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RESEARCH ARTICLE

# Identification and optimization of biosurfactant producing bacteria isolated from rag layer crude oil emulsion

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**Abstract**

Biosurfactants are surface active compounds that synthesized as secondary metabolite by wide range of bacteria and have characteristic in lowering surface and interfacial tension. This study aimed to isolate and identify biosurfactant producing bacteria from rag layer crude oil emulsion. Rag layer is considered as undesirable material as it is difficult to be separated because of the stable interaction between different components. Mineral salt medium supplemented with glucose and crude oil was used to screen the ability of isolates to produce biosurfactant. Five bacterial strains that successfully isolated from rag layer crude oil emulsion sample were screened for hydrocarbon degradation and biosurfactant production. Two isolates shown positive results in drop collapse test, surface tension measurement and emulsification index, namely P3b and P4. 16S rRNA analysis revealed P3b and P4 to be closely related to *Enterobacter xiangfangensis* while P4 was *Shewanella chilikensis*, respectively. Only isolate P3b was selected for further study. *Enterobacter xiangfangensis* SSP3b16 was found to grow optimally at 37°C, pH 7.0 and 10mM glucose. The highest reduction of surface tension was recorded when culture medium supplemented with 7 % (v/v) glucose and 2 g/L ammonium nitrate. From this study, the biosurfactant production by *Enterobacter xiangfangensis* SSP3b16 can potentially be exploited to enhance oil recovery as well as in solving the rag layer problem in oil industries.

***Keywords*:** Biosurfactant, crude oil, rag layer, *Enterobacter* sp., *Shewanella* sp.



## INTRODUCTION

Surfactants are amphiphilic molecules comprising of hydrophobic and hydrophilic moieties that can accumulate at the interface between fluid phases with different polarities such as oil/water or air/water interfaces (Souza *et al*., 2014; Moya *et al*., 2015, Varjani *et al*., 2016). Biosurfactants are surface-active compounds that excreted by a wide range of microorganisms. Microorganisms can be found in various sources such as water and land. Interestingly, they also can be found in extreme environment such as oil reservoirs and can persist at wide range of temperatures, pH values and salinity (Chirwa and Bezza, 2015). Microorganisms such as *Pseudomonas aeruginosa* are well known as biosurfactant producers (Zhang *et al*., 2012). Recently, *Achromobacter* sp., *Bacillus* sp., *Citrobacter* sp., *Lysinibacillus* sp., *Ochrobactrum* sp. and *Pseudomonas* sp. are found to yield different types of biosurfactants and exhibited the potential as hydrocarbon degraders (Joy *et al*., 2017).

Unconventional petroleum refers to crude hydrocarbons that are extracted using techniques other than the conventional (oil well) method. However, the formation of a multiphase complex layer known as oily sludge, petroleum emulsion or rag layer, is a typical problem that arises during oil recovery, transportation and treatment of unconventional crude oils (He *et al*., 2015; Khatri *et al*., 2011). As the rag layer consists of a large amount of impurities such as salts, clays, asphaltenes, resins, heavy metals and naphthenic acids, a high interfacially active properties is exhibited, leading to the formation of a thick layer of highly stable multiphase emulsions at the oil and water interface (Kralova *et al*., 2011; Langevin and Argillier, 2016; Sánchez-Lemus *et al*., 2016). Inappropriate handling and mismanagement of rag layer can be detrimental to public health and surroundings due to its high toxicity and high production quantity (Hu *et al*., 2013). Accidential spillage or improper disposal of oily sludge waste can threaten the ecosystem and living organisms because the toxic organic materials can contaminate the food chain (Reddy *et al*., 2011; Wang *et al*., 2015).

This rag layer is difficult to resolve even after several demulsification phases. Demulsification is a process to break the emulsion by disrupting the stable structure between two immiscible liquids that are suspended together. This emulsion is said to be kinetically stable but thermodynamically unstable. Water-in-oil emulsion will increase the viscosity of a liquid and thus, increase the pumping costs, causing the corrosion of equipment and ultimately the equipment failure (Kilpatrick, 2012).

Even though the production of biosurfactants has been widely studied in bacteria, continuous research is still highlighted on finding the most efficient and effective biosurfactants. Recent studies have reported on the ability of bacteria to produce biosurfactants using various types of carbon sources such as lactose, sucrose and crude oil (Hu *et al*., 2015; Antoniou *et al*., 2015; Parthipan *et al*., 2017; Patowary *et al*., 2017). However, to our knowledge, information regarding the isolation of biosurfactant producing bacteria specifically from crude oil rag layer emulsion is lacking. The present study was

thus conducted to isolate and characterize such microorganism from crude oil rag layer emulsion that capable of utilizing crude oil to produce biosurfactants. The isolated strains could be used as a source of effective biosurfactants with potential application specifically for treatment of rag layer emulsion in oil industries.

## EXPERIMENTAL

### Materials and methods

### Bacterial isolation and growth

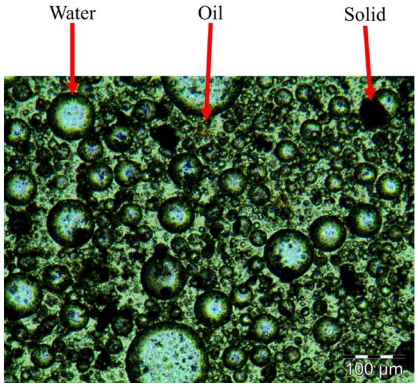


**Fig. 1** Formation of black colored rag layer in demulsified sample at 35°C.

Crude oil rag layer emulsion sample (Fig. 1) was kindly provided by PETRONAS Carigali Sdn Bhd. The properties of the emulsion were given by (Japper-Jaafar *et al*., 2014). Rag layer sample was consisted of water-in-oil (W/O) emulsion collected from slop tank oil. This sample also has no asphaltenese, low paraffin content and low viscosity (8.6 mPa.s).

### Preparation of enrichment cultures

Enrichment cultures were prepared by adding 5 mL of rag layer sample in 250 mL conical flask containing 45 mL LB broth. The culture was incubated at 37 °C, 150 rpm for 24 hours. Then, the culture was streaked aseptically onto LB agar followed by incubation at 37 °C for 24 hours. The mixed colonies formed were sub-cultured several times until few distinct single colonies were appeared. Five single colonies were selected based on their distinctive morphology. Glycerol stock 50 % (v/v) was prepared to keep each isolate at -80 °C for future use.

### Biochemical characterization of bacteria

Isolated bacteria were subjected to biochemical test such as oxidase test, catalase test, lactose fermentation test and glucose oxidative-fermentative test (Thavasi *et al*., 2011). Gram's staining and spore staining were also conducted according to standard staining protocol (Jain *et al*., 1991; Bobour and Miller-Maier, 1998). Biochemical tests were conducted to predict the genus of isolated bacteria according to Bergey's Manual of Systematic Bacteriology. Species level of identification was done via 16S rRNA sequence analysis.

### Screening for biosurfactant production

Screening for biosurfactant production was conducted using mineral salt medium (MSM) instead of LB broth. Bacteria were grown in 250 mL conical flask with 25 mL of MSM containing (g $l^{-1}$) 1.0 g of $K_2HPO_4$, 0.2 g of $MgSO_4.7H_2O$, 0.05 g of $FeSO_4.7H_2O$, 0.1 g of $CaCl_2.2H_2O$, 0.001 g of $Na_2MoO_4.2H_2O$, 30 g of NaCl (Morikawa *et al*., 2000) and supplemented with 1 % (v/v) filter-sterilized crude oil. The culture medium was incubated at 37 °C with 150 rpm agitation for 72 hours. Next, the culture medium was centrifuged at 8000 rpm at 4 °C for 15 minutes. The supernatant was collected for screening via surface tension measurement, oil spreading test, drop-collapse test and emulsification index. Positive control and negative control used in this experiment were 1 % (w/v) sodium dodecyl sulphate (SDS) and distilled water, respectively.

### Oil spreading test

The presence of biosurfactant in cell free culture could be screened using oil spreading test. About 20 mL of distilled water was poured into a petri dish and followed by adding 20 µL of crude oil to create a thin layer of oil. Then, a 10 µL of culture supernatant (after centrifugation at 8,000 rpm, 4 °C, 15 minutes) was dropped carefully onto the crude oil layer. Distilled water and 1 % (w/v) sodium dodecyl sulphate (SDS) were used as negative control and positive control, respectively. The diameter of clear zone (displacement of oil) was measured (Kigsley and Turgay, 2004).

### Drop collapse test

The method for drop collapse test was adapted from previous report by Jain *et al*. (1991). A small drop of culture supernatant was carefully placed on surface of micropipette well covered thinly with crude oil. The drop condition and stability were observed whether it remained stable or collapsed. The result was taken as positive if the drop became flat after 60 seconds. Distilled water and 1 % (w/v) sodium dodecyl sulphate (SDS) were used as negative control and positive control, respectively.

### Emulsification index

Emulsification assay was performed as described by Panjiar *et al*. (2015). 6 mL of culture supernatant was added into 15 mL Falcon tube and followed by 6 mL of filter sterilized crude oil. The suspension was vortexed vigorously for 2 minutes and allowed to stand for 24 hours. The total height of the suspension and the height of the emulsified layer were measured. The emulsification index was determined using the following formula:

$$(\text{Height of emulsified layer}/\text{Total height of suspension}) \times 100 \quad (1)$$

### Surface tension measurement

Surface tension of culture supernatant was measured using Easydyne Tensiometer (Krüss GmbH, Hamburg, Germany) using Du Noüy ring method (Youssef *et al*., 2004). Briefly, 40 mL of culture supernatant was transferred into a clean glass plate and placed on the tensiometer platform. A platinum ring was slowly immersed into the sample and the surface tension was measured automatically. The platinum ring was disinfected by rinsing with distilled water and acetone, followed by heating on flame until red hot. All measurements were done at room temperature and distilled water was used for calibration before sample measurement.

### Bacterial identification

The genomic DNA of isolates were extracted using Promega DNA Extraction Kit. Gel electrophoresis was run to validate the presence of genomic DNA. Approximately 1.5 kb long DNA fragment was amplified using universal primers [Forward primer: fD1_0815 (5' –AGA GTT TGA TCC TGG CTC AG– 3') and Reverse primer: rD1_0815 (5' – AAG GAG GTG ATC CAG CC– 3')]. Thermal cycling for amplification was conducted by one cycle of initial denaturation at 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 1 minutes, 55 °C for 1 minute, 72 °C for 2 minutes and one cycle of final extension at 72 °C for 5 minutes. The amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, Alameda, CA) and sequenced at First BASE Laboratories Sdn Bhd (Selangor, Malaysia). The DNA sequences of respective isolates were analyzed using DNA Base Assembler to obtain full length sequence of isolates before BLAST using EzBioCloud database (https://www.ezbiocloud.net/). The phylogenic relation of the isolates was determined by matching the sequencing results with the GenBank database of the EzBioCloud and phylogenetic trees were constructed using MEGA7 software with bootstrap value of 1000 replicates.

### Optimization of bacterial growth and biosurfactant production

Each isolate was aseptically tranferred into 25 mL LB broth and incubated at 37 °C with 150 rpm agitation overnight. The optical density (OD) reading at 600nm was recorded using spectrophotometer (Jenway, UK) until OD range of 0.8 – 1.0 was achieved. Then, 10 % (v/v) inoculum was added into 250 mL conical flask containing 25 mL of LB broth and OD measurement was carried out to plot reliable growth curves. The optimization of bacterial growth was subjected to three parameters; pH, temperature and initial glucose concentration. All experiments were done in triplicates.

For optimization of biosurfactant production, 10 % (v/v) of standardized inoculum was transferred into 250 mL conical flask containing 50 mL of Ramsay medium [(per liter): 2.0 g $NH_4NO_3$, 0.5 g $KH_2PO_4$, 1.0 g $K_2HPO_4$, 0.5 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.2H_2O$, 0.1 g KCl, and 0.06 g yeast extract)], 2 % (v/v) crude oil and 0.5 mL 1M glucose and incubated in shaking incubator at 150 rpm for 24 hours. The negative control used was Ramsay medium without bacterial culture.

After incubation for 24 hours, the culture was centrifuged at 8000 rpm at 4 °C for 15 minutes. The supernatant was used for surface tension measurement using automatic tensiometer. The results of surface tension measurement were shown in the form of percentage according to the following equation (Pornsunthorntawee *et al*., 2008):

$$\text{Percentage of surface tension reduction} = \frac{(\gamma m - \gamma c)}{\gamma m} \times 100 \qquad (2)$$

$\gamma$m = surface tension of the negative control
$\gamma$c = surface tension of the supernatant.

The results were analyzed using the GraphPad Prism software (version 7). The significant differences among the data were determined using one-way ANOVA and the Tukey. The result was statistically significant if $p$ value was less than 0.05.

### Effect of pH on bacterial growth

A 10 % (v/v) inoculum was inoculated into 50 mL Ramsay medium. Cultures were incubated at 37 °C and 150 rpm. The initial pH of growth medium was set at 5.0, 6.0, 6.5, 7.0, 7.5 and 8.0 (each in triplicate). Samples were taken out at regular intervals to analyze for growth, spectrophotometrically at 600nm absorbance.

### Effect of temperature on bacterial growth

A 10 % (v/v) inoculum was inoculated into 50 mL Ramsay medium at pH 7. The same experimental set up was used in this section except that the incubation temperature was varied. The temperature was set at 25 °C, 30 °C, 37 °C, 40 °C and 50 °C. Samples were taken out at regular intervals to analyze for growth, spectrophotometrically at 600 nm absorbance.

### Effect of initial glucose concentration on bacterial growth

A 10 % (v/v) inoculum was inoculated into 50 mL Ramsay medium at pH 7. The medium was supplemented with 0, 1, 3, 5, 8, 10 mM glucose (each was in triplicate). Samples were taken out at regular intervals to analyze for growth, spectrophotometrically at 600 nm absorbance.

### Incubation period for biosurfactant production

Production of biosurfactant was determined under the optimized growth medium by varying the incubation period. The samples were taken out after 1, 3, 5 and 7 days for surface tension measurement.

### Optimization of biosurfactant production

### Types and concentration of carbon source

Bacterial culture was inoculated in Ramsay medium at optimized pH and temperature with addition of different carbon sources (5 mM concentration) comprising glucose, lactose, sucrose and fructose, then incubated at 150 rpm for 24 hours. The carbon source that gave the lowest surface tension was further selected for variation in different concentrations starting from 1, 3, 5, 7 and 10 % (v/v).

### Types and concentration of nitrogen source

The original amount of nitrogen in Ramsay medium ($NH_4NO_3 \approx 2$ g/L) was replaced with different types of nitrogen. Bacterial culture was inoculated in Ramsay medium at optimized temperature and pH with addition of ammonium sulfate ($(NH_4)_2SO_4$), ammonium chloride ($NH_4Cl$), ammonium acetate ($CH_3COONH_4$), ammonium nitrate ($NH_4NO_3$) and peptone. The medium was incubated at 150 rpm for 24 hours. The nitrogen source that produced the lowest surface tension activity was further selected for different concentrations study, ranging from 0.5, 1.5, 2.0, 2.5, 3.5 and 5.0 (g/L).

## RESULTS AND DISCUSSION

Five distinctive strains were successfully isolated using streak-plate method. These bacteria were named as P1, P2, P3a, P3b and P4. Each isolate was grown at 37 °C for 24 hours in LB broth at pH 7.0 and followed by streaking on LB agar to observe the colony morphology. Table 1 summarizes the morphological characteristics of isolates. The Gram staining results showed that isolate P1, P2 and P3a were Gram negative while isolate P3b and P4 were Gram positive.

**Table 1** Colony morphology of isolated bacteria.

| Isolate | Colony morphology | Gram staining |
|---|---|---|
| P1 | Pin-point, smooth, convex, whitish, dry | +ve, branching filaments |
| P2 | Circular, smooth, raised, yellowish, moist | +ve, coccus |
| P3a | Irregular, undulate, dull, cream, butyrous | +ve, rod |
| P3b | Irregular, entire, crateriform, light whitish, moist | -ve, rod |
| P4 | Irregular, smooth, raised, light pink, moist | -ve, irregular rod |

### Screening for biosurfactant production

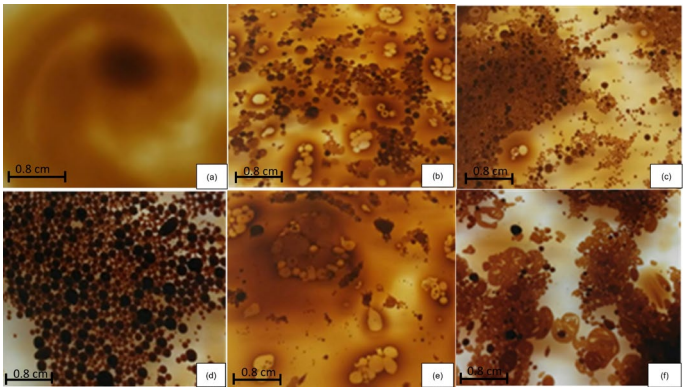


**Fig. 2** The crude oil degradation activity as showed by control (a), isolate P1 (b), isolate P2 (c), isolate P3a (d), isolate P3b (e) and isolate P4 (f).

Fig. 1 above shows the physical appearance of crude oil layer on the surface of MSM broth. Crude oil was supplemented as sole carbon source into the media. Biosurfactant producing bacteria utilize the crude oil as their carbon source and thus, producing biosurfactant to assist the uptake of hydrocarbon into their cells. Among the five isolates, only three isolates; P1 (b), P3b (e) and P4 (f) were expected to be positive for biosurfactant production as the crude oil layer was appeared to be degraded with the formation of small oil droplets. This indicated that the isolates can degrade crude oil by producing biosurfactant along with degradative enzymes. The mechanism of degradation is caused by the presence of surface-active compounds

produced by the bacteria which dissolve in oil and water due to their physical structure that consisted of hydrophobic and hydrophilic parts. This structure allows biosurfactant to accumulate at the oil-water interface which then can lowers the interfacial tension between both liquids (Soberón-Chávez and Maier, 2011). The results for biochemical tests are summarized in Table 2 below.

**Table 2** Biochemical tests of selected isolates.

| Isolate | Catalase test | Oxidase test | Glucose OF test | Lactose Fermentation test | Spore staining |
|---|---|---|---|---|---|
| P1 | +ve | +ve | -ve | Neutral | +ve |
| P3b | +ve | -ve | +ve | +ve | -ve |
| P4 | +ve | -ve | +ve | Neutral | -ve |

From the biochemical tests and by referring to Bergey's simplified identification scheme, isolate P1 was predicted as *Actinomyces* sp., isolate P3b as *Staphylococcus* sp. and isolate P4 as *Pseudomonas* sp. However, 16S rRNA gene sequence analysis was done to confirm the correct identity of isolated bacteria.

Table 3 shows the results of emulsification assay for isolates P1, P3b and P4. Isolate P4 exhibited the highest emulsification index followed by isolate P3b. The formation of stable emulsion after 24 hours indicated the existence of biosurfactant in the supernatant as the $E_{24}$ corresponded to the concentration of biosurfactant (Walter *et al*., 2000-2013). Therefore, isolates P3b and P4 were further selected for surface tension measurement. All isolates showed negative result on oil spreading test as compared to control. Isolates in this study may produce low concentration of biosurfactant and oil spreading test is more reliable for samples that contained high concentration of biosurfactant. Among the five isolates, culture broth of two isolates exhibited positive results in the drop collapse test and emulsification test, thereby indicating the existence of biosurfactant in the supernatant. The culture broth droplet was collapsed immediately or within 60 seconds after dropped onto thin crude oil layer.

**Table 3** Emulsification assay result for isolate P1, P3b and P4.

| Isolate | Height of emulsion (cm) | Height of solution (cm) | Emulsion index ($E_{24}$) |
|---|---|---|---|
| P1 | 0 | 8 | 0 |
| P3b | 0.7 | 8 | 8.75 |
| P4 | 1 | 8 | 12.5 |

Both isolates P4 and P3b that exhibited positive drop collapse test were able to decrease the surface tension values of 44.6 mN/m and 45.2 mN/m, respectively from the standard value of 72 mN/m. The lower the value of surface tension, the higher concentration of biosurfactant will be presented. Bacterium with the ability to reduce surface tension in the growth medium by more than 20 mN/m is considered as a good biosurfactant producer (Willumsen and Karlson, 1996). Joshi et al. (2008) stated that isolates with the capability of decreasing the surface tension of the medium to ≤35 mN/m can be counted as good biosurfactant producing bacteria. Therefore, it can be concluded that isolates P3b and P4 are good biosurfactant producers.

Conserved region of 16S rRNA of isolate P3b was compared against the Genbank database and the result showed that the bacterial strain has a high gene sequence resemblance with known species of *Enterobacter xiangfangensis* (100 %). Isolate P4 was closely related to *Shewanella chilikensis* with 100 % similarity. Neighbor-joining method was used to construct phylogenetic tree of isolated bacteria. Gene sequences for both strains were submitted to NCBI database and the accession numbers provided were KY828217 and KY885186, respectively. Both isolates were name as *Enterobacter xiangfangensis* SSP3b16 and *Shewanella chilikensis* SSP416.

Previous work by Sarafzadeh *et al.* (2013) showed that *Enterobacter* sp. was capable of producing surface-active compounds. They found that *Enterobacter cloacae* could efficiently reduce interfacial tension to 2.95 mN/m from the original value 32 mN/m. The reduction of interfacial tension was higher than *Enterobacter* sp. isolated in this study. In addition, You *et al.* (2015) stated that *Enterobacter* sp. was able to utilize hydrocarbons consequently by producing biosurfactant as secondary metabolites to assist the hydrocarbon uptake into cells.

*Shewanella* sp. is also known as one of the genera that capable of producing biosurfactants. Previous work by Belcher *et al*. (2012) reported that *Shewanella* sp. was able to reduce surface tension to 54 mN/m, which was less effective as compared to *Shewanella* sp. isolated in this study. Another recent study by Chikere *et al*. (2017) also revealed the ability of *Shewanella* sp. in degrading crude oil.

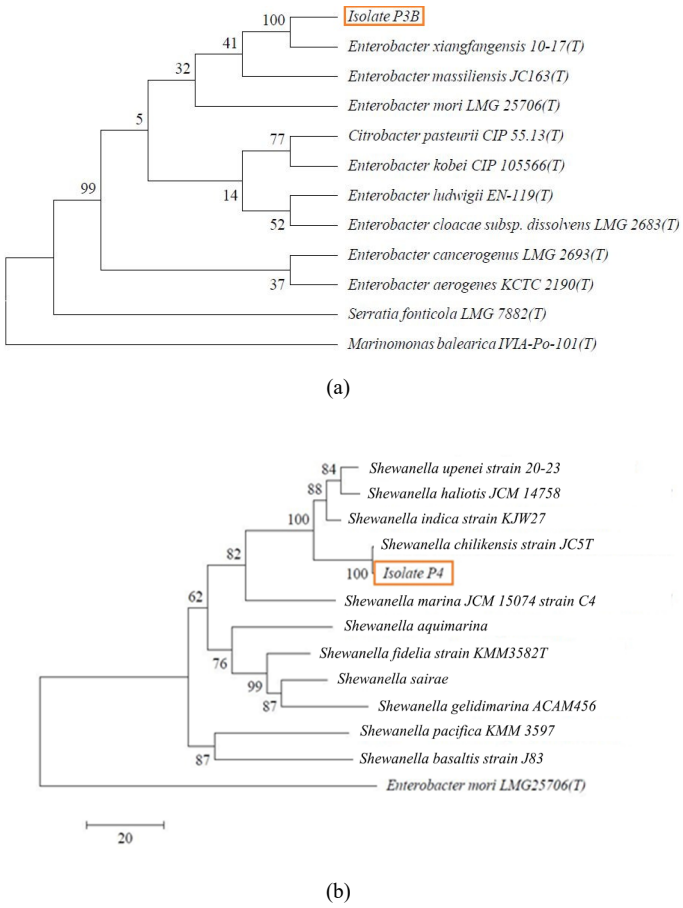


**Fig. 3** Phylogenetic tree showing the position of isolate P3b (a) and isolate P4 (b). The sequence was taken from EzBioCloud. The tree was constructed by using neighbor-joining method with a bootstrap value of 1000 replicates.

## Optimization of growth and biosurfactant production

For optimization of growth and biosurfactant production, *Enterobacter xiangfangensis* SSP3b16 was selected for further analysis as *Shewanella chilikensis* SSP416 exhibited weak growth in Ramsay medium. Optimization of growth for *Enterobacter xiangfangensis* SSP3b16 was carried out in batch culture system using Ramsay medium, supplemented with 2 % (v/v) of 5 mM glucose as carbon source. The effect of pH was studied in the pH ranging from 5.0 to 8.0. *Enterobacter xiangfangensis* SSP3b16 was found to grow well at pH ranging from 6.8 to 8.0, as shown in Fig. 4.

From the Fig. 4, pH 7.0 was chosen as optimum pH since the highest OD reading, 1.252 was recorded at this pH. Higher OD reading indicates the maximum growth achieved by bacteria in controlled environment. The pH values from 6.5 to 8.0 did not show significant difference according to statistical analysis. This result indicated that *Enterobacter xiangfangensis* SSP3b16 was tolerant to slightly alkalic environment. Acidic environment was found to be unfavorable for the bacterial growth in this study, as indicated by low absorbance reading.

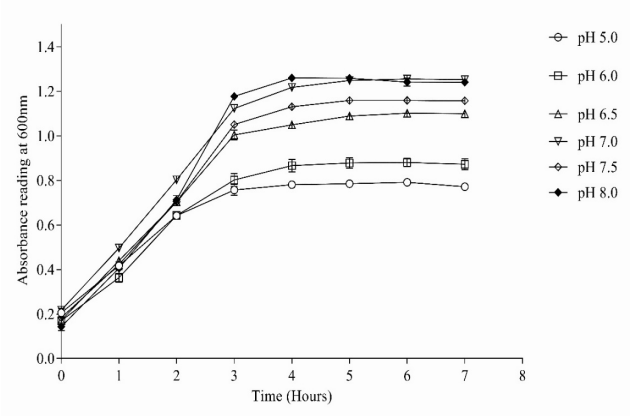


**Fig. 4** Growth profile of *Enterobacter xiangfangensis* SSP3b16 grown in Ramsay medium supplemented with 5 mM glucose at 37°C, 150 rpm with different pH values.

Fig. 5 shows the effect of different incubation temperatures on growth of *Enterobacter xiangfangensis* SSP3b16. The optimum temperature for this bacterium to grow was 37 °C. From statistical analysis, the maximum OD reading achieved by *Enterobacter xiangfangensis* SSP3b16 was found at 37 °C with value of 1.467. The OD readings were found not significantly different from 25 °C to 40 °C. These results indicated that *Enterobacter xiangfangensis* SSP3b16 was tolerant to slightly low temperature and the bacterial growth was gradually decreased as temperature was increased more than 50 °C. *Enterobacter xiangfangensis* SSP3b16 had shown very weak growth at high temperature due to incapability of bacteria to tolerate with excess heat supplied and this condition led to denaturation of cellular bodies and enzymes.

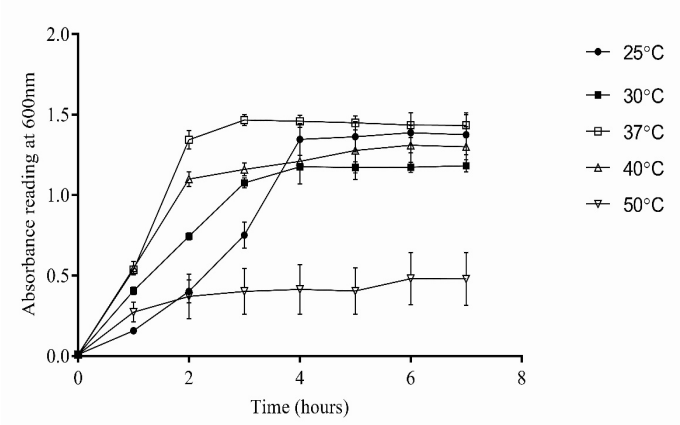


**Fig. 5** Growth profile of *Enterobacter xiangfangensis* SSP3b16 grown in Ramsay medium supplemented with 5 mM glucose, pH 7.0, 150 rpm at different temperatures.

Fig. 6 shows the effect of various initial glucose concentrations on growth of *Enterobacter xiangfangensis* SSP3b16. The glucose acted as carbon and energy source for bacteria to grow in Ramsay medium. Results indicated that the growth pattern of *Enterobacter xiangfangensis* SSP3b16 had changed according to the amount of glucose supplemented into the Ramsay medium. The highest OD reading was 1.627 when 10 mM glucose was added to the medium. The results showed no significant difference between 5 mM, 8 mM and 10 mM. However, 10mM of glucose was chosen as optimum concentration for growth of *Enterobacter xiangfangensis* SSP3b16 because the highest OD reading was achieved at this concentration.

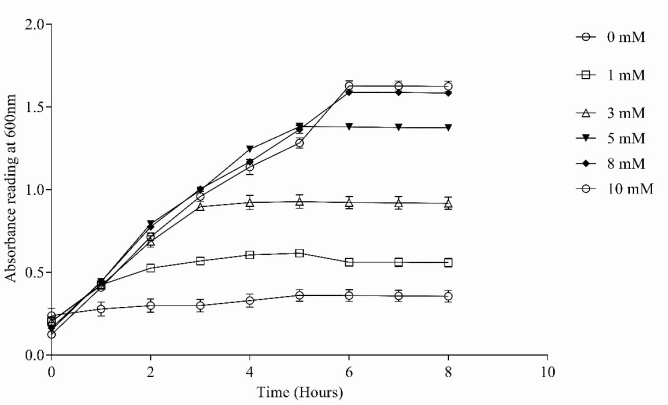


**Fig. 6** Growth profile of *Enterobacter xiangfangensis* SSP3b16 at different initial glucose concentrations.

From the table 4, the longer incubation period did not further affect the biosurfactant production of *Enterobacter xiangfangensis* SSP3b16. The highest surface tension reduction was measured after 1 day of incubation at 150 rpm, 37 °C and pH 7.0 with no significant difference compared to after 3, 5 and 7 days. The surface tension measurement was corresponded to the amount of biosurfactant produced by respective bacteria. Based on the result, the incubation period for biosurfactant production in this study was chosen as 1 day. Previous study by Jamal *et al*. (2012) grew *Klebsiella pneumoniae* strain WMF02 for 30 hours at 37 °C and 180 rpm prior to measuring surface tension activity. Hošková *et al*., (2013) incubated their cultures at 30 °C for 48 hours while working on characterization of rhamnolipids produced by *Acinetobacter calcoaceticus* and *Enterobacter asburiae*. Other related works have incubated bacterial cultures by up to 7 days (Hu *et al*., 2015; Parthipan *et al*., 2017; Patowary *et al*., 2017; Thavasi *et al*., 2011).

**Table 4** Surface tension measurement after specific incubation period by *Enterobacter xiangfangensis* SSP3b16.

| Sample | Surface tension (mN/m) |
|---|---|
| Control | 70.2 |
| 1 day | 36.8 |
| 3 days | 40.2 |
| 5 days | 38.5 |
| 7 days | 36.7 |

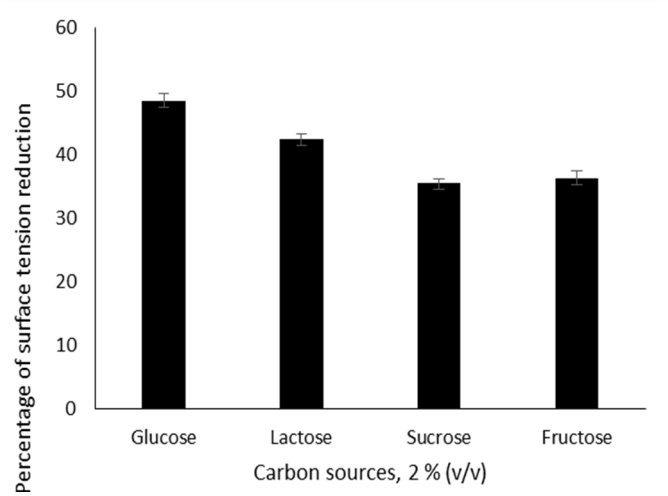


**Fig. 7** Effect of different types of carbon sources on biosurfactant production by *Enterobacter xiangfangensis* SSP3b16.

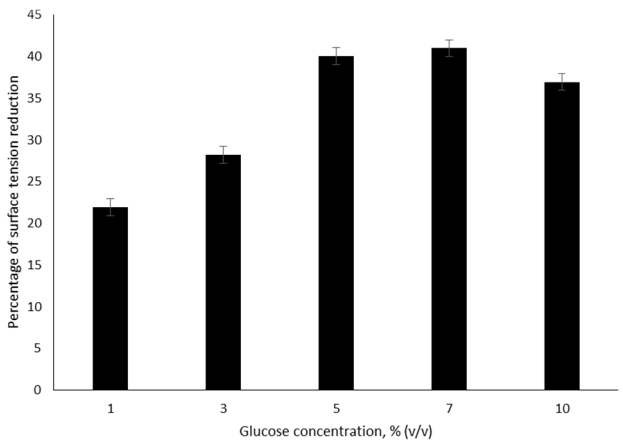


**Fig. 8** Effects of different glucose concentrations on biosurfactant production by *Enterobacter xiangfangensis* SSP3b16.

The ability of *Enterobacter xiangfangensis* SSP3b16 to utilize different types of carbon sources was studied using glucose, lactose, sucrose and fructose at 2 % (v/v) (Fig. 7). Glucose was found as carbon source that produced the lowest surface tension and the highest percentage in surface tension reduction at 36.17 mN/m (48.5 % reduction). Lactose was the second best carbon source with a surface tension of 40.4 mN/m for a 42.5 % reduction. Glucose was selected as the best carbon source as the reduction of surface tension was the highest. At different concentrations of glucose, *Enterobacter xiangfangensis* SSP3b16 showed the highest percentage of surface tension reduction with 7 % (v/v) glucose (Fig. 8). However, there was no significant difference between 5 % (v/v) and 7% (v/v) glucose concentrations.

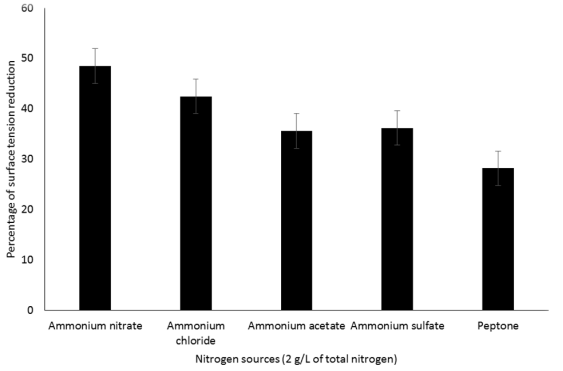


**Fig. 9** Effect of different nitrogen sources on biosurfactant production by *Enterobacter xiangfangensis* SSP3b16.

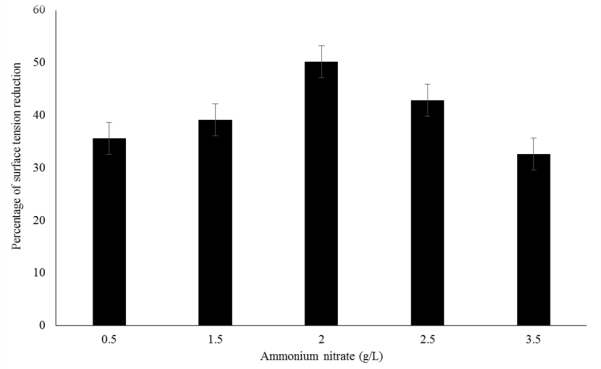


**Fig. 10** Effect of different ammonium nitrate concentrations on biosurfactant production by *Enterobacter xiangfangensis* SSP3b16.

According to Fig. 9, *Enterobacter xiangfangensis* SSP3b16 was able to consume all types of nitrogen sources whether ammonium salt or nitrate of both; $(NH_4)_2SO_4$, $NH_4Cl$, $NH_4NO_3$, $CH_3COONH_4$ and peptone at 2 g/L with 7 % (v/v) glucose. There was no significant difference between $NH_4NO_3$ and $NH_4Cl$ for surface tension reduction. However, in this study, $NH_4NO_3$ was selected as the ideal nitrogen source since it exhibited the lowest surface tension (46.77 mN/m) (48.48 % reduction).

Fig. 10 shows the effect of varying ammonium nitrate concentration supplemented to Ramsay medium on biosurfactant production by *Enterobacter xiangfangensis* SSP3b16. No significant difference was observed between 2 g/L and 3.5 g/L of ammonium nitrate on the percentage of surface tension reduction. The amount of nitrogen can be concluded as limiting condition for biosurfactant production as the percertage of surface tension reduction decreases with the addition of $NH_4NO_3$ that more than 2.0 g/L. The increase in nitrogen source concentration changed the C/N ratio of 7:2 thus possibly interfering with the biosurfactant production by bacteria. The production of biosurfactant by *Pseudomonas aeruginosa* was reportedly enhanced when grown in medium supplemented with C/N ratio of 52:1 using glycerol and sodium nitrate (Wu *et al*., 2008). Heryani and Putra (2017) reported a C/N ratio of 12:4 as the optimum condition for *Bacillus* sp. BMN14 to produce biosurfactant.

Biosurfactants production is greatly influenced by the physical and nutrient factors such as pH, temperature, carbon sources and nitrogen sources. Batool *et al*. (2017) found that the maximum growth of biosurfactant producing bacteria was at 37 °C and pH 7.0. Similar findings also reported by Guerra-Santos *et al*. (1986) and Hamzah (2013). Batool *et al*. (2017) found that *Enterobacter clocae* produced highest amount of biosurfactant when its growth was supplemented with yeast extract and glucose. They also reported that Gangrene oil gave the highest emulsification index rather than kerosene, olive oil, cooking oil and coconut oil. Duvnjak and Kosaric (1985) demonstrated that when grown on simple sugars such as glucose or sucrose, *Cornyebacterium lepus* was able to produce large amount of biosurfactant.

## CONCLUSION

In this study, biosurfactant producing bacteria, P3b and P4 were successfully isolated from rag layer emulsion using streak-plate technique. Emulsification test and surface tension measurement indicated that both isolates were biosurfactant producers. The 16S rRNA gene sequence analysis discovered that isolates P3b and P4 were closely related to *Enterobacter xiangfangensis* and *Shewanella chilikensis*, respectively. *Enterobacter xiangfangensis* SSP3b16 was found to grow optimally at 37 °C, pH 7.0 and 10mM glucose. The highest reduction of surface tension was recorded when culture medium was supplemented with 7 % (v/v) glucose and 2 g/L ammonium nitrate with 1 day incubation period. Work is currently focusing on extraction, purification and characterization of biosurfactant from *Enterobacter xiangfangensis* SSP3b16. The purified biosurfactant can potentially be used in rag layer treatment as an alternative to synthetic surfactants.

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