestingly, the factors which proved to affect the resulting amounts of alginate and proteins (g/l) significantly were not necessarily effective with respect to alginate and protein specific yields (g/OD$_{600}$) by FRD1 and PAO1 strains, respectively. Similarly, some variables that have negative effects on the formation of alginate or virulence proteins showed positive effects on the specific yields of alginate and/or extracellular proteins. For instance, the results of this work together with previously published data (Lotfy et al., 2018) showed contradicting effects of hydrogen peroxide and magnesium sulfate on the alginate total concentration and on the amount of alginate expressed by each PAO1 biomass unit.

A fermentation study was performed in a 5 L stirred tank bioreactor to facilitate a culture at its fastest growth rate so that more bacterial generations and a consistent environment were achieved. In general, the excessive aeration and the pH-stat condition introduced by the bioreactor resulted in higher growth and more production of extracellular proteins. Similarly, it has been previously reported that excessive aeration in oxygen- and pH-controlled bioreactors result in higher yield of proteinaceous virulence factors by the same strain (Kim et al., 2003; Sabra et al., 2003). Our findings also demonstrated that magnesium sulfate, trace elements and hydrogen peroxide favoured EPS production by FRD1. Whereas, ferrous sulfate, culture volume and casamino acids were found to be critical for the synthesis of extracellular proteins by PAO1. Interestingly, the presence of ferrous sulfate in the growth medium down regulated the expression of both alginate and extracellular proteins by *P. aeruginosa* at the cellular level. Parallel to these observations, it has been reported that iron limitation was preferential for maximum specific yields of alginate and extracellular proteins by the mucoid and non-mucoid strains, respectively (Banin et al., 2005; Kim et al., 2003; Wiens et al., 2014; Wood et al., 2006).

The production of alginate and the formation of macrocolonies in the lungs of patients are the most characteristic features of persistent *P. aeruginosa* infection. These macrocolonies represent a survival biofilm strategy in chronic *P. aeruginosa* infections (Harmsen et al., 2010; Yang et al., 2007). Mucoid *P. aeruginosa* has an advantage over the non-mucoid strains against phagocytes (Holby et al., 2011; Matz and Girskov, 2007), antibodies and antibiotics (Lee et al., 2000; Yang et al., 2011; Yang et al., 2008). These mucoid strains from chronic lung infections in CF patients are generally non-motile and express lower levels of protease, exotoxins and siderophores (Gasser et al., 2015; Pritt et al., 2007; Qiu et al., 2007; Wood et al., 2006).

In general, many metabolic processes in prokaryotic cells are controlled in response to variations in the surrounding environment. For a greater understanding of the environmental factors that control the expression of alginate and proteins, the yield of these products per cell density was considered. Categorization of a metabolite as primary or secondary is still questionable because the distinction between primary and secondary metabolism is not always easy (Luckner, 2013; Sonneleitner and Haas, 2011). The nomenclature of either a primary or a secondary metabolite is based on different theories. The first one is the overproduction theory which assumed that primary products of metabolism do not accumulate to substantial levels in cells, since, generally, the steady state concentrations of such materials are low (Luckner, 2013). When significant accumulations do occur, the phenomenon may be termed “overproduction” and the metabolite that accumulates, even an intermediate in a primary metabolic pathway, is often reclassified as a secondary metabolite. A second theory is the growth phase dependent theory, in which the onset of microbial secondary metabolism is often associated with the “cessation” of growth. Indeed, the logarithmic/stationary phase distinction is a useful way of describing this relationship (Bennett and Bentley, 1989; Luckner, 2013). According to this theory, the term secondary metabolite refers to those organic compounds that are not
directly involved with the normal growth, development, or reproduction of bacteria.

In the current study, specific growth rate (h⁻¹) and specific productivity of EPS and extracellular proteins (calculated as g/OD₆₀₀/h) were used for quantitative metabolic profiling. The results suggested that under the bioreactor controlled conditions, both alginate and extracellular proteins secreted by P. aeruginosa cells are expressed parallel to biomass increase during the first three hours of growth. It is likely that the cells of the mucoid P. aeruginosa strain tend to secrete an enormous amount of this polysaccharide to be used as a matrix sufficient for further increase in cell numbers during the latter stages of the exponential growth. In addition, the present results also indicated that the formation of extracellular proteinaceous virulence factors by PAO1 was mainly associated with growth.

Disclosure of Interest

The authors report no conflict of interest.

References

Banin, E., Vasil, M. L., and Greenberg, E. P. (2005) Iron and Pseudomonas aeruginosa biofilm formation. Proc. Natl. Acad. Sci. USA, 102, 11076–11081.

Bennett, J. and Bentley, R. (1989) What’s in a name?—Microbial secondary metabolism. Adv. Appl. Microbiol., 34, 1–28.

Bjarnsholt, T., Jensen, P. Ø., Burmølle, M., Hentzer, M., Haagensen, J. A. et al. (2005) Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiol., 151, 373–383.

Carval, D. and Ferriere, R. (2010) A unified model for the coevolution of resistance, tolerance, and virulence. Evolution, 64, 2988–3009.

Cezairliyan, B. and Ausubel, F. M. (2017) Investment in secreted enzymes during nutrient-limited growth is utility dependent. Proc. Natl. Acad. Sci. USA, 114, E7796–E7802.

Clementi, F., Moresi, M., and Parente, E. (1999) Alginate from a marine brown alga. J. Carbohydr. Biotechnology Protocols, ed. by Bucke, C., Humana Press, Totowa, NJ, pp. 23–42.

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

Gasser, V., Guillou, L., Cunrath, O., and Schallk, I. J. (2015) Cellular organization of siderophore biosynthesis in Pseudomonas aeruginosa: evidence for siderosomes. J. Inorg. Biochem., 148, 27–34.

Grobe, S., Wingender, J., and Fleming, H.-C. (2001) Capability of mucoid Pseudomonas aeruginosa to survive in chlorinated water. Int. J. Hyg. Environ. Heal., 204, 139–142.

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

Harrison, F., Browning, L. E., Vos, M., and Buckling, A. (2006) Cooperation and virulence in acute Pseudomonas aeruginosa infections. BMC Biol., 4, 21.

Höbl, N., Ciofu, O., Johansen, H. K., Song, Z.-J., Moser, C. et al. (2011) The clinical impact of bacterial biofilms. Int. J. Oral Sci., 3, 55.

Kim, E.-J., Sabra, W., and Zeng, A.-P. (2003) Iron deficiency leads to inhibition of oxygen transfer and enhanced formation of virulence factors in cultures of Pseudomonas aeruginosa PAO1. Microbiology, 149, 2627–2634.

Lee, D. J., Cox, D., Li, J., and Greenberg, S. (2000) Rac1 and Cdc42 are required for phagocytosis, but not NF-kB-dependent gene expression, in macrophages challenged with Pseudomonas aeruginosa. J. Biol. Chem., 275, 141–146.

Lee, J. H., Cho, M. H., and Lee, J. (2011) 3-Indolylacetonitrile decreases Escherichia coli O157: H7 biofilm formation and Pseudomonas aeruginosa virulence. Environ. Microbiol., 13, 62–73.

Leid, J. G., Willson, C. J., Shurtleff, M. E., Hassett, D. J., Parsek, M. R. et al. (2005) The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-γ-mediated macrophage killing. J. Immunol., 175, 7512–7518.

Lotfy, W. A., Abd-El-Karim, N. M., El-Sharouny, E. E., and El-Helou, E. R. (2017) Isolation and characterization of a haloalkaliphilic protease producer bacterium from Wadi Natrun in Egypt. Afri. J. Biotechnol., 16, 1210–1220.

Lotfy, W. A., Atalla, R. G., Sabra, W. A., and El-Helou, E. R. (2018) Expression of extracellular polysaccharides and proteins by clinical isolates of Pseudomonas aeruginosa in response to environmental conditions. Int. Microbiol., 21, 129–142.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.

Luckner, M. (2013) Secondary Metabolism in Microorganisms, Plants and Animals. Springer Science & Business Media.

Ma, L., Wang, S., Wang, D., Parsek, M. R., and Wozniak, D. J. (2012) The roles of biofilm matrix polysaccharide PsI in mucoid Pseudomonas aeruginosa biofilms. FEMS Immunol. Med. Microbiol., 65, 377–380.

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

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

Pier, G. B., Coleman, F., Grout, M., Franklin, M., and Ohman, D. E. (2001) Role of alginate O acetylation in resistance of mucoid Pseudomonas aeruginosa to opsonic phagocytosis. Infect. Immun., 69, 1895–1901.

Plackett, R. L. and Burman, J. P. (1946) The design of optimum multifactorial experiments. Biometrika, 33, 305–325.

Pritt, B., O’Brien, L., and Winn, W. (2007) Mucoid Pseudomonas aeruginosa in cystic fibrosis. Am. J. Clin. Pathol., 128, 32–34.

Quo, D., Eisinger, V. M., Rowen, D. W., and Hongwei, D. Y. (2007) Regulated proteolysis controls mucoid conversion in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA, 104, 8107–8112.

Ramsey, D. M. and Wozniak, D. J. (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.

Sabra, W., Kim, E.-J., and Zeng, A.-P. (2002) Physiological responses of Pseudomonas aeruginosa PA01 to oxidative stress in controlled microaerobic and aerobic cultures. Microbiology, 148, 3195–3202.

Sabra, W., Lünsdorf, H., and Zeng, A.-P. (2003) Alterations in the formation of lipopolysaccharide and membrane vesicles on the surface of Pseudomonas aeruginosa PA01 under oxygen stress conditions. Microbiology, 149, 2789–2795.

Sarhan, H. A., El-Garhy, O. H., Ali, M. A., and Youssef, N. A. (2016) The efficacy of nebulized magnesium sulfate alone and in combination with salbutamol in acute asthma. Drug Des. Devel. Ther., 10, 1927.

Soheili, V., Bazzaz, B. S. F., Abdollahpour, N., and Hadizadeh, F. (2015) Investigation of Pseudomonas aeruginosa quorum-sensing signaling system for identifying multiple inhibitors using molecular docking and structural analysis methodology. Microb. Pathog., 89, 73–78.

Sonnleitner, E. and Haas, D. (2011) Small RNAs as regulators of primary and secondary metabolism in Pseudomonas species. Appl. Microbiol. Biotechnol., 91, 63–79.

Starkey, M., Hickman, J. H., Ma, L., Zhang, N., De Long, S. et al. (2009) Secondary metabolism in Microorganisms, Plants and Animals. Springer Science & Business Media.

Soheili, V., Bazzaz, B. S. F., Abdollahpour, N., and Hadizadeh, F. (2015) Investigation of Pseudomonas aeruginosa quorum-sensing signaling system for identifying multiple inhibitors using molecular docking and structural analysis methodology. Microb. Pathog., 89, 73–78.

Sonnleitner, E. and Haas, D. (2011) Small RNAs as regulators of primary and secondary metabolism in Pseudomonas species. Appl. Microbiol. Biotechnol., 91, 63–79.
Pseudomonads. In *Pseudomonas*: Volume 1 Genomics, Life Style and Molecular Architecture, ed. by Ramos, J.-L., Springer U.S., pp. 547–571.

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

Toyofuku, M., Zhou, S., Sawada, I., Takaya, N., Uchiyama, H. et al. (2014) Membrane vesicle formation is associated with pyocin production under denitrifying conditions in *Pseudomonas aeruginosa* PAO1. *Environ. Microbiol.*, 16, 2927–2938.

Wang, S., Hao, Y., Lam, J. S., Vlahakis, J. Z., Szarek, W. A. et al. (2015) Biosynthesis of the common polysaccharide antigen of *Pseudomonas aeruginosa* PAO1: characterization and role of GDP-α-rhamnose: GlcNAc/GalNAc-diphosphate-lipid α1-3-α-rhamnosyltransferase WbpZ. *J. Bacteriol.*, 197, 2012–2019.

Wood, L. F., Leech, A. J., and Ohman, D. E. (2006) Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of α22 (AlgT) and the AlgW and Prc proteases. *Mol. Microbiol.*, 62, 412–426.

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

Toyofuku, M., Zhou, S., Sawada, I., Takaya, N., Uchiyama, H. et al. (2014) Membrane vesicle formation is associated with pyocin production under denitrifying conditions in *Pseudomonas aeruginosa* PAO1. *Environ. Microbiol.*, 16, 2927–2938.

Vital-Lopez, F. G., Reifman, J., and Wallqvist, A. (2015) Biofilm formation mechanisms of *Pseudomonas aeruginosa* predicted via genome-scale kinetic models of bacterial metabolism. *PLoS Comput. Biol.*, 11, e1004452.

Wang, S., Hao, Y., Lam, J. S., Vlahakis, J. Z., Szarek, W. A. et al. (2015) Biosynthesis of the common polysaccharide antigen of *Pseudomonas aeruginosa* PAO1: characterization and role of GDP-α-rhamnose: GlcNAc/GalNAc-diphosphate-lipid α1-3-α-rhamnosyltransferase WbpZ. *J. Bacteriol.*, 197, 2012–2019.

Wiens, J. R., Vasil, A. I., Schurr, M. J., and Vasil, M. L. (2014) Iron-regulated expression of alginate production, mucoid phenotype, and biofilm formation by *Pseudomonas aeruginosa*. *MBio.*, 5, e01010-01013.

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

Zaborina, O., Dhiman, N., Chen, M. L., Kostal, J., Holder, I. A. et al. (2000) Secreted products of a nonmucoid *Pseudomonas aeruginosa* strain induce two modes of macrophage killing: external-ATP-dependent, P2Z-receptor-mediated necrosis and ATP-independent, caspase-mediated apoptosis. *Microbiology*, 146, 2521–2530.