Modelling production of extracellular polymeric substances in a *Pseudomonas aeruginosa* chemostat culture

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Abstract Formation of extracellular polymeric substances (EPSs) by mucoid *Pseudomonas aeruginosa* was investigated using literature data from chemostat cultures. The data were used to calibrate a product formation regime allowing substrate sufficient and endogenous EPS formation. Yield coefficients for both formation conditions were elucidated based on metabolic pathway analysis. Growth and non-growth related specific formation rates of 0.18 g C_{EPS}/g C_{cell}/h and 0.03 1/h were estimated, respectively. The exogenous and endogenous EPS yield was found to be 0.77 g C_{EPS}/g C_{glu} and 0.79 g C_{EPS}/g C_{cell}, respectively. Being structurally equivalent to comprehensive maintenance models, this model allows for non-growth related product formation, showing the same quality of fit as previous models restricted to exogenous EPS precursors.

Keywords Chemostat; EPS; extracellular polymeric substances; modelling; Pseudomonas aeruginosa

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Introduction

Mathematical modelling has proven to be an important tool for the understanding of sessile microbial growth. Most models have been focused on the heterogeneity of substrate concentration and cellular biomass distribution, in 1-D (perpendicular to substratum; Reichert, 1998), and lately 2- and 3-dimensions (Picioreanu *et al.*, 1998). Less attention has been paid to the fundamental biofilm processes of attachment and detachment, and extracellular polymeric substance (EPS) dynamics. Alginate, an extracellular polysaccharide composed of mannuronic acid and guluronic acid monomers, is thought to play a key role in the initial adhesion and/or permanent establishment of *Pseudomonas aeruginosa* to surfaces (Marcus and Baker, 1985; Rampal and Pier, 1985; Davis *et al.*, 1993). Alginate formation in *P. aeruginosa* has been studied in detail due to its role in chronic infections in the cystis fibrosis lung. Most of the biosynthetic pathway has been elucidated (Boyd and Chakrabarty, 1995), and the biosynthetic genes have been identified and characterised (Gacesa, 1998; Rehm and Valla, 1997). Transcription of the alginate genes is thought to be environmentally inducible (Gacesa and Russell, 1990), resulting, however, in a relatively low production rate of alginate. Copious amounts are, on the contrary, formed if environmentally triggered transcription coincides with a mutation in one of the genetic regulators. Energy-, N- or P-poor growth media select for the mucoid phenotype, indicating substrate repression of alginate biosynthesis (DeVault *et al.*, 1991). However, Ma *et al.* (1997) showed that availability of glucose and gluconate increased the formation of alginate, both in mucoid and non-mucoid *P. aeruginosa*.

Trulear (1983) measured alginate formation in a *P. aeruginosa* chemostat culture using glucose as sole carbon source. Using the Leudeking/Piret equation (Leudeking and Piret, 1959) a growth and non-growth formation rate was suggested on exogenous substrate only.

The stoichiometry used for this model was based on glucose as the sole carbon source for alginate formation (Robinson *et al.*, 1984), structurally similar to the maintenance concept of Pirt (1965). This limits the model by excluding non-growth associated alginate formation. Hseih *et al.* (1994) included growth and non-growth EPS formation as part of the cellular maintenance requirements. In this work we present a calibrated mass balance model for the formation of extracellular alginate by *P. aeruginosa* for substrate sufficient and endogenous conditions, including maintenance. EPS anabolism is explicitly defined and incorporated into the mass balance of substrate and biomass.

The main purpose is to develop a model for EPS production that covers all conditions expected to be experienced by cells in a biofilm. The goal is to develop a mechanistic model for biofilm detachment, of which EPS is assumed to be a key constituent.

Methods

Combining the stoichiometric models by Pirt (1965) and Herbert (1958), Beeftink *et al.* (1991) suggested a maintenance model for both substrate sufficient and starvation conditions. The carbon flux for this model, including product (EPS) formation, is presented in Figure 1. Figure 2 shows the system's carbon mass balances, consistent with the stoichiometry in Figure 1. Steady state *P. aeruginosa* data from Trulear (1983) were used for parameter estimation of growth and EPS formation rate.

Yield coefficient was estimated by studying the biosynthetic pathways, followed by identification of the limiting factor (carbon, energy or reducing equivalents). During substrate (glucose) sufficient conditions, fructose-6-phosphate, the precursor of alginate biosynthesis, is generated via the Pentose Phosphate (PP) pathway from the Entner-Doudoroff (ED) pathway intermediate gluconate-6P (White, 2000). Various sources for biosynthesis may be used during non-growth conditions. Fats are assumed to follow β-oxidation to form acetyl-CoA, followed by formation of the TCA cycle intermediate oxaloacetate via glyoxylate (Narbad *et al.*, 1988; Beale and Foster, 1996), and entering the reversed ED pathway through phosphoenol pyruvate. Storage compounds, such as poly-β-hydroxybutyrate are assumed to follow a similar pathway, forming acetyl-CoA and oxaloacetate via the glyoxylate cycle (Matthews and van Holde, 1990). Amino acids generally follow transamination pathways, forming TCA cycle intermediates followed by formation of oxaloacetate. In this study, four intracellular precursors for alginate synthesis were used (poly-β-hydroxybutyrate, fatty acids, cysteine, serine and alanine amino acids

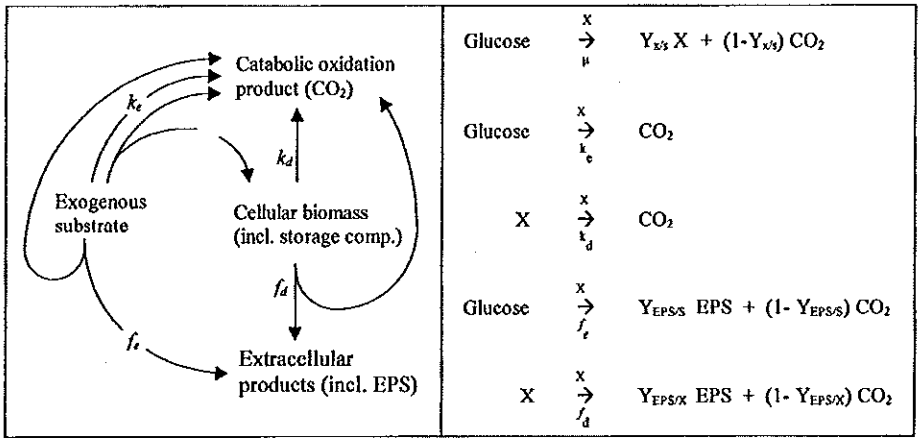

Figure 1 Carbon flow and stoichiometry of the *P. aeruginosa* product formation model

(forming approximately 20% of protein (Matthews and van Holde, 1990) and aspargine and aspartate (forming approximately 10% of protein (Matthews and van Holde, 1990) to estimate an average alginate to biomass yield coefficient. *O*-acetylation of mannuronic acid monomers was assumed to occur at the *O*-2 and *O*-3 positions at an average frequency of 1.2 (Tatnell *et al.*, 1996). Using average values for monomer composition (approximately 65% mannuronic acids, Tatnell *et al.*, 1996), this gives one *O*-acetylation at three out of four monomers. Based on data from Roels (1983), the maintenance substrate consumption rate for *Pseudomonas* was estimated to be 0.02 g glucose/g biomass/h at 20°C, or 0.0165 g C_{gluc}/g C_{cell}/h. Endogenous respiration was related to maintenance via the cellular yield, as $k_d = Y_{X/S} \cdot k_e$ (Roels, 1983). The mass balance model was implemented in Aquasim (Reichert, 1998), and growth and EPS formation parameters were estimated.

Results and discussion

Substrate (glucose) sufficient theoretic yield was found to be 0.77 g C_{alg}/g C_{Glu}. Yields based on intracellular precursors were estimated to be 0.65 g C_{alg}/g C_{fat} (as C_{12}), 0.65 g $C_{alginate}$/g C_{PHB} and 0.71 g $C_{alginate}$/g $C_{protein}$ (weighted average of serine, cysteine and alanine, and aspargine and aspartate). All aggregated pathways for alginate synthesis showed to be carbon limited. This gives an average alginate to biomass yield coefficient of 0.67 g C_{alg}/g $C_{biomass}$.

The system's state variables, measured and simulated, as a function of dilution rate, are presented in Figure 3. Data points are measured values, presented as mean and standard deviation from two independently operated chemostats at the same dilution rate. Figure 4

Cellular growth:
$$\frac{dX}{dt} = \left[\frac{\mu_{max} \cdot S}{K_S + S} - D - \left(k_d + f_d\right)\left(1 - \frac{S}{K_S + S}\right)\right] \cdot X$$

Substrate conversion:
$$\frac{dS}{dt} = D\left(S_{in} - S\right) - \left[\frac{\mu_{max} + k_d}{Y_{x/s}} + f_e\right] \cdot \frac{S}{K_S + S} \cdot X$$

EPS formation:
$$\frac{dEPS}{dt} = \left[f_d \cdot Y_{EPS/X} \frac{K_S}{K_S + S} + f_e \cdot Y_{EPS/S} \cdot \frac{S}{K_S + S}\right] \cdot X - D \cdot EPS$$

Figure 2 Carbon mass balances for the proposed *P. aeruginosa* chemostat model

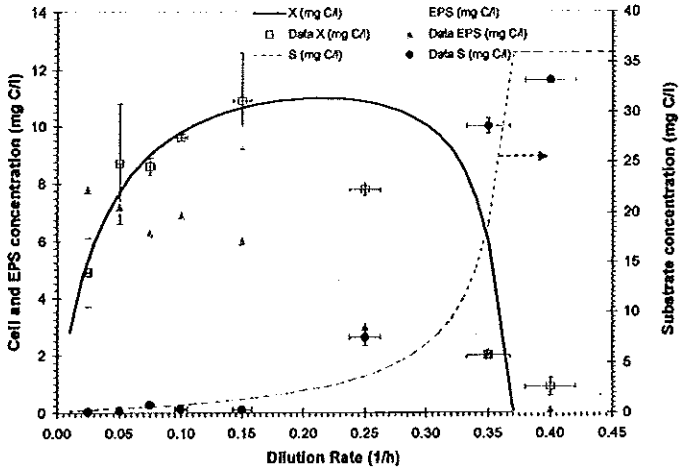

Figure 3 System state variables (cell mass, *X*, extracellular polysaccharides, *EPS*, and substrate (glucose), *S*, as functions of dilution rates

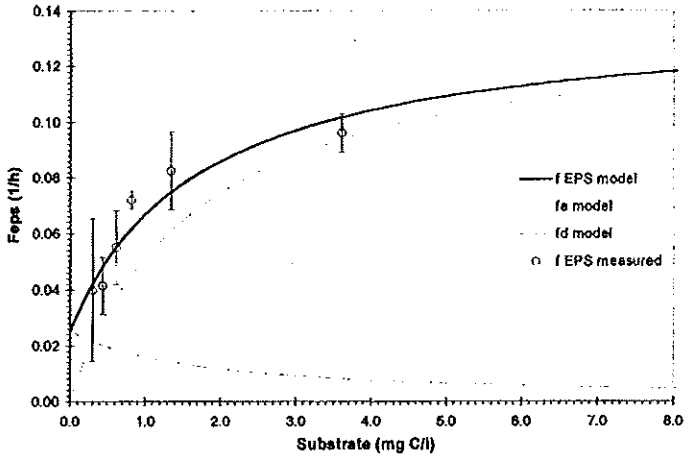

Figure 4 EPS formation rate as a function of bulk phase substrate concentration

show the EPS formation rate (1/h) plotted against substrate concentration, while the same rate is plotted against specific cellular growth rate in Figure 5. Data close to wash out (D = 0.35 1/h) were not included in the plot due to large uncertainties of the steady state approximation, and the presumably high contribution by sessile bacteria growing on reactor surfaces under these conditions (Trulear, 1983). The cellular specific growth parameters were estimated to be $\mu_{max} = 0.39$ 1/h, and the half saturation constant $K_S = 1.7$ mg C/l. The cellular yield was found to be 0.44 g C_{cell}/g C_{glu}. The EPS growth- and non-growth dependent formation rates were estimated to be 0.18 g C_{glu}/g C_{cell}/h and 0.03 1/h, respectively. The lines in Figures 3 to 5 represent the results of simulations using the proposed model and the parameters estimated above.

As mentioned in the Introduction, this model does not take into account the shift from non-mucoid to the mucoid phenotype. This may, however, be easily incorporated using a shift factor representing the regulation of phenotype transition. The model suggests an infinite EPS formation during starvation conditions. The identification of a non-growth related formation rate of EPS seems surprising and is, however, not likely to be the case during long term starvation. Thus, the non-growth related formation should be interpreted as a transition to starvation formation rate, rather than a permanent process.

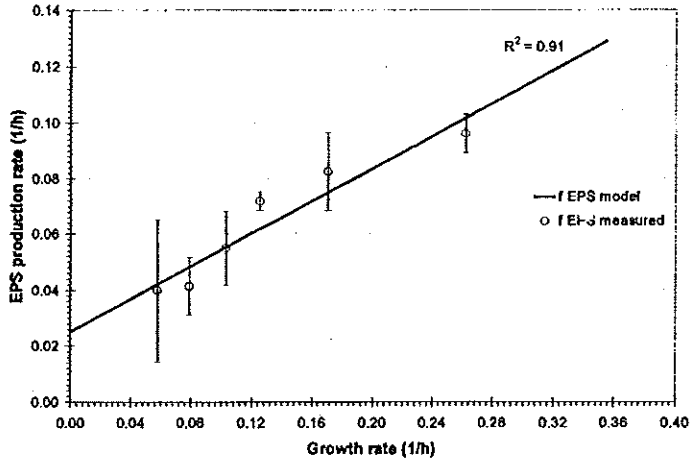

Figure 5 EPS formation as a function of specific cellular growth rate

The results presented here are an important step in improving our ability to predict EPS accumulation in biofilms. However, we have primarily concentrated on the formation of EPS. To make the model more complete we will also have to include EPS degradation, a process almost completely uncharacterised, or quantified, experimentally. Preliminary simulations of biofilm behaviour based on the model presented here suggest, for example, that EPS degradation must also be included in the biofilm model to avoid unrealistic EPS and cell gradients in the biofilm. Developing a model for EPS degradation that covers all conditions expected to be experienced in a biofilm should therefore be pursued. The model may then at some point be able to relate EPS production and degradation to the formation and detachment of biofilm structures and be able to predict how a biofilm may behave under various nutritional conditions.

Conclusions

A *P. aeruginosa* product formation (EPS or alginate) model for both substrate sufficient and endogenous conditions is proposed and calibrated based on chemostat data. The model includes maintenance, endogenous product formation and respiration. The model showed the same quality of fit as the Leudeking/Piret approach used by Robinson *et al.* (1984), and displayed similar dynamics. Cellular growth parameters were not significantly different from literature data (Trulear, 1983; Robinson *et al.*, 1984, Bakke *et al.*, 1984, Jenkins, 1980). Specific product (EPS) formation rates differed, however, significantly from these previously published models of *P. aeruginosa* EPS production. A growth and non-growth related specific formation rate of 0.18 g C_{gluc}/g C_{cell}/h and 0.03 1/h respectively was estimated here.

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