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EFFECTS OF CULTIVATION MEDIA COMPONENTS ON BIOSURFACTANT AND PIGMENT PRODUCTION FROM *Pseudomonas aeruginosa* PAO1

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Abstract - Biosurfactant and pigment production by *P. aeruginosa* have been known to be associated with its selfish growth and colonization. However, according to recent studies these products can be exploited for human benefits. In the present work the effects of culture media components on yield of these two products from *P. aeruginosa* PAO1 have been studied with statistical screening design experiments. Biosurfactant yield was found to be increased by two times in a modified medium. This study will help in further modifying the media composition for cheaper media development, kinetic modelling and fermentation strategy development.
Keywords: *Pseudomonas aeruginosa* PAO1; Biosurfactant; Pigment; Culture medium; Screening design.

INTRODUCTION

P. aeruginosa, a Gram-negative opportunistic human pathogen, produces biosurfactants which belong to rhamnolipids for colonization (Perfumo *et al.*, 2006; Arutchelvi and Doble, 2010; Müller *et al*. 2010) and toxins, including pigment like blue-green pyocyanin, to kill other competitor microorganisms (Norman *et al*., 2004; Ozyurek *et al*., 2011). While biosurfactants are used in textile industries, leather processing, in bioremediation, agriculture, and in the food and beverage industry (Coelho *et al*., 2010), pyocyanin can be exploited against other pathogens to reduce crude oil degradation (Norman *et al*., 2004) and for quantifying ammonium ion concentration (Iida and Satoh, 2013). In this work complex media components for cultivation of *P. aeruginosa* PAO1 have been screened for identifying their specific roles to produce biosurfactant and pigment. Screening of components of complex cultivation media with statistical analysis helps us to understand the effects of each component on biomass growth, product formation and underlying metabolic changes responsible for such variation (Das *et al*., 2009; Moussa *et al*., 2014). As carbon source is essential for growth and product formation, glycerol was used since it is available in excessive amount at a cheap price as a by-product from the biodiesel industry (Xu *et al*., 2012). This knowledge would further help us to minimize number of components in the media, leading to economization in large scale production, subsequent optimization and process modelling (Mukherjee *et al*., 2008).

MATERIALS AND METHODS

Isolation of Microorganism and Culture Conditions

A bacterial isolate from crude oil was used in the present study. The culture was maintained on Luria

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Bertanni (LB) agar plates and LB medium was used for the preparation of the primary inoculum. 1% inoculum (approximately 0.02 g) from LB was then transferred to peptone glycerol ammonium salts (PGAS) medium for biosurfactant production. The medium consisted of 0.02 M NH_4Cl, 0.02 M KCl, 0.12 M Tris-HCl (pH 7.2), 0.0016 M $MgSO_4$, 1% glycerol and 1% peptone. After inoculation the media were incubated for a week at 37 °C with an agitation speed of 200 rpm. An uninoculated medium was also incubated as a sterility control.

Studies on Growth and Biosurfactant Production

Fermentation broth samples were collected twice daily and checked for OD_{600nm} and biosurfactant concentration. Biomass was estimated by the dry weight and also by the optical density of the fermentation broth at 600 nm measured with a UV-Visible spectrophotometer (Eppendorf, Germany).

Biosurfactant Recovery and Pigment Estimation

Biosurfactant was isolated from the culture broth obtained after the completion of each fermentation cycle by the standard technique (Das *et al*., 2008). Briefly, the fermentation broth was acidified and kept at 4 ºC overnight for complete precipitation of the biosurfactant. The precipitate was then centrifuged to get the crude biosurfactants as a pellet.

Pigment concentration was determined by multiplying the optical density of the acidified culture supernatant at 520 nm with 17.072 (Raoof and Latif, 2010).

Statistical Screening of Media Compositions

For statistical screening of effective media components (A: NH_4Cl, B: KCl, C: Tris-HCl with pH 7.2, D: $MgSO_4$, E: Glycerol, F: Peptone) in *P. aeruginosa* PAO1 broth for biosurfactant and pigment production 'screening design of mixture' experiments were chosen since the medium was complex and the number of medium components (i.e., six) was too large for employing response surface methodology and too small for running the statistical optimization method involving Plackett-Burman design. Moreover, screening experiments allows us to find effects of each component in media very precisely. Mere visualization of the results based on the experiments designed can help to make important decisions like which components have negative or no effects and can be removed from the medium and which component has a positive effect on increasing yield. The concentrations of media components were 0.02 M NH_4Cl, 0.02 M KCl, 0.12 M Tris-HCl (pH 7.2), 0.0016 M $MgSO_4$, 1% glycerol and 1% peptone as mentioned earlier and were coded as central points (+0.1667) of reference, whereas the lowest level (0 level) was set for zero concentrations of all components. A total of 23 experiments was performed, where there were six axial points (each experiment representing a medium composition having +1 level for one component and 0 level for the other five components), 12 (=2×6) points of vertices and five centroids (each components at +0.1667 level) for finding pure error in the whole set of experiments (Table 1).

Assay of Emulsification

The cell free supernatants obtained from cultures grown in different media combinations were checked for their ability to emulsify petrol and diesel. Equal volumes of aqueous biosurfactant solution (1 mg mL^{-1}) and hydrocarbons were mixed and vortexed at high speed for 5 min. The resulting mixture was incubated at 25 °C for 24 h and then the emulsification index (EI) value was calculated using the formula:

$$EI = \left(\frac{\text{Height of emulsion layer}}{\text{Height of the total mixture}} \right) \times 100 \quad (1)$$

The emulsification of petrol and diesel by chemical surfactants like SDS and Tween 20 was also observed as a positive control.

RESULTS AND DISCUSSION

The time course of biosurfactant production in batch cultivation of *P. aeruginosa* PAO1 in medium containing 0.02 M NH_4Cl, 0.02 M KCl, 0.12 M Tris-HCl (pH 7.2), 0.0016 M $MgSO_4$, 1% glycerol and 1% peptone was observed to follow a growth associated pattern, whereas pigment production followed a semi-growth associated profile (Figure 1). Biomass and biosurfactant were observed to reach maxima at about 6-7 h. Biosurfactant concentration in the media remained almost same, whereas pigment production continued beyond the growth phase (not shown in Figure 1).

As far as screening design is concerned, it allows us to visualize the effect of each individual component very clearly and gives us quite an understanding about them. The effects of media components on product yield are shown in Table 2 (and Table 1 as

reference). Without carbon source the microorganism could not even grow (Table 2). With only glycerol (6%) in the medium, biomass growth was observed to some extent and also biosurfactant (Expt. 2, Tables 1 and 2). Medium containing 6% peptone showed growth as well as product formation, but not at the highest level, indicating that peptone contains all necessary ingredients for synthesis of proteins involved in the synthesis of the two products—biosurfactant and pigment (Expt. 6, Table 1 and 2). This also proved that peptone in the absence of glycerol could work as carbon source. Since the yields of biomass and products were not the highest among all experiments, it can be inferred that peptone at this much higher concentration is not desired by the microorganism and other media components also need to be supplemented. The maximum amount of biomass was obtained with a medium containing KCl 0.024 M, Tris-HCl (pH 7.2) 0.144 M, $MgSO_4$ 0.00129 M, Glycerol 1.2%, Peptone 1.2%, but no ammonium chloride (Experiment No. 13 Tables 1 and 2), indicating that more alkalinity than normal that could be conferred by the presence of ammonium chloride in the medium is not good for initial multiplication of the bacterium.

The maximum amount of biosurfactant was obtained when all media components were of half concentration compared to the original medium, except peptone, which was 3.5 times higher. A high amount of biosurfactant production was also reflected in high emulsification indices with respect to emulsification of petrol, diesel and hexadecane.

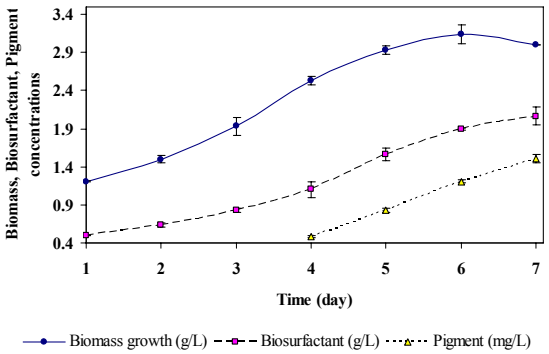

Figure 1: Time course of Biomass, biosurfactant and pigment production in batch cultivation.

Table 1: Design of screening experiments showing real values for media components.

Expt. No.	NH_4Cl (M)	KCl (M)	Tris-HCl (M)	$MgSO_4$ (M)	Glycerol (%)	Peptone (%)
1	0.12	0	0	0	0	0
2	0	0.12	0	0	0	0
3	0	0	0.72	0	0	0
4	0	0	0	0.0096	0	0
5	**0**	**0**	**0**	**0**	**6**	**0**
6	0	0	0	0	0	6
7	0.07	0.01	0.06	0.0008	0.50	0.50
8	0.01	0.07	0.06	0.0008	0.50	0.50
9	0.01	0.01	0.42	0.0008	0.50	0.50
10	0.01	0.01	0.06	0.0056	0.50	0.50
11	**0.01**	**0.01**	**0.06**	**0.0008**	**3.50**	**0.50**
12	0.01	0.01	0.06	0.0008	0.50	3.50
13	0	0.024	0.144	0.002	1.20	1.20
14	**0.0024**	**0**	**0.144**	**0.002**	**1.20**	**1.20**
15	0.0024	0.024	0	0.002	1.20	1.20
16	0.0024	0.024	0.144	0	1.20	1.20
17	**0.0024**	**0.024**	**0.144**	**0.002**	**0.00**	**1.20**
18	**0.0024**	**0.024**	**0.144**	**0.002**	**1.20**	**0.00**
19	0.02	0.02	0.12	0.0016	1	1
20	0.02	0.02	0.12	0.0016	1	1
21	0.02	0.02	0.12	0.0016	1	1
22	0.02	0.02	0.12	0.0016	1	1
23	0.02	0.02	0.12	0.0016	1	1

Table 2: Results of 23 screening experiments showing biomass, biosurfactant, pigment yields.

Expt. No.	Biomass (g/L)	O.D. (600 nm)	Biosurfactant concentration (g/L)	Pigment concentration (µg/ml)	Petrol emulsification (E24)	Diesel emulsification (E24)	Hexadecane emulsification (E24)
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0
5	0.18	0.08	0.1	0	0	0	0
6	2.25	0.98	0.7	6.62	0.5	0.54	0.54
7	3.36	1.46	1.1	7.84	0.5	0.52	0.55
8	3.63	1.58	0.45	2.71	0.5	0.47	0.47
9	0.87	0.38	0.5	2.78	0.31	0.33	0.41
10	3.31	1.44	0.35	4.51	0.47	0.47	0.47
11	2.25	0.98	**0.5**	0.72	**0**	**0**	**0**
12	1.33	0.58	**2.4**	7.49	**0.6**	**0.5**	**0.52**
13	3.73	**1.62**	0.55	11.35	0.6	0.52	0.47
14	3.08	1.34	0.7	**17.25**	0.5	0.52	0.47
15	2.12	0.92	0.7	9.49	0.47	0.5	0.52
16	2.67	1.16	0.25	1.88	0.25	0.4	0.35
17	1.52	0.66	**0.65**	0.92	**0**	**0**	**0**
18	1.24	0.54	**0.8**	0	**0**	**0**	**0**
19	2.53	1.1	0.85	**25.69**	0.6	0.3	0.42
20	2.94	1.28	1.35	**15.36**	0.5	0.52	0.47
21	3.27	1.42	0.88	**21.34**	0.5	0.48	0.45
22	2.85	1.24	1.2	**18.25**	0.54	0.5	0.45
23	2.53	1.1	0.98	**22.53**	0.6	0.5	0.5

Therefore, peptone had a pivotal role in producing biosurfactant, whereas other components were also necessary, but at low concentration. This high amount of biosurfactant showed high emulsification of petrol, diesel and hexadecane (Expt. 12, Table 2). It was noteworthy that biosurfactant production in all experiments was not accompanied by proportional values of emulsification indices. In experiments 5, 11, 17, 18 (Tables 1 and 2) where the media were incomplete, the biosurfactant, although low, was synthesized. However, the biosurfactant thus formed could not emulsify petrol, diesel or hexadecane, indicating that the biosurfactant was different in composition from that obtained from complete medium. Hence, neither too high, nor zero glycerol was desired by the bacterium for biosurfactant production. Without peptone the bacterium could also not produce good quality biosurfactant with respect to emulsification potential. The variation in emulsification ability may be due to variation in production of different homologues, with different physicochemical properties depending on media composition (Arutchelvi and Doble, 2010).

For the best yield of pigment the original medium seemed to be the best, although in the absence of potassium ion, pigment yield was high as well (Table 2 and 1, expt. 14). In the absence of peptone no pigment was produced, indicating some particular peptides or microelements or vitamins that could be present in peptone were essential for pigment synthesis. As far as dependence of pigment production on a single medium component is concerned in this study, only peptone emerged as a potential supporter for product formation (Table 2 and 1, expt. 6). The optimum medium components for maximum pigment production were not same as those for biosurfactant, because the two products were produced in different phases of growth.

The statistical analysis of screening design experimental data cannot predict the optimum medium composition so precisely, but does indicate which media component greatly affects the outcome(s) either positively or negatively. Since much information is clear from the experimental data in Table 2, a detailed discussion of the positive or negative effects of the components on product yield with respect to the statistical analysis would be redundant. Hence, we keep our discussion on data statistics as brief as possible. For describing the biomass, biosurfactant and pigment data; mean, linear and mean models could be employed respectively (Table 3), according to the p-values of comparative model fitting. In all the cases, quadratic and higher model terms were aliased and could not be selected. Mean models are employed only when linear or other higher order models cannot be employed and any single effector parameter (media component) does not affect the outcome(s) greatly. A visual overview of a normal probability plot shows the nature of the deviation of experimental results from the predicted values (Figure 2).

Table 3: Model selection for statistical screening of media components for production of biomass (OD_{600nm}), biosurfactant, and pigment.

	Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	
Biomass (OD600)	Mean	< 0.0001				Suggested
	Linear	0.91	0.003	-0.19	-4.21	
	Quadratic	0.05	0.007	0.33	-8.14	Aliased
Biosurfactant	Linear	0.40	0.03	0.02	-2.62	Suggested
	Quadratic	0.17	0.04	0.25	-6.91	Aliased
Pigment	Mean	0.0002				Suggested
	Linear	0.97	0.04	-0.23	-1.68	
	Quadratic	0.31	0.04	-0.09	-6.56	Aliased

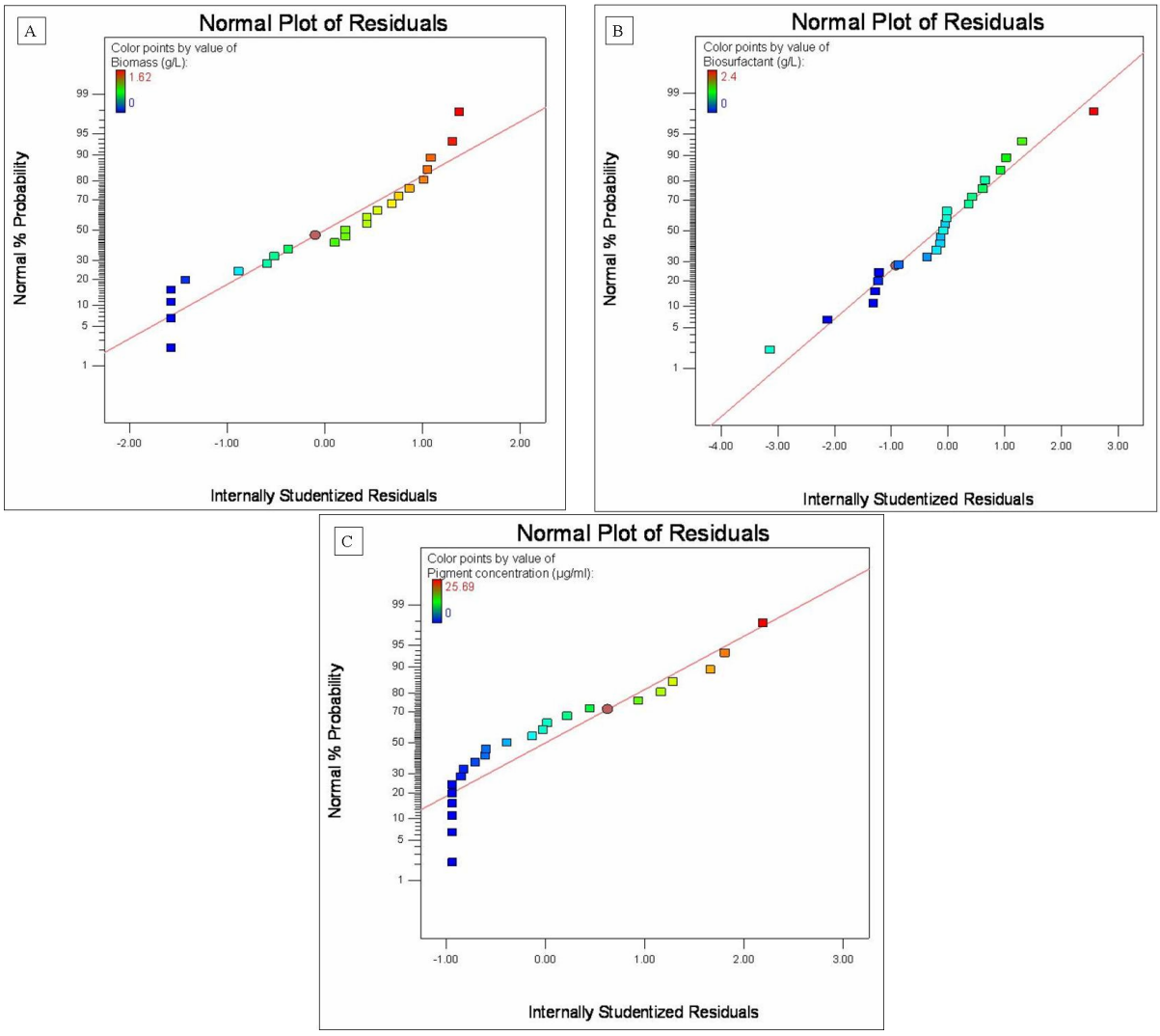

Figure 2: Normal probability plot of (A) biomass (g/L), (B) biosurfactant (g/L), and (C) pigment (µg/ml) data.

In an ideal case, the points denoting all the deviations would pass through a straight line; in other words, the distribution will be normal. In the present study, for all 3 responses, deviations from the ideal case were observed, although biosurfactant data seemed to be better than that of the other two responses. For biomass and pigment the plots were slightly sigmoidal, indicating that a power transformation of the experimental data could improve the sequential p-value and would make the lack of fit somewhat insignificant (by increasing the lack of fit p-value) in model fitting. However, in practice no

significant improvement was observed in model fitting of transformed data values. An analysis of variance (ANOVA) of the mean, linear and mean models of biomass, biosurfactant, and pigment, respectively, is documented in Table 4. In the models for biomass and biosurfactant significant lack of fit was observed; hence, some insignificant parameters can be omitted from further experiment and subsequent analysis. Since a linear model was employed for biosurfactant, to understand which media-component affects biosurfactant production and which does not, a Trace (Piepel) plot was drawn (Figure 3). For biosurfactant production peptone had a huge positive effect while other components, except ammonium chloride, had a slightly negative effect. Therefore, increasing the concentrations of salts (except NH_4Cl) and glycerol above the concentrations in the reference (i.e., original), the biosurfactant yield will decrease. NH_4Cl had no effect on biosurfactant yield as such. Hence, in the optimum medium the peptone concentration was more whereas other components had lower concentrations than that of the reference medium composition. For pigment the mean model did not have a significant lack of fit (Table 4) and hence the mean model could describe experimental outcomes well in 23 experiments. However, as the model was the mean value model, none of the media components could affect pigment formation in a greater way than one another.

Table 4: Analysis of variance (ANOVA) of mean, linear and mean models of biomass (OD_{600nm}), biosurfactant, and pigment respectively.

	Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Biomass (OD600) (Mean model)	Model	0	0				
	Residual	6.91	22	0.314			
	Lack of Fit	6.84	18	0.38	20.97	0.005	Significant
	Pure Error	0.07	4	0.018			
	Cor Total	6.91	22				
Biosurfactant (Linear model)	Model	1.61	5	0.32	1.09	0.403	Not significant
	Linear Mixture	1.61	5	0.32	1.09	0.403	
	Residual	5.02	17	0.30			
	Lack of Fit	4.83	13	0.37	7.99	0.03	Significant
	Pure Error	0.19	4	0.05			
	Cor Total	6.63	22				
Pigment (Mean model)	Model	0	0				
	Residual	1552.13	22	70.55			
	Lack of Fit	1488.98	18	82.72	5.24	0.06	Not significant
	Pure Error	63.15	4	15.79			
	Cor Total	1552.13	22				

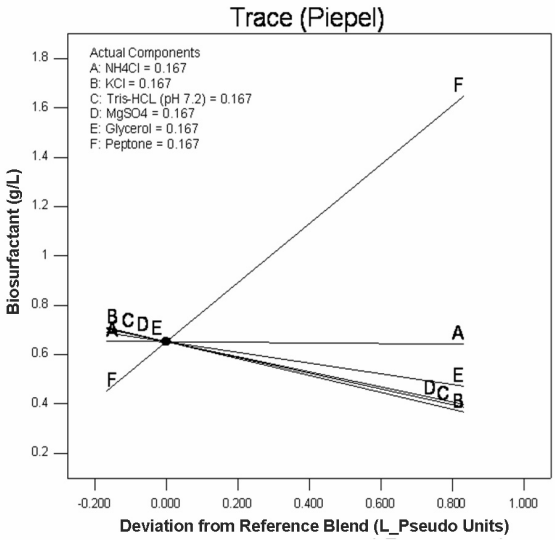

Figure 3: Piepel's trace plot for media components affecting biosurfactant production.

As far as literature about biosurfactant production by *P. aeruginosa* is concerned, some recent reports would be relevant to mention. Kaya *et al.* (2014) reported that out of 26 *Pseudomonas* isolates from petroleum industry waste in Turkey, two *P. auruginosa* strains 78 and 99 produced 0.287 and 0.286 g/L rhamnolipid, respectively, in nutrient broth. Patil *et al*. (2014) optimized complex media for cultivating *P. aeruginosa* F23 by a conventional method and could maximize rhamnolipid production up to 2.8 g/L. However, literature on production and quantification of pyocyanin pigment production from *Pseudomonas* is rare. To the best of our knowledge, there is no report till date on pigment production from *P. aeruginosa*, either on a laboratory scale or commercial scale.

CONCLUSION

A modified medium with 3.5 times higher concentration of peptone and half concentration of glycerol and salts compared to the original medium increased biosurfactant concentration by two-fold. Peptone had a very important role in biomass growth and biosurfactant production. Modification of the original medium composition did not increase pigment yield. This is the first report on optimization of medium composition for pigment production from *P. aeruginosa* PAO1.

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