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Optimizing C:N Ratio, C:P Ratio, and pH for Biosurfactant Production by *Pseudomonas fluorescens*

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Authors' contributions

This work was carried out in collaboration between both authors. Author PLP designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author GOA managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: To optimize the combination of selected culture medium parameters so as to achieve maximum biosurfactant production from *Pseudomonas fluorescens*.
Study Design: (1) Culturing *P. fluorescens* for biosurfactant production using a glycerol-mineral salt media with variations in ratio, carbon and phosphorus ratio (C:P ratio), and pH, (2) Screening for biosurfactant activity, (3) use of Response Surface Methodology in determining the combination of the factors that will lead to maximum biosurfactant production.
Place and Duration of Study: Department of Microbiology, Faculty of Science, University of Port Harcourt, Nigeria, between September 2016 and June 2017.
Methodology: *Pseudomonas fluorescens* was cultured for biosurfactant production using glycerol-mineral salt media with variations in carbon and nitrogen ratio(C:N ratio), C:P ratio, and pH.

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Combination of these factors was optimized via the use of Response Surface Methodology. The range of values of the factors investigated was C:N: 20–60, C:P: 10–16, pH: 5.5–8.5. Fifteen experimental runs were carried out.

Results: At the end of the experimental runs, the surface tensions of the culture broths ranged from 30.66 – 51.90 $mN.m^{-1}$. The surface tensions were fitted into the generalized polynomial model for 3-factor design. The model was worked out to be $Y = 296.5533 – 34.4456X_1 + 0.602833X_2 – 23.4019X_3 – 0.0545\ X_1X_2 + 1.088889X_1X_3 + 0.020417\ X_2X_3 + 1.663333X_1^2 – 0.00596X_2^2 + 0.551944X_3^2$. Prediction profiles generated from this model showed that the lowest surface tension, indicating maximum biosurfactant production, was achievable at a combination of pH 5.5, C:N = 20, and C:P = 16. Use of this combination for biosurfactant production resulted in reduction of the surface tension of the broth culture from 60.04 $mN.m^{-1}$ to 30.64 $mN.m^{-1}$. This almost tallied with the predicted value of 30.57 $mN.m^{-1}$.

Conclusion: The optimized combination avoided wastage of the carbon source. It is thus economical to carry out optimization procedures before proceeding to commercial production of biosurfactants.

Keywords: Pseudomonas fluorescens; biosurfactant; response surface methodology; surface tension.

1. INTRODUCTION

Biosurfactants, surface-active compounds mainly produced by microorganisms [1], can be applied in any industrial processes where modification of interfacial activity is required [2,3]. Biosurfactants are potential replacements for chemical surfactants due to their biodegradability and non-toxic nature.

Among *Pseudomonas* species, numerous studies are abounding for bio-surfactant production by *P. aeruginosa* [4,5,6,7,8]. However, *P. aeruginosa* is a bacterium frequently isolated from clinical specimens and is responsible for many cases of nosocomial infections [9,10,11]. The use of *P. aeruginosa* in the production of biosurfactant for application in food and pharmaceutical industries has become a subject of health concern. In a study carried out to compare the pathogenic potential of selected *Pseudomonas* strains using functional virulence and toxicity assays, it was found that almost all strain of *P. aeruginosa* used for the study induce inflammatory responses, and cause cytotoxicity towards cultured human HT29 cells [12]. This was not the case for *P. fluorescens* and some other *Pseudomonas* strains used in the study. In another study conducted by the Ministers of the Environment and of Health of the Government of Canada on a *Pseudomonas fluorescens* strain (ATCC 13525), it is stated that there have been no reports regarding the pathogenic or toxigenic potential of *P. fluorescens* ATCC 13525, nor has it been associated with toxins or metabolites that may lead to adverse effects [13]. It is also stated in the study that the inability of *P. fluorescens* ATCC 13525 to grow at normal human body temperature may limit its ability to invade and cause disease in immune competent individuals. Thus *P. fluorescens* isolated from the environment may not cause disease in immune competent individuals. *P. fluorescens* is capable of producing biosurfactant like *P. aeruginosa* [14,15]. It may thus serve as a replacement for *P. aeruginosa* in the production of biosurfactant for use in food and pharmaceutical industries.

Biosurfactant production by *P. fluorescens* has been achieved by using selected sources of carbon, e.g. olive oil [16,17]. However, reports which contain information on influence of different combinations of media components on the extent of biosurfactant production by *P. fluorescens* are not yet globally available as of the time of this research. A combination of nutrient ratios, and other factors which have been shown to optimize biosurfactant production by a close relative, *P. aeruginosa*, may also lead to optimization of biosurfactant production by *P. fluorescens*. Glycerol (3% v/v), carbon and nitrogen ratio (C:N ratio) of 18:1 - 60:1, carbon and phosphorus ratio (C:P ratio) of 10-16:1, and C:Fe ratio of 72,400:1 has been shown to result in optimum yield of biosurfactant by *P. aeruginosa* [4,18]. Other parameters which have been shown to influence biosurfactant production by selected microorganisms include pH, temperature, duration of incubation, and agitation [8,19,20,21].

Optimizing the combination of selected parameters which influences biosurfactant production so as to achieve maximum biosurfactant yield can help in minimizing the overuse of nutrients. Optimization can be

achieved via the use of Response Surface Methodology (RSM). RSM is a popular methodology applied in the optimization of biosurfactant production [6,8,22]. It involves the use of mathematical/statistical design/model such as the Box-Behnken design.

The aim of this research is to optimize the combination of C:N and C:P ratio, and pH via the use of RSM so as to achieve maximum biosurfactant production from *P. fluorescens*. The outcome of the study will aid in the commercial production of biosurfactant using *P. fluorescens*.

2. MATERIALS AND METHODS

2.1 Source of *Pseudomonas fluorescens*

Pseudomonas fluorescens bv. 3 (EU543578.1) isolated by the researchers in a previous study [23] was used for this study.

2.2 Culturing for Biosurfactant Production and Optimization

Broth cultures of the *P. fluorescens* were prepared by transferring colonial growth obtained from stock cultures into 350 ml sterile nutrient broth and incubated at 35°C for 48 hrs in anaerobic condition.

Glycerol-Mineral salt media with variations in the concentration of nitrate and phosphate (Table 1 and Table 2), and variations in pH were used in culturing the bacterium for biosurfactant production. The constituent as reported by the media is based on the glucose-mineral salt medium that is as follows in Bodour et al. [24]; glucose is replaced with glycerol.

Table 1. Composition of the glycerol-mineral salt medium

Composition	Concentration
Glycerol (% v/v)	3
KH_2PO_4	*
$MgSO_4.7H_2O$ ($g.L^{-1}$)	0.4
NaCl ($g.L^{-1}$)	1.0
$CaCl_2.2H_2O$ ($g.L^{-1}$)	0.1
$NaNO_3$	*
TES (% v/v)	0.1

*TES - Trace elements solution; * - the concentration was varied in other to achieve a particular C:N and C:P ratio*

The Response Surface Methodology was applied in the optimization of biosurfactant production. Three factors were selected for the optimization; C:N ratio, C:P ratio, and pH. The range of values of these factors and the chosen level of response surface analysis, based on the Box-Behnken design [22], is presented in Table 3. The ranges were chosen based on works already carried out on a closely related bacterium, *P. aeruginosa* [4,8,18]. The Box-Behnken experimental design matrix [8,22] based on the number of selected factors with two extra centre points is presented in Table 4. Thus, a total of 15 runs of experiments were carried out.

Table 2. Composition of the trace element solution

Trace element salts	$g.L^{-1}$
$MnSO_4.H_2O$	1.5
$FeSO_4.7H_2O$	0.5
$CuSO_4.5H_2O$	0.2
$Na_2MoO_4.2H_2O$*	0.1
$ZnSO_4.7H_2O$	1.5
H_3BO_3	0.3

$Na_2MoO_4.2H_2O$ - Sodium molybdate dehydrate*

Table 3. Factors and levels for response surface analysis

	Level		
Factor	**-1**	**0**	**+1**
pH	5.5	7.0	8.5
C:N ratio	20	40	60
C:P ratio	10	13	16

(Design adapted from Zhang and Dequan, 2013)

The determined nitrate and phosphate concentrations of the different Glycerol-Mineral salt media for the experimental runs so as to achieve the different C:N and C:P ratio is presented in Table 5 (the calculations used in achieving the concentrations is presented in the next section). About 100 ml of the different glycerol-mineral salt broth making up the 15 runs of the experiment were placed in 250 ml capacity conical flasks and sterilized in an autoclave. After sterilization and cooling, 10 ml of the broth culture of the bacterium was transferred separately into the contents of the different flasks. The flasks and their contents were then incubated at ambient temperature (27ºC – 31ºC) for seven days on a PSU-20i Multi-functional Orbital Shaker (Keison Products, UK) operated at 150 rpm for 8 hrs per day. At the end of the incubation period, the pH, surface tension, and oil spread diameter of broth in the experimental runs were determined.

Table 4. Combination of the factors for experimental runs using the box-behnken experimental design matrix

Run number	pH (X_1)	C:N (X_2)	C:P (X_3)
1	- 1 (5.5)	- 1 (20)	0 (13)
2	- 1 (5.5)	+ 1 (60)	0 (13)
3	+ 1 (8.5)	- 1 (20)	0 (13)
4	+ 1 (8.5)	+ 1 (60)	0 (13)
5	- 1 (5.5)	0 (40)	- 1 (10)
6	- 1 (5.5)	0 (40)	+ 1 (16)
7	+ 1 (8.5)	0 (40)	- 1 (10)
8	+ 1 (8.5)	0 (40)	+ 1 (16)
9	0 (7)	- 1 (20)	- 1 (10)
10	0 (7)	- 1 (20)	+ 1 (16)
11	0 (7)	+ 1 (60)	- 1 (10)
12	0 (7)	+ 1 (60)	+ 1 (16)
13	0 (7)	0 (40)	0 (13)
14	0 (7)	0 (40)	0 (13)
15	0 (7)	0 (40)	0 (13)

(Design adapted from Kumar et al., 2015)

Results generated from the determination of surface tensions of the experimental runs were fitted using a generalized polynomial model (Eq. 1) for 3-factor design [8]. Model fitting was achieved using second order polynomial regression equations. The equations were resolved using matrices via the aid of Microsoft excel®.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{1,2}X_1X_2 + \beta_{1,3}X_1X_3 + \beta_{2,3}X_2X_3 + \beta_{1,1}X_1^2 + \beta_{2,2}X_2^2 + \beta_{3,3}X_3^2 \quad \text{(Eq. 1)}$$

Y is the predicted response; X_1, X_2, and X_3 represents the values for the three factors (pH, C:N ratio, and C:P ratio); β_0 is the value of fitted response at the centre point of the design; β_1, β_2, and β_3 are the linear coefficients.; $\beta_{1,2}$, $\beta_{1,3}$, and $\beta_{2,3}$ are the interaction coefficients.; and $\beta_{1,1}$, $\beta_{2,2}$, and $\beta_{3,3}$ are the quadratic coefficients.

A combination of the values of the independent variables that led to the least surface tension (indicating maximum biosurfactant production) as predicted using the polynomial model was used in a new experimental run for biosurfactant production.

Table 5. Experimental runs and the nitrate and phosphate concentration of their glycerol-mineral salt media with their C:N and C:P ratio

R/N	CC g/L	NN g/L	cN g/L	C:N $\frac{CC}{cN}$	KP g/L	Cp g/L	C:P $\frac{CC}{cP}$
1	14.7	4.46	0.74	20	4.96	1.13	13
2	14.7	1.49	0.25	60	4.96	1.13	13
3	14.7	4. 46	0.74	20	4.96	1.13	13
4	14.7	1. 49	0.25	60	4.96	1.13	13
5	14.7	2.23	0.37	40	6.45	1.47	10
6	14.7	2.23	0.37	40	4.03	0.92	16
7	14.7	2.23	0.37	40	6.45	1.47	10
8	14.7	2.23	0.37	40	4.03	0.92	16
9	14.7	4. 46	0.74	20	6.45	1.47	10
10	14.7	4. 46	0.74	20	4.03	0.92	16
11	14.7	1. 49	0.25	60	6.45	1.47	10
12	14.7	1. 49	0.25	60	4.03	0.92	16
13	14.7	2.23	0.37	40	4.96	1.13	13
14	14.7	2.23	0.37	40	4.96	1.13	13
15	14.7	2.23	0.37	40	4.96	1.13	13

R/N – Run number, CC - carbon concentration (3 % v/v glycerol is used in all the media, and the concentration of carbon in 1 L of media containing 3 % v/v glycerol is 14.7 g/L), NN - $NaNO_3$ concentration, cN – calculated nitrogen concentration, C:N – carbon nitrogen ratio, KP - KH_2PO_4 concentration, cP – calculated phosphorus concentration, C:P – carbon phosphorus ratio

2.3 Determination of the Nitrate and Phosphate Concentrations of the Different Glycerol-Mineral Salt Media so as to Achieve the Different C:N And C:P Ratios

The density of glycerol, the sole carbon source is 1.26 g/cm^3 [25]. This implies that 1 cm^3 (or 1 ml) of glycerol is equivalent to 1.26 g of glycerol.

Molecular formula of glycerol is $C_3H_8O_3$ [25].

Molecular weight = 3 (12) + 8 (1) + 3 (16) = 92 g/mol

Percentage of C in glycerol = $\frac{3\,(12)\times 100}{92}$ = 39.1 %

Therefore 1 ml glycerol contains $\frac{39.1\times 1.26}{100}$ = 0.49 g C

3% v/v glycerol is used in all the media. Therefore in the preparation of 1 L of each media, 30 ml of glycerol is added (to 969 ml of distilled water plus 1 ml of the trace element solution to make up to 1 L before addition of the various salts).

1 ml glycerol contains 0.49 g C. Therefore 30 ml glycerol contains $\frac{30\ ml\times 0.49\ g}{1\ ml}$ = 14.7 g. Thus the concentration of C in each media is 14.7 g/L.

2.3.1 C:N ratio determination

Sole nitrogen source = $NaNO_3$

Molecular weight of $NaNO_3$ = 23 + 14 + 3 (16) = 85 g/mol

Molecular weight of N in $NaNO_3$ = 14 g/mol

For C:N = 20, in 1 L of media; $\frac{14.7\ g}{N}$ = 20; therefore N = $\frac{14.7\ g}{20}$ = 0.735 g

There are 14 g of N in 85 g of $NaNO_3$.

Therefore 0.735 g of N will be present in $\frac{85\ g\times 0.735\ g}{14\ g}$ $NaNO_3$ = 4.46 g $NaNO_3$

Thus 4.46 g $NaNO_3$ will be added to 1 L of media containing 3 % v/v glycerol to obtain a C:N ratio of 20.

For C:N = 60, in 1 L of media; $\frac{14.7\ g}{N}$ = 60; therefore N = $\frac{14.7\ g}{60}$ = 0.245 g

There are 14 g of N in 85 g of $NaNO_3$.

Therefore 0.245 g of N will be present in $\frac{85\ g\times 0.245\ g}{14\ g}$ $NaNO_3$ = 1.49 g $NaNO_3$

Thus 1.49 g $NaNO_3$ will be added to 1 L of media containing 3 % v/v glycerol to obtain a C:N ratio of 60.

For C:N = 40, in 1 L of media; $\frac{14.7\ g}{N}$ = 40; therefore N = $\frac{14.7\ g}{40}$ = 0.3675 g

There are 14 g of N in 85 g of $NaNO_3$.

Therefore 0.3675 g of N will be present in $\frac{85\ g\times 0.3675\ g}{14\ g}$ $NaNO_3$ = 2.23 g $NaNO_3$

Thus 2.23 g $NaNO_3$ will be added to 1 L of media containing 3 % v/v glycerol to obtain a C:N ratio of 40.

2.3.2 C:P ratio determination

Sole phosphorus source = KH_2PO_4

Molecular weight of KH_2PO_4 = 39 + 2 (1) + 31 + 4 (16) = 136 g/mol

Molecular weight of P in KH_2PO_4 = 31 g/mol

For C:P = 13, in 1 L of media; $\frac{14.7\ g}{P}$ = 13; therefore **P** = $\frac{14.7\ g}{13}$ = 1.1308 g

There are 31 g of P in 136 g of KH_2PO_4.

Therefore 1.1308 g of P will be present in $\frac{136\ g\times 1.1308\ g}{31\ g}$ KH_2PO_4 = 4.96 g KH_2PO_4

Thus 4.96 g KH_2PO_4 will be added to 1 L of media containing 3 % v/v glycerol to obtain a C:P ratio of 13.

For C:P = 10, in 1 L of media; $\frac{14.7\ g}{P}$ = 10; therefore **P** = $\frac{14.7\ g}{10}$ = 1.47 g

There are 31 g of P in 136 g of KH_2PO_4.

Therefore 1.47 g of P will be present in $\frac{136\ g\times 1.47\ g}{31\ g}$ KH_2PO_4 = 6.45 g KH_2PO_4

Thus 6.45 g KH_2PO_4 will be added to 1 L of media containing 3 % v/v glycerol to obtain a C:P ratio of 10.

For C:P = 16, in 1 L of media; $\frac{14.7\ g}{P}$ = 16; therefore **P** = $\frac{14.7\ g}{16}$ = 0.9188 g

There are 31 g of P in 136 g of KH_2PO_4.

Therefore 0.9188 g of P will be present in $\frac{136\ g \times 0.9188\ g}{31\ g}$ KH_2PO_4 = 4.03 g KH_2PO_4

Thus 4.03 g KH_2PO_4 will be added to 1 L of media containing 3 % v/v glycerol to obtain a C:P ratio of 16.

2.4 Culturing for Biosurfactant Production Using Optimized Conditions

A new experimental run, in duplicate, was carried out using the optimized parameters determined from the prediction profile. In the new experimental run, broth media volumes were increased to 1000 ml while inoculum volumes were increased to 100 ml. Due to the increased volume and weight the speed of the orbital shaker on which the new experimental run was incubated on was reduced to 100 rpm as a safety measure. To compensate for this reduction, the duration of incubation was increased to eight days. The bacterial population in the new experimental run was determined at day 0, and at two days interval. At the end of the experimental period, the broth cultures were screened for biosurfactant activity. After the screening, the broth cultures were subjected to a procedure for biosurfactant extraction.

2.5 Screening for Biosurfactant Activity

The surface tension, oil spread diameter, and drop collapse activity of the culture broths were determined. This was also carried out for a positive control (T-POL, a commercial available surfactant) and negative controls (un-inoculated broth and distilled water).

2.5.1 Surface tension measurements

The capillary rise method was used in measuring the surface tensions of the culture broth. The rise in height was then used to calculate the surface tension with the aid of Equation 2 [26].

γ = ½.rhdg (mN.m^{-1}) (Eq. 2)

'r' is the radius of the capillary tube in cm; 'h' is the rise in height in cm of the liquid; 'd' is the broth density in g.ml^{-1}; and 'g' is the acceleration due to gravity in cm.s^{-2}, i.e. 980 cm.s^{-2}.

2.5.2 Oil spread diameter

A modification of the method outlined in Almansoory et al. (2014) [27] was used in determining the oil spread diameter. About 40 ml of water were poured into Petri dishes and oil films generated on the surface of the water by applying several drops of diesel oil. A drop of broth culture was placed in the centre of the oil films, and the diameter of the ensuing zone of clearance was measured.

2.5.3 Drop collapse activity

A modification of the method described by Almansoory et al. [27] was also used in determining the drop collapse activity. Each well in a ceramic well plate were coated with a drop of used engine oil. The well plate was then incubated at 37ºC for about 1 hr. After incubation, two drops of the different culture broths were transferred into the different oil-coated wells. After 1 minute, the shapes of the drops were observed.

2.6 Extraction and Quantification of Biosurfactant

Extraction of biosurfactant was carried out using a modification of the method described in Dhail and Jasuja (2012) [28]. Cells were removed from the broth cultures by centrifugation at 5000 rpm for 18 minutes. The liquid supernatant was collected and the sediment materials (cell pellets) discarded. Extracellular materials in the liquid supernatant were obtained via acid precipitation followed by overnight refrigeration; in the acid precipitation, 5 M hydrochloric acid was added until the pH was reduced to about 2. After refrigeration, the precipitates that formed were collected through centrifugation at 5000 rpm for 35 minutes. The precipitate, now considered the crude biosurfactant, was weighed and subjected to chemical analysis for determination of carbohydrate, lipid, and protein concentrations as outlined by Nielson (2002) [29].

3. RESULTS

The surface tension of culture broths of *P. fluorescens* making up the experimental runs in the optimization experiment is presented in

Table 6. From the Table it can be seen that the surface tensions ranged from 30.66-51.90 mN.m^{-1}.The oil spread diameter of the culture broths is presented in Table 7. On determination of the oil spread diameter, when a drop of each broth culture was placed on the oil films, they spread the oil to a diameter range of 4 mm to 6 mm. After a few seconds a spontaneous increase in the size of the diameter occurred, like a bursting phenomenon. Immediately after the spontaneous increase, the enlarged diameter gradually reduced till it disappeared. Thus the measured oil spread diameter is a quick estimated measurement taken. In the case of the positive control (T-POL), a large clearing zone occurred immediately its drop was applied on the oil film. However, the enlarged diameter also gradually reduced till it disappeared.

The change in pH of the experimental runs is depicted in Fig. 1. From the Figure it can be seen that there was increase in the pH of all the culture broth making up the experimental runs, except for Run number 4 in which the pH remained stable.

Fitting of the surface tensions obtained from the different combinations of the factors used into the generalized model ($Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{1,2}X_1X_2 + \beta_{1,3}X_1X_3 + \beta_{2,3}X_2X_3 + \beta_{1,1}X_1^2 + \beta_{2,2}X_2^2 + \beta_{3,3}X_3^2$) is presented in Table 8. The calculations were done with the aid of Microsoft excel ®. In determining the coefficients (β_0, β_1, β_2, β_3, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$, $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$) in the generalized model, the matrix equation $\hat{\beta} = (X^TX)^{-1}X^TY$ [30] was used. The matrix equation was also resolved with the aid of the Microsoft excel ® platform. From the calculations, the coefficients β_0, β_1, β_2, β_3, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$, $\beta_{1,1}$, $\beta_{2,2}$, and $\beta_{3,3}$ were deduced to be 296.5533, - 34.4456, 0.602833, - 23.4019, - 0.0545, 1.088889, 0.020417, 1.663333, - 0.00596, and 0.551944 respectively. Thus, the generalized model can now be written as,

$$Y = 296.5533 - 34.4456X_1 + 0.602833X_2 - 23.4019X_3 - 0.0545\ X_1X_2 + 1.088889X_1X_3 + 0.020417\ X_2X_3 + 1.663333X_1^2 - 0.00596X_2^2 + 0.551944X_3^2 \quad \text{(Eq. 3)}$$

Table 6. Surface tension of the culture broths of *P. fluorescens* making up the experimental runs

	pH	C:N	C:P	S.T.
R/N	**X_1**	**X_2**	**X_3**	**(mN.m^{-1})**
1	5.5	20	13	30.66
2	5.5	60	13	38.83
3	8.5	20	13	37.20
4	8.5	60	13	38.83
5	5.5	40	10	51.90
6	5.5	40	16	33.93
7	8.5	40	10	43.73
8	8.5	40	16	45.36
9	7.0	20	10	40.46
10	7.0	20	16	38.83
11	7.0	60	10	33.93
12	7.0	60	16	37.20
13	7.0	40	13	37.20
14	7.0	40	13	33.93
15	7.0	40	13	33.93
PC	-	-	-	29.57
DW	-	-	-	71.50

R/N – Run number; S.T. – surface tension; PC – positive control (T-POL); DW – distilled water

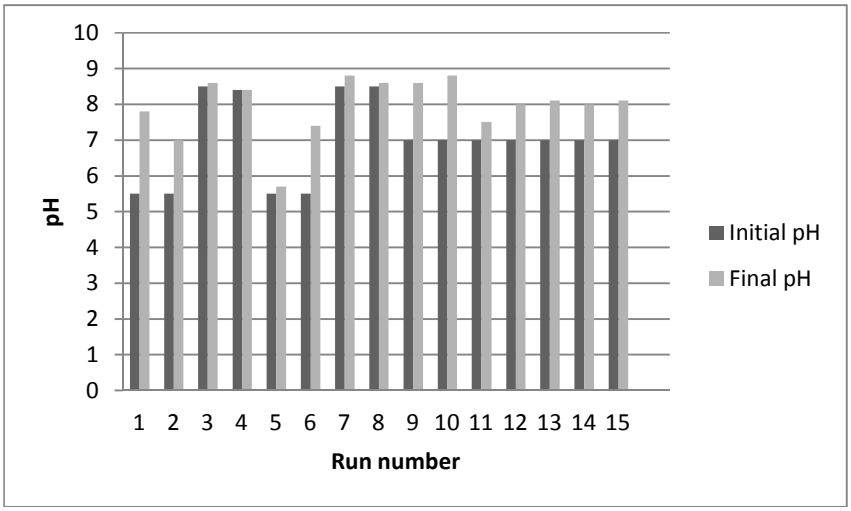

Fig. 1. Change in pH of the experimental runs

Table 7. Zone of clearance on oil film of culture broths from the experimental runs

R/N	iDm_1 (mm)	eDm_1 (mm)	iDm_2 (mm)	eDm_2 (mm)	iDm_3 (mm)	eDm_3 (mm)	iDm_{av} (mm)	eDm_{av} (mm)
1	6	40	6	40	5	35	5.7	38
2	5	35	4	25	5	30	4.7	30
3	4	25	5	30	4	25	4.3	27
4	5	35	5	30	4	-	4.7	33
5	(3)	-	(3)	-	(3)	-	(3)	-
6	5	-	5	-	5	35	5	35
7	5	-	5	-	4	25	4.7	25
8	4	-	5	35	5	35	4.7	35
9	6	40	5	30	6	-	5.7	35
10	5	-	5	-	5	40	5	40
11	6	40	5	-	5	-	5.3	40
12	6	7	5	10	5	-	5.3	9
13	5	40	5	-	5	-	5	40
14	5	45	5	-	5	-	5	45
15	4	20	5	30	4	-	4.3	25
PC	55	55	60	60	60	60	58	58
DW	(3)	-	(3)	-	(3)	-	(3)	-

R/N – Run number; PC – positive control (T-POL); DW – distilled water; iDm – initial diameter of clear zone; eDm – estimated enlarge diameter of clear zone; Dm_{av} – average diameter of clear zone; (3) – actually a bubble on the oil film, not a clearing

Equation 3 was used to calculate the predicted values of the surface tensions resulting from the different combination of factors. The result of the calculation is presented in Table 9.

The analysis of variance (ANOVA) of the regression model indicates that at least one coefficient out of β_0, β_1, β_2, β_3, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$, $\beta_{1,1}$, $\beta_{2,2}$, and $\beta_{3,3}$ is significant. In other words a regression model exists between surface tension and one or more of the factors (pH, C:N, and C:P). A summary of the ANOVA is presented in Table 10.

The prediction profiles are presented in Table 11. The values in the profiles were derived using equation 4. In deriving the prediction profiles, one of the variables (pH) was kept constant, while the others were varied. From the prediction profiles, the lowest surface tension (approximately 30.32 $mN.m^{-1}$) is achievable at the combination of pH 5.5, C:N = 20, and C:P = 16. This combination was used in the new experimental run.

The population growth of *P. fluorescens* in the new experimental run is depicted in Fig. 2. From the Figure it can be seen that the bacterium attained stationary phase in two days.

The surface tension of the culture broth from the new experimental run at the end of the incubation period is presented in Table 12. From the Table it can be seen that there is no much difference between the experimental and predicted surface tension values. Also, it can be seen from the Table that the experimental surface tension of broth from the new experimental run is about two times lower than the negative control (un-inoculated broth).

The oil spread diameter of the culture broth from the new experimental run ranged from 30 to 40 mm. The initial oil spread diameter was about 5 mm. The drop collapse test of culture broth from the new experimental run at the end of the incubation period was positive (Plate 1). At the end of the incubation period, the pH of the broth in the new experimental run increased from 5.5 to 8.3.

The total weight of the extracellular materials obtained from about 1,800 ml cell free culture broth through acid precipitation, which is considered as the crude biosurfactant was about 7.3 g, the yield is thus calculated to be about 0.004 g/L. The concentration of carbohydrate, lipid, and protein of the crude biosurfactant, are 2.54%, 25.54%, and 36.48% respectively.

Table 8. Factors and surface tension values fitted into the generalized model

	pH	C:N	C:P							S.T.
	X_1	X_2	X_3	X_1*X_2	X_1*X_3	X_2*X_3	X_1^2	X_2^2	X_3^2	
					[X]					Y
1	5.5	20	13	110	71.5	260	30.25	400	169	30.66
1	5.5	60	13	330	71.5	780	30.25	3600	169	38.83
1	8.5	20	13	170	110.5	260	72.25	400	169	37.20
1	8.5	60	13	510	110.5	780	72.25	3600	169	38.83
1	5.5	40	10	220	55	400	30.25	1600	100	51.90
1	5.5	40	16	220	88	640	30.25	1600	256	33.93
1	8.5	40	10	340	85	400	72.25	1600	100	43.73
1	8.5	40	16	340	136	640	72.25	1600	256	45.36
1	7	20	10	140	70	200	49	400	100	40.46
1	7	20	16	140	112	320	49	400	256	38.83
1	7	60	10	420	70	600	49	3600	100	33.93
1	7	60	16	420	112	960	49	3600	256	37.20
1	7	40	13	280	91	520	49	1600	169	37.20
1	7	40	13	280	91	520	49	1600	169	33.93
1	7	40	13	280	91	520	49	1600	169	33.93

S.T. – surface tension; [X] – design matrix

4. DISCUSSION

The cost associated with producing biosurfactants may be quite high, thus there is need to economize nutrient sources used in biosurfactant production. Wastage of nutrients sources can be avoided by investigating the proportion in which the nutrients should be combined to achieve maximum biosurfactant production. The best possible proportion can be determined via the use of the Response Surface Methodology (RSM).

In this study, optimum combination of C:N ratio, C:P ratio, and pH values that would lead to maximum biosurfactant production by a *P. fluorescens* isolate was investigated. On culturing the bacterium for biosurfactant production, via optimization of C:N ratio, C:P ratio, and pH, using RSM, it was found that a combination of C:N = 20, C:P = 16, and pH = 5.5 resulted in the least surface tension value indicating maximum biosurfactant production. Maximum biosurfactant production by a close relative (*P. aeruginosa*) via the use of two (glycerol and sodium nitrate) out of the three major substrates used in this study have been shown by Santa-Anna et al. [18], and Rashedi et al. [31] to occur at a C:N value of about 60. The authors however did not use a statistical combinatory approach in achieving maximum biosurfactant production, and thus did not take into account the involvement of other important media parameters, e.g. pH, which can affect biosurfactant production. The use of a statistical approach in this study taking into account the input of other parameters apparently resulted in the disagreement observed between the C:N values.

Table 9. Predicted values of the surface tensions resulting from the different combination of factors using equation 3

	pH	C:N	C:P	S.T. ($mN.m^{-1}$)	
R/N	X_1	X_2	X_3	E	P
1	5.5	20	13	30.66	33.314
2	5.5	60	13	38.83	36.982
3	8.5	20	13	37.20	39.034
4	8.5	60	13	38.83	36.162
5	5.5	40	10	51.90	49.237
6	5.5	40	16	33.93	35.762
7	8.5	40	10	43.73	41.887
8	8.5	40	16	45.36	48.012
9	7.0	20	10	40.46	40.461
10	7.0	20	16	38.83	34.336
11	7.0	60	10	33.93	38.409
12	7.0	60	16	37.20	37.185
13	7.0	40	13	37.20	35.014
14	7.0	40	13	33.93	35.014
15	7.0	40	13	33.93	35.014

S.T. – surface tension; E – experimental values; P – predicted values

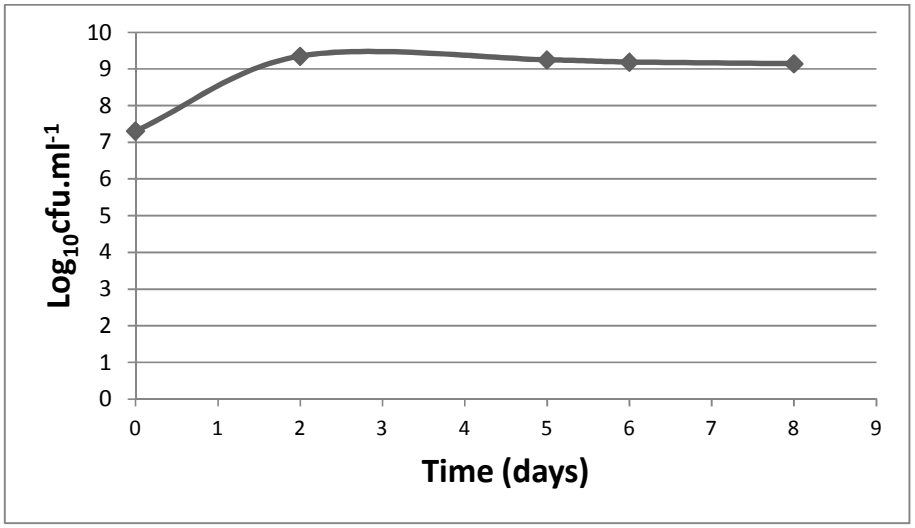

Fig. 2. Population growth of *P. fluorescens* in the new experimental run

Table 10. Summary of the ANOVA of the regression model

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F statistics	*P* value
Regression	3	318.8459	106.282	13.10599	0.1
Error	11	89.20365	8.109423		
Total	14	408.04955			

The tabulated statistic $F_{\alpha,k,n-(k+1)}$, i.e. $F_{0.1,3,11}$ is 2.66

At the optimized combination of C:N = 20, C:P = 16, and pH = 5.5, the surface tension of the culture broth of *P. fluorescens* at the end of the fermentation period was 30.64 $mN.m^{-1}$. Using *P. fluorescens* in biosurfactant production, Persson et al. [32] obtained a surface tension value of 27 $mN.m^{-1}$; and Abouseoud et al. [33] obtained a surface tension value of 33.5 $mN.m^{-1}$. The deviation in the surface tension obtained in this study and those obtained in the other studies is not wide. It can thus be said that there is an agreement in the surface tension results. The oil spread diameter of the biosurfactant from the optimized broth was about 40 mm, while the average oil spread diameter of the positive control (Table 7) was 58 mm. This along with the positive drop collapse test (Plate 1) indicated high biosurfactant activity.

The pH of the optimized broth for biosurfactant production at the end of the incubation period was 8.3. This is in close agreement with the result obtained by Persson et al. [32] while studying biosurfactant production by *P. fluorescens*; their biosurfactant yield was optimal at pH 8.0.

Table 11. The prediction profiles

X_2 (C:N)	X_3 (C:P)		
	10	**13**	**16**
At pH 5.5			
20	46.24368	33.3138	30.31892
40	49.23674	37.53188	35.76202
60	47.4618	36.98196	36.43712
At pH 7.0			
20	40.46111	32.43123	34.33635
40	41.81917	35.01431	38.14445
60	38.40923	32.82939	37.18455
At pH 8.5			
20	42.16353	39.03366	45.83878
40	41.88659	39.98174	47.01188
60	36.84165	36.16182	45.41698

Lowest value = 30.31892

The yield of the crude biosurfactant was about 0.004 $g.L^{-1}$. A biosurfactant yield of 0.437 $g.L^{-1}$ has been reported by Peter and Singh [34] for *P. fluorescens*. A yield of about 2 $g.L^{-1}$ has been obtained by Abouseoud et al. [33]. In a related study, biosurfactant yield obtained via utilization of glycerol by *P. aeruginosa*, a close relative of *P. fluorescens*, was found to be 3.16 $g.L^{-1}$ [18]. The wide disparity in the yield obtained in this

study and those obtained in other study could be attributed to the substrate used, or the method of biosurfactant extraction employed. In this study, glycerol, sodium nitrate, and potassium dihydrogen phosphate was used as the major substrate for biosurfactant production. The yield reported by Peter and Singh (2014) [34] was obtained via the use of soybean oil as the major substrate. The yield obtained by Abouseoud et al. [33] was via the utilization of Olive oil. The relatively high yield obtained for the close relative of *P. fluorescens*, using the same substrate used in this study by Santa-Anna et al. [18] can be attributed to the "superiority" of *P. aeruginosa* to *P. fluorescens* in some aspects. For instance, among *P. aeruginosa*, *P. fluorescens*, and *P. putida*, *P. aeruginosa* has been shown to have the highest level of cell adhesion to hydrocarbons [35]. It should be noted that "adhesion to hydrocarbons" is one of the test used to screen cells for biosurfactant production [36].

Even though biosurfactant yield obtained via the use of *P. fluorescens* is low compared to that obtained via the use of *P. aeruginosa* it would be safer to use *P. fluorescens* for bio-product generation for use in food, pharmaceutical, and environmental applications. This is because *P. fluorescens* is not known to be pathogenic against humans, unlike *P. aeruginosa* [12, 13]. Moreover, the critical micelle concentration (CMC) of biosurfactant produced by *P. fluorescens* is low compared to that of SDS, a known chemical surfactant; biosurfactant produced by *P. fluorescens* have CMC ranging from 290 mg/L – 715 mg/L [37,38], while CMC of SDS is 2,310 mg/L [39]. Thus a relatively small quantity of biosurfactant obtained from *P. fluorescens* can be effective in achieving a desired modification in interfacial activity.

Table 12. Surface tension of the culture broth from the new experimental run

	pH	C:N	C:P	S.T. ($mN.m^{-1}$)	
	X_1	X_2	X_3	E	P
NE	5.5	20	16	30.64	30.56881
NC	5.5	-	-	60.04	-
DW	7.4	-	-	71.48	-
PC	-	-	-	29.55	-

NE – New Experimental Run; NC – negative control (un-inoculated broth); DW – distilled water; PC – positive control (T-POL); S.T. – surface tension; E – experimental value; P – predicted value

Plate 1. Drop collapse activity of culture broth of *P. fluorescens* (P); C - negative control

The procedure used for biosurfactant extraction in this study was adapted from the procedure used by Dhail and Jasuja [28]. Dhail and Jasuja [28] used a speed of 8,000 rpm for 20 min to sediment the precipitate formed after acid precipitation. Due to the inability of the centrifuged used in this study (L-600 Centrifuge; Shinova Systems Co., Ltd., Shanghai) to attain such speed, its highest speed 5,000 rpm was used, however the time was increased to 35 min. This increase in time may have had no effect on compensating for the additional speed required to sediment adequate quantity of the precipitates.

The concentration of carbohydrate, lipid, and protein of the crude biosurfactant obtained in this study, are 2.54%, 25.54%, and 36.48% respectively. Biosurfactant from *P. fluorescens* have been identified as a rhamnolipid-type biosurfactant [33], i.e. they are glycolipids in nature. However, Persson et al. [32] have identified a biosurfactant from *P. fluorescens* that consisted mainly of carbohydrate and protein. Comparing the works of Abouseoud et al. [33] and Persson et al. [32], it could be seen that biosurfactants produced by *P. fluorescens* have carbohydrate, and either protein or lipid. The presence of both lipid and protein in the biosurfactant extracted in this study is in variant with this idea. The presence of high protein concentration could be attributed to incomplete removal of the cells during extraction. In the removal of cells from broth cultures to obtain cell-free broth for biosurfactant quantification, a centrifugal speed of 7,000 rpm–8,500 rpm for 10–30 min have been used [18;28,34]. In this study cells were removed from the broth cultures

by centrifugation at 5,000 rpm for 18 min. It could be that this speed and time used was not adequate to remove all the cells. However, due to the fact that some microorganisms produce biosurfactants which remain attached to their cells [40,41], high protein concentration can be encountered during chemical analysis. Factoring this into the scenario observed it could be said that the biosurfactant extracted in this study is a glycolipid-type biosurfactant.

5. CONCLUSION

P. fluorescens was able to produce biosurfactant using glycerol, sodium nitrate, and potassium dihydrogen phosphate as the major substrates. The substrate combinations were expressed in terms of C:N and C:P ratio. An important culture parameter, pH, was factored into the combination. The C:N ratio investigated ranged from 20–60, C:P ranged from 10–16, and pH ranged from 5.5 – 8.5. An Optimized combination of C:N = 20, C:P = 16, and pH = 5.5 achieved using the Response Surface Methodology resulted in maximum biosurfactant production as indicated by the low surface tension value. It can be seen from the optimized combination that wastage of carbon source could be avoided, since C:N is determined to be 20, rather than arbitrary using a C:N ratio of 30, 55, or 60, such as have been used by other researchers. Due to the high production cost of biosurfactants, it would thus be economical to carry out optimization procedures so as to determine the best combination of substrate concentration and culture conditions that would lead to maximum biosurfactant production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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