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RESEARCH ARTICLE

# Pseudosolubilized *n*-alkanes analysis and optimization of biosurfactants production by *Pseudomonas* sp. DG17

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**Abstract** The pseudosolubilized medium-chain-length *n*-alkanes during biodegradation process, and optimization of medium composition and culture conditions for rhamnolipid production by *Pseudomonas* sp. DG17 using Plackett–Burman design and Box–Behnken design, were examined in this study. The results showed that pseudosolubilized concentration of $C_{14}$ to $C_{20}$ *n*-alkanes was higher than that of $C_{24}$ to $C_{26}$. After incubation for 120 h, pseudosolubilized $C_{16}H_{34}$ increased to 2.63±0.21 mg. Meanwhile, biodegradation rates of *n*-alkanes decreased along with the increase of carbon chain length. Carbon-14 assay suggested that nonlabeled $C_{14}H_{30}$, $C_{16}H_{34}$, and $C_{20}H_{42}$ inhibited the biodegradation of $^{14}C$ *n*-octadecane, and *Pseudomonas* sp. DG17 utilized different alkanes simultaneously. Three significant variables (substrate concentration, salinity, and C/N) that could influence rhamnolipid production were screened by Plackett–Burman design. Results of Box–Behnken design suggested that rhamnolipid concentration could be achieved at 91.24 mg $L^{-1}$ (observed value) or 87.92 mg $L^{-1}$ (predicted value) with the optimal levels of concentration, salinity, and C/N of 400 mg $L^{-1}$, 1.5 %, and 45, respectively.

**Keywords** *n*-alkanes · Pseudosolubilization · *Pseudomonas* · Biosurfactants · Biodegradation · Optimization

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## Introduction

Bioremediation, which exploits the metabolic potential of microbes for the cleanup of recalcitrant xenobiotic compounds, has come up as a promising alternative (Chauhan et al. 2008). In this technology, biosurfactants produced by many bacterial strains that can degrade or transform petroleum products can greatly improve the soil bioremediation (Cubitto et al. 2004; Obayori et al. 2009). Biosurfactants can enable the formation of microemulsions, which are very small droplets surrounded by surfactants within the aqueous phase, a process often referred to as pseudosolubilization (Goma et al. 1973). Thus, microorganisms can utilize pseudosolubilized oil droplets with the regard to enhance biodegradation of hydrocarbons (Sotirova et al. 2008; Lee et al. 2006), which was a well-reported uptake mechanism of hydrocarbons (Hua and Wang 2012; Watkinson and Morgan 1990). Biosurfactants have been tested in environmental applications such as bioremediation and dispersion of oil spills, enhanced oil recovery, and transfer of crude oil. Many of the biosurfactants known today have been investigated with a view toward possible technical applications (Nayak et al. 2009). Biosurfactants are amphiphilic compounds with hydrophobic and hydrophilic domains. The lipophilic moiety can be a protein or a peptide with a high proportion of hydrophobic side chains, but is usually the hydrocarbon chain of a fatty acid with 10–18 carbon atoms (Sim et al. 1997). The hydrophilic moiety can be an ester, a hydroxyl, a phosphate or carboxylate group, or a carbohydrate (Bonnolo 1999). In this case, perhaps the most important roles for biosurfactants in bioremediation are the reduction of interfacial tension between aqueous and organic phases, and micellization or pseudosolubilization of

hydrocarbons so that a hydrophilic microbe may interact with a hydrophilic micelle in which hydrophobic substrate is found (Beal and Betts 2000; Abalos et al. 2004; Prabhu and Phale 2003). Moreover, *n*-alkane content seems to be a good compound-related indicator for defining the occurrence of the biodegradation process (da Cunha et al. 2006). Particularly, the alkanes of intermediate length ($C_{10}$–$C_{20}$) are biodegraded more readily (Subarna et al. 2002). Many studies have shown that microorganisms have high bioavailability of *n*-alkanes (Alexander et al. 2007; Lindley and Heydeman 1986; Gyorgy et al. 2001). For these microorganisms, solubilization and lowering of the surface and interfacial tension are thought to be the main reasons for facilitating the transport of pollutants adsorbed on solid phases to the surfactant-containing aqueous phases (Kim et al. 2002; Zhang and Miller 1995; Doong and Lei 2003).

Bioremediation technologies generally aim at providing favorable conditions of oxygen, temperature, nutrients, aqueous solubility, and the concentration of microorganisms in the impacted site to enhance biological hydrocarbon breakdown (Rahman et al. 2002; Rosa et al. 2013; Ferreira et al. 2012; Mrozik and Piotrowska-Seget 2010). For example, low concentration of substrates was one likely reason for failure of biodegradation process for the acclimation period before mineralization of substrates was lengthened (Goldstein et al. 1985; Wiggins and Alexander 1988). Meanwhile, supplementation of nutrients, especially nitrogen and phosphorus, could meet the biomass increase during the metabolism process of hydrocarbons with regard to improve the bioremediation efficiancy (Chaillan et al. 2006; Romina et al. 2012). In this case, factors that influence the biosurfactant production by bacteria should be investigated before land-scale bioremediation. Every microorganism evidences its own idiosyncratic physicochemical and nutritional requirements for growth and biosurfactant secretion. Many researchers have attempted to induce the effect of different growth conditions, including pH, salt concentration, temperature, substrate, and nitrogen source, on its own biosurfactant production (Abouseoud et al. 2010; Seghal Kiran et al. 2009; Ilori et al. 2005). In this case, as for *Pseudomonas* sp. DG17, it was needed to analyze the effect of environmental factors on the production of biosurfactant and hydrocarbon biodegradation. The Plackett–Burman design allows the screening of main factors from a large number of process variables and is quite useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated in further optimization processes (Reddy et al. 2008). In addition, response surface methodology (RSM), most widely applied to develop, improve, and optimize the processes and evaluate the relative significant affecting factors, is an efficient strategic experimental tool to select the optimal conditions of a multivariable system (Li et al. 2014).

The aim of this study was to evaluate the pseudosolubilization of *n*-alkanes, a major component of crude oil, by *Pseudomonas* sp. DG17 during the biodegradation process. Medium- and long-chain-length alkanes, including $C_{14}H_{30}$, $C_{16}H_{34}$, $C_{18}H_{38}$, $C_{20}H_{42}$, $C_{24}H_{46}$, and $C_{26}H_{54}$, were selected as the model compounds. Also, another aim was to screen significant factors that influence biosurfactant production by using Plackett–Burman design and Box–Behnken design. The optimal levels of factors were useful for implying *Pseudomonas* sp. DG17 to the bioremediation of crude-oil-polluted soil.

## Materials and methods

### Culture and growth conditions

*Pseudomonas* sp. DG17 (CGMCC, No. 5051; NCBI Accession No. JN 216879) and mineral salt medium used in this study were as described by Hua and Wang (2012). Before biodegradation assay, bacterial inoculum from the crude oil solid medium was transferred into 100 mL of lysogeny broth (LB) culture medium with 0.5 % yeast extract in a 250-mL Erlenmeyer flask and enriched at 25±1 °C on a gyratory shaker at 140 rpm for 48 h. Cells were collected by centrifugation at 4000 rpm for 10 min, washed two times with mineral salt medium (MSM, pH 7.0), and re-dissolved in sterile MSM with equal volume. The final $A_{600}$ value measured at 600 nm on a spectrophotometer (Varian, Palo Alto, CA, USA) was 1.5.

### Biodegradation and pseudosolubilization of n-alkane assay

For one group, the ability of *Pseudomonas* sp. DG17 to degrade aerobically *n*-alkanes was determined in 250-mL Erlenmeyer flasks containing 100 mL of pH-neutral MSM after incubation for 120 h. In the second group, cell growth of DG17 was analyzed at 0, 6, 12, 24, 36, 48, 72, 96, and 120 h. In the third group, samples were used to analyze pseudosolubilized *n*-alkanes at 12, 24, 48, 72, 96, and 120 h. The MSM was amended with single *n*-alkanes $C_{14}H_{30}$, $C_{16}H_{34}$, $C_{18}H_{38}$, $C_{20}H_{42}$, $C_{24}H_{50}$, and $C_{26}H_{52}$ (each 200 mg $L^{-1}$) as the sole carbon source. The biodegradation and pseudosolubilization analyses were as described by Hua and Wang (2013), and the initial cell density ($A_{600}$) was 0.15. Three different samples were conducted simultaneously for standard deviation analysis. Standard curve of alkane concentration was as follows: $A_i$=364274594.595 $X$ for $C_{14}H_{30}$, $A_i$=378076756.757 $X$ for $C_{16}H_{34}$, $A_i$=426208108.108 $X$ for $C_{18}H_{38}$, $A_i$=372563566.567 $X$ for $C_{20}H_{42}$, $A_i$=365404864.865 $X$ for $C_{24}H_{50}$, and $A_i$=43007189.189 $X$ for $C_{26}H_{54}$, where $A_i$ was peak area and $X$ was alkane concentration (mg $L^{-1}$).

### Selection of significant variables by Plackett–Burman design

The Plackett–Burman design was used to screen and evaluate environment factor variables that influence rhamnolipid production by *Pseudomonas* sp. DG17. Quantitative analysis of rhamnolipids was as described by Hua and Wang (2012). As shown in Table 1, a total of seven parameters were selected for investigation, with each variable parameter represented at three levels: the maximum value corresponds to +1, the center point correspond to 0, and the minimum one to −1. The variables were as follows: (1) crude oil concentration, (2) temperature, (3) pH, (4) salinity (%, *w*/*v*), (5) inoculum size (%, *v*/*v*), (6) carbon–nitrogen ratio (weight of crude oil and nitrogen), and (7) carbon–phosphorus ratio (weight of crude oil and phosphorus). Response value was measured in terms of rhamnolipid concentration. In the present study, seven variables were analyzed in 17 experimental trials (Table 2). All the trials were carried out in duplicate. Regression analysis was used to determine the significant variables that have significant effect on rhamnolipids production ($p<0.05$).

### Response surface methodology

The Box–Behnken design was used to determine the optimum condition of variables as follows: concentration (300, 400, and 500 mg $L^{-1}$), salinity (1, 1.5, and 2 %), and C/N (10, 45, 80). A total of 17 experiment trials with the actual and predicted values were conducted as shown in Table 3. The central values of all variables were coded as zero. The minimum and maximum ranges of the variables were used. Three-dimensional surface plots were used to express the fitted polynomial equation. The combination of different optimized variables was determined to verify the model. The statistical software package Design-Expert, Version 8.0.6 was used for the experimental designs and analysis of the central composite design. The information of model fitting was estimated by the analysis of variance (ANOVA) and multiple linear regressions. The quality of fit explained by the model was given by the multiple coefficient of determined $R^2$ value; a good coefficient value accepted for biological sample was $R^2>0.7$ (Torres et al. 2012). The statistical significance of the model equation was determined via Fisher's test ($F$ test) value (Table 4).

## Results

### Biodegradation of *n*-alkanes

Biodegradability of *n*-alkanes by *Pseudomona*s sp. DG17 was as shown in Table 5. The control group showed the abiotic loss of different *n*-alkanes during the biodegradation process. The initial contents of $C_{14}H_{30}$ to $C_{26}H_{54}$ in the medium were 19.36±1.05, 19.67±1.18, 19.69±0.88, 19.81±1.26, 19.66±1.57, and 19.73±0.69 mg, respectively. After incubation for 12 h, the biodegradation ability of $C_{14}H_{30}$ to $C_{26}H_{54}$ by *Pseudomona*s sp. DG17 was 15.18, 1.59, 4.28, 1.83, 0.36, and 0.51 %, respectively. Moreover, during the first 12 h, the biodegradation rates of alkanes were lower than 0.1 mg $h^{-1}$. For $C_{14}H_{30}$ and $C_{16}H_{34}$, biodegradation ability increased to 48.41 and 43.83 % after incubation for 48 h, respectively. Accordingly, the biodegradation rate was achieved at the max values with 0.19 and 0.17 mg $h^{-1}$. Similarly, as for $C_{18}H_{38}$ and $C_{20}H_{42}$, the biodegradation ability increased to 50.22 and 46.50 % at 72 h with the max biodegradation rates of 0.13 and 0.12 mg $h^{-1}$. As for $C_{24}H_{50}$ and $C_{26}H_{54}$, the biodegradation ability increased to 47.71 and 43.63 % after incubation for 120 h, respectively. Accordingly, the biodegradation rate was achieved at the max values with 0.07 and 0.067 mg $h^{-1}$. After incubation for 120 h, the residue contents of $C_{14}H_{30}$ to $C_{26}H_{54}$ in the medium were 3.64±0.57, 4.84±0.38, 6.35±0.85, 6.38±0.73, 9.25±0.75, and 10.35±0.66 mg, respectively. During the biodegradation process, the abiotic loss was lower than 10.32 %.

Moreover, cell growth of *Pseudomonas* sp. DG17 on different alkanes was shown in Fig. 1. The results indicated that biomass content of *Pseudomonas* sp. DG17 grown on $C_{14}H_{30}$ was more than that grown on other kinds of alkanes. After incubation for 6 h, cells grown on $C_{14}H_{30}$ entered into

**Table 1** Experimental variables at different levels used for rhamnolipids production by *Pseudomonas* sp. DG17 using Plackett–Burman design

| Variables | Units | Symbol code | Experimental values | | | *F* value | *p* value |
|---|---|---|---|---|---|---|---|
| | | | Low (−1) | Center (0) | High (+1) | | |
| Concentration | mg $L^{-1}$ | $X_1$ | 100 | 300 | 500 | 13.58 | 0.005 |
| Temperature | °C | $X_2$ | 10 | 20 | 30 | 3.13 | 0.1106 |
| pH | | $X_3$ | 5 | 7 | 9 | 0.26 | 0.6207 |
| Salinity | % | $X_4$ | 0.5 | 1.75 | 3 | 20.74 | 0.0014 |
| Inoculum size | % | $X_5$ | 1 | 3 | 5 | 3.28 | 0.1036 |
| C/N | | $X_6$ | 10 | 105 | 200 | 9.55 | 0.0129 |
| C/P | | $X_7$ | 10 | 105 | 200 | 5.72 | 0.0404 |

**Table 2** Seventeen-trial Plackett–Burman design matrix for seven variables with observed and predicted rhamnolipid concentration

| Std order | Experimental values | | | | | | | Rhamnolipids concentration (mg $L^{-}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $X_6$ | $X_7$ | Observe | Predicted |
| 1 | 500 | 30 | 5 | 3 | 5 | 200 | 10 | 22.82 | 10.28 |
| 2 | 100 | 30 | 9 | 0.5 | 5 | 200 | 200 | 32.47 | 38.22 |
| 3 | 500 | 10 | 9 | 3 | 1 | 200 | 200 | 20.63 | 32.09 |
| 4 | 100 | 30 | 5 | 3 | 5 | 10 | 200 | 20.38 | 23.59 |
| 5 | 100 | 10 | 9 | 0.5 | 5 | 200 | 10 | 13.16 | 7.41 |
| 6 | 100 | 10 | 5 | 3 | 1 | 200 | 200 | 12.48 | 1.02 |
| 7 | 500 | 10 | 5 | 0.5 | 5 | 10 | 200 | 59.66 | 71.48 |
| 8 | 500 | 30 | 5 | 0.5 | 1 | 200 | 10 | 44.86 | 57.4 |
| 9 | 500 | 30 | 9 | 0.5 | 1 | 10 | 200 | 122.57 | 101.78 |
| 10 | 100 | 30 | 9 | 3 | 1 | 10 | 10 | 11.26 | 23.08 |
| 11 | 500 | 10 | 9 | 3 | 5 | 10 | 10 | 26.35 | 23.85 |
| 12 | 100 | 10 | 5 | 0.5 | 1 | 10 | 10 | 43.48 | 39.9 |
| 13 | 300 | 20 | 7 | 1.75 | 3 | 105 | 105 | 33.38 | 31.12 |
| 14 | 300 | 20 | 7 | 1.75 | 3 | 105 | 105 | 29.26 | 31.12 |
| 15 | 300 | 20 | 7 | 1.75 | 3 | 105 | 105 | 28.69 | 31.12 |
| 16 | 300 | 20 | 7 | 1.75 | 3 | 105 | 105 | 32.46 | 31.12 |
| 17 | 300 | 20 | 7 | 1.75 | 3 | 105 | 105 | 31.83 | 31.12 |

exponential stage. The max biomass content $A_{600}$ was achieved at 0.43±0.0071 after incubation for 48 h. Meanwhile, cells grown on $C_{16}H_{34}$, $C_{18}H_{38}$, and $C_{20}H_{42}$ entered into exponential stage after incubation for 24 h. The max biomass of $A_{600}$ values was 0.39±0.013, 0.33±0.0085, and 0.32±0.016, respectively. As for $C_{24}H_{50}$ and $C_{26}H_{54}$, the biomass contents were lower than those of short-carbon-chain-length alkanes during the whole growth process, and cell growth entered into exponential stage at 36 h.

## Pseudosolubilized *n*-alkanes in the water phase

The quantity of pseudosolubilized *n*-alkanes in the water phase was analyzed as shown in Table 6. Under the emulsification effect of DG17, concentrations of pseudosolubilized *n*-alkanes in the water phase increased as time went on during the 120-h incubation time. During the first 12 h, only pseudosolubilized $C_{14}H_{30}$ was detected with the content at 0.14±0.068 mg, which accounted for 0.76 % of total $C_{14}H_{30}$. As for $C_{26}H_{54}$, pseudosolubilized carbon source could be detected after incubation for 48 h. The results indicated that short-chain-length alkanes were pseudosolubilized easier than long-chain-length alkanes. Moreover, the pseudosolubilized rate of $C_{14}H_{30}$ increased to 0.039 mg $h^{-1}$ at 48 h, after which the pseudosolubilized rate declined as time went on. Similarly, the pseudosolubilized rates of $C_{16}H_{34}$, $C_{18}H_{38}$, and $C_{20}H_{42}$ increase to 0.027, 0.024, and 0.024 mg $h^{-1}$ at 72 h, respectively. The pseudosolubilized rates of $C_{24}H_{50}$ and $C_{26}H_{54}$ were achieved at 0.018 and 0.016 mg $h^{-1}$ at 120 h, respectively. Many studies have shown that biosurfactants can emulsify hydrocarbons and decrease surface tension, thus enhancing their water solubility, increasing the displacement of oily substances from soil particles, assisting the transport and translocation of the insoluble substrates across cell membranes, and helping detach the bacteria from the oil droplets after the utilizable hydrocarbon has been depleted (Abalos et al. 2004; Prabhu and Phale 2003). In our study, after incubation for 120 h, the pseudosolubilized $C_{14}H_{30}$, $C_{16}H_{34}$, $C_{18}H_{38}$, $C_{20}H_{42}$, $C_{24}H_{50}$, and $C_{26}H_{54}$ achieved to 1.94±0.53, 2.63±0.21, 1.91±0.17, 2.43±0.18, 2.11±0.22, and 1.87±0.16, respectively. Thus, the "biosurfactant-mediated uptake" was with substrate entrapped in hydrophobic micelles, thereby increasing contaminant aqueous solubility and hence the amount available for microbial uptake.

## Screening of significant variables using Plackett–Burman design

As shown in Tables 1 and 2, 17 runs were carried out and the variables evidencing statistically significant effects were determined through ANOVA. Factors with *p* value less than 0.05 were considered to have significant effects on rhamnolipids production. The lower probability values indicate more significance on the rhamnolipid production. Salinity, with a probability value of 0.0014, was considered to be the most significant factor, followed by concentration (0.0050), C/N (0.0129), and C/P (0.0404). All insignificant variables were neglected, and the optimum levels of the three significant variables (concentration, salinity, and C/N) were further determined by RSM design.

**Table 3** Box–Behnken optimization matrix for the experimental design and predicted responses for rhamnolipids concentration

| Std order | Experimental values | | | Rhamnolipid concentration (mg $L^{-}$) | |
|---|---|---|---|---|---|
| | $X_1$ | $X_4$ | $X_6$ | Observe | Predicted |
| 1 | 300 | 1 | 45 | 112.58 | 106.61 |
| 2 | 500 | 1 | 45 | 186.24 | 183.34 |
| 3 | 300 | 2 | 45 | 72.51 | 75.41 |
| 4 | 500 | 2 | 45 | 84.53 | 90.5 |
| 5 | 300 | 1.5 | 10 | 120.36 | 114.68 |
| 6 | 500 | 1.5 | 10 | 212.61 | 203.86 |
| 7 | 300 | 1.5 | 80 | 40.57 | 49.32 |
| 8 | 500 | 1.5 | 80 | 46.26 | 51.94 |
| 9 | 400 | 1 | 10 | 185.63 | 197.28 |
| 10 | 400 | 2 | 10 | 106.35 | 109.13 |
| 11 | 400 | 1 | 80 | 65.29 | 62.51 |
| 12 | 400 | 2 | 80 | 38.26 | 26.61 |
| 13 | 400 | 1.5 | 45 | 86.32 | 87.92 |
| 14 | 400 | 1.5 | 45 | 88.52 | 87.92 |
| 15 | 400 | 1.5 | 45 | 91.81 | 87.92 |
| 16 | 400 | 1.5 | 45 | 84.38 | 87.92 |
| 17 | 400 | 1.5 | 45 | 88.55 | 87.92 |

## Optimization of significant variables using response surface methodology

Box–Behnken design was construed to determine the optimum levels of the three most significant variables, concentration ($X_1$), salinity ($X_4$), and C/N ($X_6$). The variables were with three appropriate levels. The design matrix, including 17 runs with five replicate center points, and the predicted values are shown in Table 3. The variance between observed and predicted rhamnolipid concentration was shown in Fig. 2. Analysis of diagnostics indicated that the model was considered to be reasonable. $p$ values less than 0.0500 indicate that model variations are significant. As shown in Table 4, the ANOVA analysis indicated that the model terms, $X_1$, $X_4$, $X_6$, $X_1X_4$,$X_4X_6$, $X_1X_6$, and $X_1^2$, were significant ($p<0.05$). Values greater than 0.1000 indicate that the model terms are not significant. The interaction between concentration and C/P was more significant. The model $F$ value was 50.69, and the $F$ value for lack of fit was 25.49. The $p$ values for the model (<0.0001) and value of lack of fit (0.0046) also suggested that the obtained experimental data was a good fit with the model. The regression equation was calculated and the data was fitted to a second-order polynomial equation. In response, rhamnolipid concentration ($Y$) can be by the following equation using the coded factor:

$$Y = 87.92 + 22.95X_1 - 31.01X_4 - 54.32X_6 - 15.41X_1X_4 - 21.64X_1X_6 + 13.06X_4X_6 + 16.06X_1^2 + 9.99X_4^2 + 0.98X_6^2$$

The regression equation obtained from the ANOVA showed that the coefficient $R^2$ was 0.9849, which applied that the model was capable of explaining 98.49 % of the variation in response. The "Predicted $R^2$" of 0.7691 is in reasonable agreement with the "Adjusted $R^2$" of 0.9655, which suggested that the model was good. For a good statistical model, the $R^2$ value should be in the range of 0 to 1.0, and the more the value is nearer to 1.0, the more the model is fit (Hua and Wang 2013). The "adequate precision value" of the model, which measures the signal-to-noise ratio, was 24.491. A ratio greater than 4 is desirable for a model to be a good fit. Thus, this model can be used to navigate the design space.

In the Design-Expert, the optimal levels of each significant variable for maximum rhamnolipid production can be determined by constructing three-dimensional response surface plots on the $z$-axis against any two independent variables, while maintaining other variables at their optimal levels (Reddy et al. 2008). As shown in Table 4, the interactions between concentration and salinity, concentration and C/N, and salinity and C/N were significant ($p<0.05$). As shown in Fig. 3a, a decrease in rhamnolipid concentration was observed when the crude oil concentration was decreased and salinity was increased. A similar profile was observed in Fig. 3b with substrate concentration and C/N. Meanwhile, it was found that rhamnolipid concentration was decreased when salinity and C/N were increased. The design indicated that the optimum levels of each variable were determined to be as follows: salinity 1.5 % ($w/v$), C/N 45, and crude oil concentration 400 mg $L^{-1}$. Under these optimized conditions, the predicted response for rhamnolipid concentration was 87.92 mg $L^{-1}$. Based on the results, to confirm the model adequacy for predicating the maximum rhamnolipid production, the

**Table 4** Results of regression analysis of the Box–Behnken design for optimization of rhamnolipid production

| Factors | Coefficient | df | MS | $F$ value | $p$ value |
|---|---|---|---|---|---|
| Model | 87.92 | 9 | 4214.54 | 47.33 | 0.0002 |
| $X_1$ | 22.95 | 1 | 7693.58 | 86.4 | <0.0001 |
| $X_4$ | −31.01 | 1 | 23606.39 | 265.1 | <0.0001 |
| $X_6$ | −54.32 | 1 | 949.87 | 10.67 | 0.0137 |
| $X_1X_4$ | −15.41 | 1 | 1873.16 | 21.04 | 0.0025 |
| $X_1X_6$ | −21.64 | 1 | 682.52 | 7.66 | 0.0278 |
| $X_4X_6$ | 13.06 | 1 | 1085.76 | 12.19 | 0.0101 |
| $X_1^2$ | 16.06 | 1 | 420.27 | 4.72 | 0.0664 |
| $X_4^2$ | 9.99 | 1 | 4.01 | 0.045 | 0.838 |
| $X_6^2$ | 0.98 | 1 | 4214.54 | 47.33 | 0.0002 |
| Residual | | 7 | 89.05 | | |
| Lack of fit | | 3 | 197.45 | 25.49 | 0.0046 |
| Pure error | | 4 | 7.75 | | |
| Total | | 16 | | | |

**Table 5** Biodegradation of *n*-alkanes by *Pseudomonas* sp. DG17

| Substrate | 12 h | | 24 h | | 48 h | | 72 h | | 96 h | | 120 h | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RE (mg) | C (mg) | RE (mg) | C (mg) | RE (mg) | C (mg) | RE (mg) | C (mg) | RE (mg) | C (mg) | RE (mg) | C (mg) |
| $C_{14}H_{30}$ | 18.32±1.38 | 19.24±1.43 | 14.86±1.91 | 18.72±0.61 | 9.56±0.86 | 18.53±1.52 | 5.38±0.48 | 18.16±0.54 | 4.48±0.34 | 17.44±1.46 | 3.64±0.57 | 17.59±0.52 |
| $C_{16}H_{34}$ | 19.22±0.90 | 19.53±0.68 | 16.95±0.80 | 19.22±1.44 | 10.38±1.18 | 18.48±0.93 | 8.15±0.85 | 18.69±0.68 | 6.36±0.51 | 18.21±0.52 | 4.84±0.38 | 17.64±0.77 |
| $C_{18}H_{38}$ | 18.78±1.25 | 19.62±0.52 | 17.66±1.16 | 19.43±0.64 | 12.52±1.08 | 18.88±0.88 | 9.25±0.92 | 18.58±0.45 | 8.18±1.15 | 18.52±0.54 | 6.35±0.85 | 18.13±0.62 |
| $C_{20}H_{42}$ | 19.36±0.73 | 19.72±0.82 | 18.24±0.58 | 19.36±0.84 | 14.16±0.68 | 18.73±0.51 | 9.87±0.56 | 18.45±0.76 | 7.62±0.88 | 18.37±0.89 | 6.38±0.73 | 18.04±0.61 |
| $C_{24}H_{50}$ | 19.51±1.05 | 19.58±0.83 | 18.43±0.68 | 18.92±0.61 | 17.48±0.74 | 18.36±0.52 | 14.52±0.96 | 17.98±0.54 | 11.67±1.21 | 17.64±0.86 | 9.25±0.75 | 17.69±0.83 |
| $C_{26}H_{54}$ | 19.56±0.62 | 19.66±0.51 | 18.44±0.47 | 19.41±0.44 | 17.86±0.58 | 19.12±0.62 | 15.58±0.65 | 18.84±0.48 | 12.36±0.64 | 18.73±0.75 | 10.35±0.66 | 18.36±0.61 |

*RE* residue alkanes, *C* control group

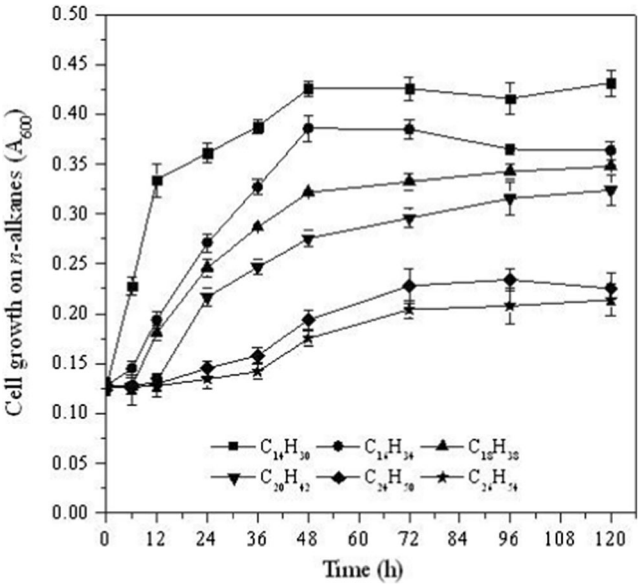


**Fig. 1** Cell growth of *Pseudomonas* sp. DG17 on *n*-alkanes

additional experiment was conducted in triplicate. The results showed an average rhamnolipid concentration of 91.24 mg·$L^{-1}$, which was quite close to the predicted value (87.92 mg $L^{-1}$). These results confirmed the validity of the mode.

## Discussion

In our studies, low-molecular-weight alkanes were more easily used by *Pseudomonas* sp. DG17. Similarly, strain *Acinetobacter venetianus* 6A2 was capable of utilizing *n*-alkanes with chain lengths ranging from decane ($C_{10}H_{22}$) to tetracontane ($C_{40}H_{82}$) as a sole carbon source, and cell growth was measured as an increase in protein concentration in cultures over time (Throne-Holst et al. 2006). Biodegradation of different *n*-alkanes was done sequentially in order of increasing molecular weight, each at about the same rate as during growth on it as single alkane (Lindley and Heydeman 1986). Microorganisms have established effective strategies involving specialized enzyme systems and metabolic pathways to access long-chain *n*-alkanes as a carbon and energy source (Alexander et al. 2007). Biosurfactants produced by *Pantoea* sp. A-13 when grown on kerosene or n-paraffins increased cell hydrophobicity and enhanced both the surface-tension-lowering capacity and the emulsifying potential. This suggested the occurrence of both modes of biosurfactant-enhanced growth on tested hydrocarbons: (1) direct contact with large alkane droplets and (2) alkane transfer mechanism, which involves solubilization and emulsification of hydrocarbons at higher concentrations of biosurfactants in the culture medium (Vasileva-Tonkova and Gesheva 2007). In our

**Table 6** Pseudosolubilized *n*-alkanes by *Pseudomonas* sp. DG17

| Substrate | 12 h | | 24 h | | 48 h | | 72 h | | 96 h | | 120 h | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PS (mg) | % | PS (mg) | % | PS (mg) | % | PS (mg) | % | PS (mg) | % | PS (mg) | % |
| $C_{14}H_{30}$ | 0.14±0.068 | 0.86 | 0.92±0.065 | 6.19 | 1.86±0.081 | 19.46 | 2.15±0.14 | 39.96 | 2.31±0.17 | 51.56 | 1.94±0.53 | 53.30 |
| $C_{16}H_{34}$ | 0 | 0.00 | 0.62±0.015 | 3.72 | 1.15±0.084 | 11.08 | 1.93±0.17 | 23.68 | 2.47±0.16 | 38.84 | 2.63±0.21 | 54.34 |
| $C_{18}H_{38}$ | 0 | 0.00 | 0.39±0.016 | 2.44 | 0.82±0.064 | 6.55 | 1.71±0.13 | 17.54 | 2.27±0.15 | 27.75 | 1.91±0.17 | 30.08 |
| $C_{20}H_{42}$ | 0 | 0.00 | 0.34±0.024 | 1.86 | 0.65±0.11 | 4.59 | 1.76±0.12 | 17.83 | 1.58±0.16 | 20.73 | 2.43±0.18 | 38.09 |
| $C_{24}H_{50}$ | 0 | 0.00 | 0.32±0.031 | 1.74 | 0.64±0.054 | 3.66 | 1.23±0.14 | 8.47 | 1.56±0.13 | 13.37 | 2.11±0.22 | 22.81 |
| $C_{26}H_{54}$ | 0 | 0.00 | 0 | 0.00 | 0.35±0.024 | 1.96 | 0.58±0.087 | 3.77 | 1.36±0.098 | 11.00 | 1.87±0.16 | 18.42 |

*PS* pseudosolubilized, *%* the percentage of pseudosolubilized *n*-alkanes account for total alkanes in the medium

studies, pseudosolubilized *n*-alkanes were detected in the water phase during the biodegradation process by *Pseudomonas* sp. DG17. Thus, it was inferred that biosurfactant production was an important process that influences alkane biodegradation. Similarly, studies have shown that the solubility of phenanthrene was proportional to the concentration of the single surfactants when above the critical micellar concentration (CMC). The solubilization capacity for phenanthrene was in the order TW80 > Brij35 > TX100 > SDS. Solubilization and lowering of the surface and interfacial tension are thought to be main reasons for facilitating the transport of pollutants adsorbed on solid phase to the surfactant-containing aqueous phases (Zhao et al. 2005). As for a *Pseudomonas putida* strain, the addition of surfactant was found to enhance the bioavailability of naphthalene, phenanthrene, and pyrene with efficiencies ranging from 21.1 to 60.6 %, 33.3 to 62.8 %, to 26.8 to 70.9 %, respectively (Doong and Lei 2003). Similarly, the addition of biosurfactant produced by *Pseudoxanthomonas* sp. PNK-04 facilitates the degradation of 2-chlorobenzoic acid, 3-chlorobenzoic acid, and 1-methyl naphthalene by *Pseudoxanthomonas* sp. PNK-04. This strain is able to degrade 2-chlorobenzoic acid (75 %) and 1-methyl naphthalene (60 %) efficiently in the presence of biosurfactants (Nayak et al. 2009).

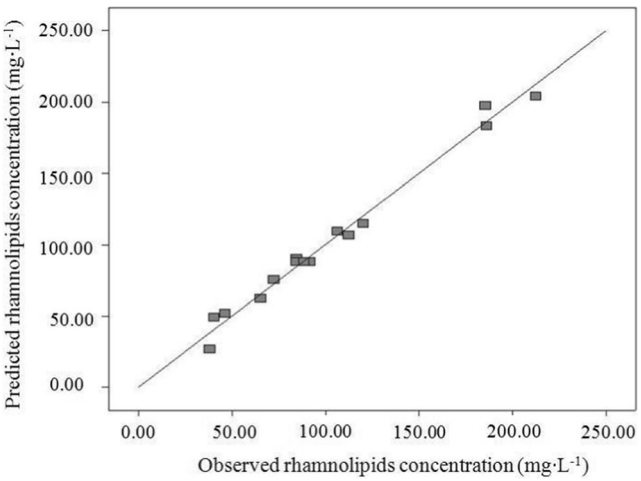


**Fig. 2** Variance of observed rhamnolipid concentration and regression analysis of the Box–Behnken optimization experiments

Bioavailability and biodegradation kinetics of the hydrophobic pollutants are affected variably by the surfactants. Both stimulating and inhibiting effects of surfactants on bioremediation of pollutants are known depending on the chemical characteristics of the surfactant, pollutant, and physiology of the microorganism (Banat et al. 2000; Van Hamme et al. 2003). In some cases, an increase in degradation rate was observed, whereas in other cases, a decrease in degradation rates was noted after addition of surfactants. Added rhamnolipids above critical micellar concentration (CMC) enhanced the apparent aqueous solubility of hexadecane, enhanced biodegradation of hexadecane, octadecane, n-paraffins, creosotes, and other hydrocarbon mixtures in soil, and promoted bioremediation of petroleum sludges (Maier and Soberon-Chavez 2000; Noordman et al. 2002; Rahman et al. 2002). However, Witconol SN70 (anonionic, alcohol ethoxylate), at a concentration below its CMC, did not affect mineralization rates of hexadecane or phenanthrene, whereas above the CMC, it inhibited mineralization of both hydrocarbons (Colores et al. 2000). Surfactant concentrations, greater than or equal to the CMC for all four surfactants tested, inhibited mineralization of phenanthrene by *Pseudomonas aeruginosa* in soil–water cultures, and lower surfactant concentrations had no effect (Bramwell and Laha 2000). Thus, it was inferred that above the CMC of biosurfactant produced by DG 17, the formation of micelles occurs, and hydrocarbons can partition into the hydrophobic micellar core, increasing their apparent aqueous solubility.

Optimizing factors that affect growth in biosurfactant producing organisms with potential for commercial exploitation are of paramount importance (Ilori et al. 2005). For example, as for *Streptomyces* sp. MAB36, the Plackett–Burman design found four key ingredients and conditions for the best yield of glycolipid biosurfactant production and central composite design showed that the optimized concentrations of the four significant variables were starch (1.582 g), casein (0.7819 g), crude oil (1.6002 mL), and incubation time (3.2914 days)

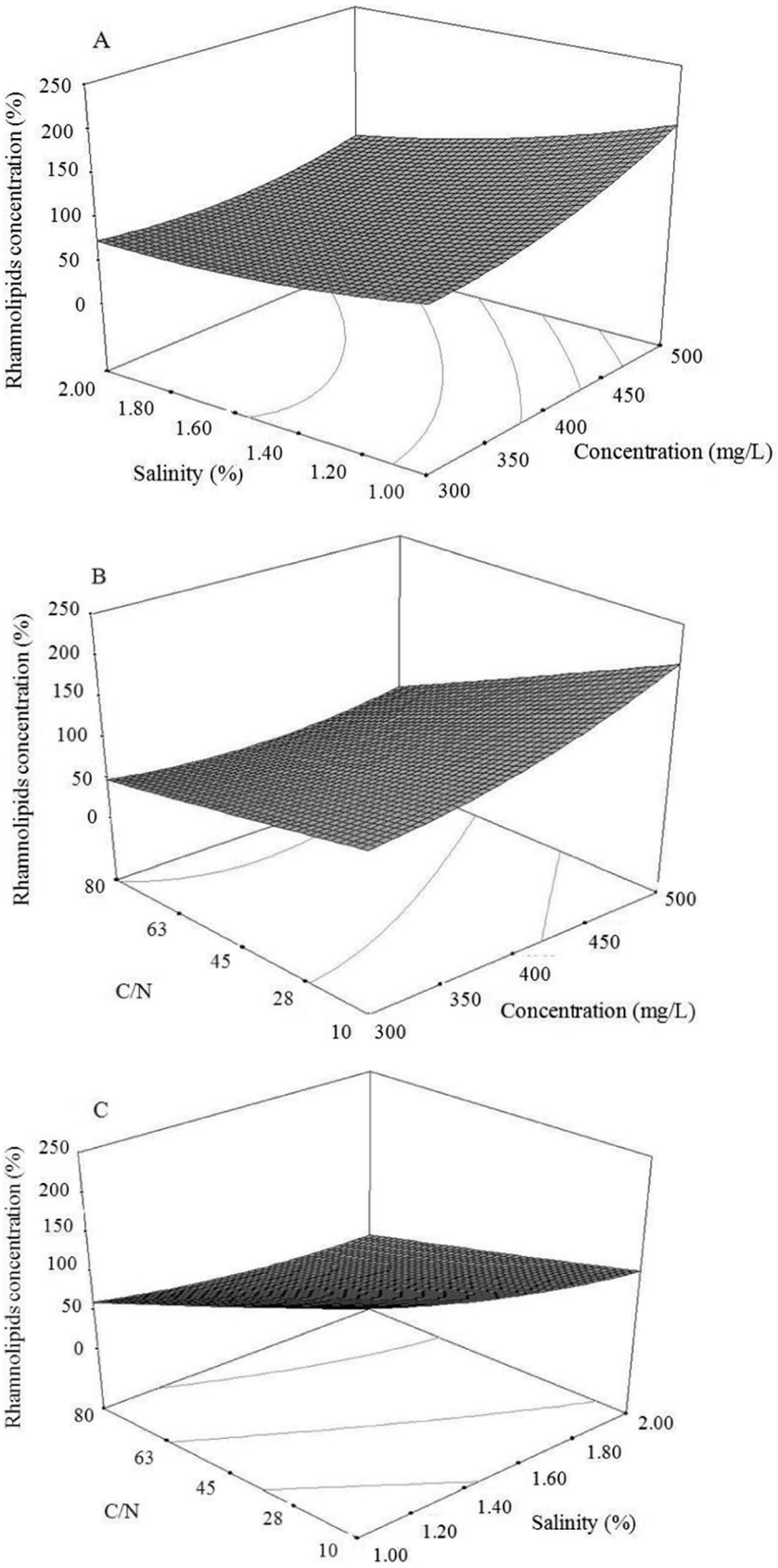


**Fig. 3** Three-dimensional response surface plots for crude oil biodegradation optimization showing the interactive effects of **a** concentration and salinity, **b** concentration and C/N, and **c** salinity and C/N. Hold value: C/N of 45 (**a**), salinity of 1.50 (**b**), and concentration of 400 (**c**)

(Panchanathan et al. 2014). Meanwhile, as for fungi *Aspergillus ustus* MSF3, the optimized biosurfactant production conditions was pH 7.0, temperature 20 °C, salt concentration 3 %, and glucose and yeast extract as carbon source and nitrogen sources, respectively. Meanwhile, the RSM experiment suggested that the peak yield of biosurfactant appeared at the carbon–nitrogen ratio of 3:2. The carbon–nitrogen ratio is one of the most important factors which induce the secondary metabolite production (Seghal Kiran et al. 2009). Moreover, as for a *Pseudomonas fluoresce* strain, effect of pH and salinity on the naphthalene solubility suggested that naphthalene solubility was about 7-fold its aqueous solubility for 0.5–1.5 g $L^{-1}$ of biosurfactant, pH 7, and no salinity. The solubility reached a saturation value (205 mg $L^{-1}$), when biosurfactant concentration exceeded 1.5 g $L^{-1}$. The structure of the biosurfactant molecule, which has not been identified yet, and its interaction with physicochemical factors such as pH and salinity seem to affect micelle formation and shape and therefore affect the solubility of hydrocarbons (Abouseoud et al. 2010). In order to optimize low-cost fermentation conditions for biosurfactant production by *Pseudomonas* sp. DG17, we conducted Plackett–Burman design and RSM experiments using the screened variables. The optimum levels of each variable were as follows: 400 mg $L^{-1}$ of crude oil concentration, 1.5 % of salinity, and C/N of 45. The introduction of biosurfactant for use in various industries applications is not easy because its production is a complex process that is influenced by several factors, being both extremely exclusive and time-consuming (Lotfabad et al. 2008). Taken together, our results suggested the optimal conditions for biosurfactant production by *Pseudomonas* sp. DG17, results of which might be useful in the bioremediation process.

## Conclusions

In our study, pseudosolubilized content and biodegradation rate of low-molecular-weight alkanes were higher than those of high-molecular-weight alkanes. Carbon-14 assay showed that biodegradability of $^{14}C$ *n*-octadecane decreased to 68.87, 59.61, and 6.93 %, respectively, with excessed non-labeled *n*-eicosane, *n*- hexadecane, and *n*-tetradecane, which indicated that *Pseudomonas* sp. DG17 utilized different *n*-alkanes simultaneously. Three variables, including concentration, salinity, and C/N, were screened by Plackett–Burman design and exerted significant effects on rhamnolipid concentration. Box–Behnken design suggested the optimum levels of each variable as follows: 400 mg $L^{-1}$ of substrate concentration, 1.5 % of salinity, and 45 of C/N.

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**Conflict of interest** All author consent is given for publication in Environmental Science and Pollution Research, if accepted.

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