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Research paper

Production, functional stability, and effect of rhamnolipid biosurfactant from *Klebsiella* sp. on phenanthrene degradation in various medium systems

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ABSTRACT

The present study investigated the stability and efficacy of a biosurfactant produced by *Klebsiella* sp. KOD36 under extreme conditions and its potential for enhancing the solubilization and degradation of phenanthrene in various environmental matrices. *Klebsiella* sp. KOD36 produced a mono-rhamnolipids biosurfactant with a low critical micelle concentration (CMC) value. The biosurfactant was stable under extreme conditions (60 °C, pH 10 and 10% salinity) and could lower surface tension by 30% and maintained an emulsification index of > 40%. The emulsion index was also higher (17–43%) in the presence of petroleum hydrocarbons compared to synthetic surfactant Triton X-100. Investigation on phenanthrene degradation in three different environmental matrices (aqueous, soil-slurry and soil) confirmed that the biosurfactant enhanced the solubilization and biodegradation of phenanthrene in all matrices. The high functional stability and performance of the biosurfactant under extreme conditions on phenanthrene degradation show the great potential of the biosurfactant for remediation applications under harsh environmental conditions.

1. Introduction

Microbially produced compounds that have the capability to reduce surface tension in aqueous systems are considered as biosurfactants (Rodrigues et al., 2006a). Microbial biosurfactants belong to different groups based on molecular structure, which include lipopeptides, glycolipids, phospholipids, lipopolysaccharides-protein complexes, neutral lipids, and long and short-chain fatty acids (Ahmad et al., 2016; Banat and Rengathavasi, 2018; Thavasi and Banat, 2019; Płaza and Achal, 2020). In general, biosurfactants are superior to the synthetic surfactants in that they are less toxic, active at high temperature, pH and salt concentrations and their critical micelle concentration (CMC) is mostly lower than chemical surfactants (Santos et al., 2013). These properties contribute to the environmental applicability of biosurfactants in particularly harsh environments (Schultz and Rosado, 2019; Marcelino et al., 2020).

Research on the production of biosurfactants and their biotechnological applications has raised significant attention over the last two decades, as they can be used in the field of bioremediation, food, biomedical, pharmaceuticals, oil, and petroleum industries (Silva et al., 2017). For such a reason, intensive research has been conducted with a focus on the selection of novel microorganisms that produce biosurfactants with low CMC value using low cost substrate (Borah et al., 2019). Rhamnolipids, which are mainly produced by *Pseudomonas aeruginosa*, are the most studied glycolipid biosurfactants (Mnif and Ghribi, 2016; Haloi et al., 2020). However, several other microorganisms, such as *Acinetobacter calcoaceticus* (Nitschke et al., 2005b), *Enterobacter* sp., (Nitschke et al., 2005a) *Pantoea* sp., *Burkholderia, E. coli* (Wang et al., 2007; Gong et al., 2015) and *Pseudomonas putida* (Silva et al., 2010a), have been reported to produce rhamnolipids using various hydrophobic substrates. It is worthy to mention that there are several other bacterial genera that have the potential to produce biosurfactants but they are rarely reported, such as *Klebsiella* species. Jain et al. (2012, 2013) demonstrated the ability of *Klebsiella* sp. to utilize various carbon

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sources (starch, sucrose, xylose, and soybean oil) and produced biosurfactant with excellent surface tension reduction capability and emulsification activity that makes it suitable candidate for future application in remediation of hydrocarbon contaminated environment. Similarly, Lee et al. (2008) isolated *Klebsiella* sp. Y6–1 from waste soybean oil and demonstrated that produced biosurfactant had good emulsification activity and stable under various environments. Although these studies are limited, they showed great promises of biosurfactant produced by *Klebsiella* species. Non-renewable resources like plant and vegetable oils have been used for producing biosurfactants and many different plant/vegetable oils (e.g. soybean oil, olive oils, sunflower oil) have been successfully used for producing rhamnolipids. It has been well documented that plant and vegetable oils are effective substrates for induction of rhamnolipid production. Moreover, the final concentration and substrate-to-product conversion yields are generally higher than those for non-hydrophobic substrates (Kandasamy et al., 2019). However, the functionality of biosurfactants under extreme conditions is important and varies among different bacteria. For instance, the functional activity loss of biosurfactant produced by *Pseudomonas* sp. strain LP1 and *Rhodococcus* sp. strain TA6 were 100% and 34%, respectively at pH 10 (Obayori et al., 2009; Li et al., 2014) Similarly, biosurfactant activity of *Pseudomonas* sp. strain LP1 and *Bacillus subtilis* strain JA-1 was lost by 82% and 85%, respectively in a saline environment (Costa et al., 2006; Obayori et al., 2009). The loss in functional activity reduces usefulness of such biosurfactants and biosurfactant producing bacteria in diverse environments. Therefore, it is hypothesized that the bacterium *Klebsiella* sp. KOD36 may produce biosurfactant that is functionally stable under extreme environments and have potential as facilitating molecules in biodegradation of hydrocarbons.

In our previous study, we isolated a new biosurfactant producing bacterial strain, *Klebsiella* sp. KOD36 (Ahmad et al., 2016). A particular focus of this study was the activity and stability of the biosurfactant produced by the isolate *Klebsiella* sp. KOD36 under extreme conditions of temperature, pH and salinity and its effect on phenanthrene solubilization and degradation of phenanthrene in three representative environmental matrices.

2. Material and methods

2.1. Bacterium and culture media

The bacterial strain KOD36, previously isolated and reported as a biosurfactant producer was used in the present study. The strain was sequenced through 16s RNA and identified as *Klebsiella* sp. The sequence was submitted to gene bank with accession number KT364873 (Ahmad et al., 2016). The specific strain was also found non-pathogen (Ahmad et al., 2016; Gudiña et al., 2016). For inoculum preparation, the culture of strain KOD36, which was previously stored in 20% glycerol at −80 °C, was first streaked twice on the fresh tryptic soy agar plates and then inoculated in 100 mL minimal salt media (MSM) liquid culture. The composition of the MSM used was (g L^{-1}): NaCl (1.0), $CaCl_2{\cdot}2H_2O$ (0.1), $MgSO_4{\cdot}7H_2O$ (0.5), KH_2PO_4 (1.0), Na_2HPO_4 (1.0) and yeast extract (4.0) (Nwaguma et al., 2016). The flask was incubated under shaking conditions at 200 RPM to create aerobic conditions for growing the bacterium. In biosurfactant production studies, ten milliliters of the inoculum having a uniform cell density were inoculated to 500 mL MSM and 20 g L^{-1} soybean oil was used as carbon source. The flasks were placed on rotary shaker at 250 RPM and 30 °C for 11 days. Later it was centrifuged at 10,000 RPM and 4 °C for ten min. to get a cell-free supernatant.

2.2. Purification and identification of the biosurfactant produced by the strain KOD36

Initially, the crude biosurfactant as viscous brown material was obtained from the cell-free culture using modified method described by Abbasi et al. (2013). The determination of CMC was done by dissolving crude biosurfactant at various concentrations (0–150 mg L^{-1}) in phosphate buffer solution (PBS), and surface tension was measured using a du Noüy ring method (Amani et al., 2010). Critical micelle concentration was determined by the relation between surface tension and logarithm of concentration of biosurfactant, and the concentration at the intersection of the two lines fitting the sub CMC and super CMC data is the CMC.

Separation of different rhamnolipid fractions was done employing liquid chromatography, and then these fractions were subjected to thin layer chromatography (TLC) for identification of different rhamnolipid fractions. For visualization of spots, TLC plates were sprayed evenly with anthrone reagent and were placed in an oven at 110 °C for 20 min (Déziel et al., 2000; Satpute et al., 2010). Mono-rhamnolipids standard (Sigma Aldrich, 99% pure) was used to identify different fractions of rhamnolipids on TLC plates. For purification purposes, the different spots on TLC were scrapped and were dissolved in $CHCl_3/CH_3OH$ (1:2 v/v). The purified fraction was obtained by evaporating the solvent under a vacuum. This purified biosurfactant was further used for identification.

2.3. Identification of the biosurfactant

2.3.1. Fourier transform infrared (FTIR) spectroscopy analysis of crude biosurfactant

Fourier transform infrared spectroscopy analysis of crude biosurfactant was done using FTIR Bruker, Impact 400 IR spectrophotometer taking the sample dispersed in KBr. Crude biosurfactant was mixed with KBr (solid spectral grade) to form pellets using KBr press ATLAS T25 (ATS). Spectral measurements were taken at 16 cm^{-1} resolution with five scans in transmittance mode in the range of 200–4000 cm^{-1} (Ibrar and Zhang, 2020). The data taken were corrected considering background spectrum.

2.3.2. X-ray diffractometry analysis

The biosurfactant was also subjected to X-ray diffractometry analysis (JDX 3532, JEOL, Japan), and measurements were taken at diffraction angle 5–60° at room temperature with a scan step 0.01 (Banat et al., 2010).

2.3.3. SEM with energy-dispersive X-ray spectroscopy

To analyze the surface morphology of the purified biosurfactant sample, Scanning Electron Microscopy (Hitachi S-2380) was performed by method as described by Zia et al. (2013).

2.4. Numerical modeling for rhamnolipids production and soybean oil consumption

The bacterium KOD36 was grown in MSM amended with soybean oil at 20 g L^{-1} for ten days as described above. After each interval of 12 h, a sample was taken and centrifuged at 10,000 RPM to obtain supernatant and pellet. The supernatant was used for analysis of rhamnolipids produced by the bacterium and analysis of soybean content, whereas bacterial pellet was used to measure bacterial biomass.

2.4.1. Rhamnolipids production

Rhamnose concentration as a measure of rhamnolipid concentration was calculated as the method described by Vanavil and Rao (2018). 4.5 mL dilute sulfuric acid was mixed with 1 mL of cell-free supernatant and heated at 100 °C for 10 min. 100 μL 3% thioglycolic acid was mixed with cell-free culture and cooled in dark incubator for 3 h. Using spectrometer the absorbance was measured at 420 nm (Shimadzu UV-1601). The standard curve of L-rhamnose (99% pure, Sigma Aldrich, USA) was developed to calculate the rhamnose concentration (Du et al., 2019; Zhu et al., 2012). The amount of rhamnolipid was calculated by multiplying rhamnose concentration by a factor 3.4 (Benincasa et al., 2004). The mathematical modeling approach was used to calculate rhamnolipid

production over time by *Klebsiella* sp. KOD36 following the modified Eq. (1) as proposed by Rodrigues et al. (2006b):

$$P = \frac{P_o P_{max} e^{p_r t}}{P_{max} - P_o + P_o e^{p_r t}} \quad (1)$$

In Eq. (1), P represents the yield of microbially produced surfactant, P_o the initial biosurfactant concentration, P_{max} is the maximum biosurfactant concentration, P_r represents the ratio between *rp* (initial biomass formation) and P_o, and t represents time.

These parameter values were estimated by non-linear regression analysis. The model was employed to calculate predicted biosurfactant production at any given time, and results were compared with the actual biosurfactant concentration measured at the time.

2.4.2. Bacterial biomass production and soybean oil consumption

Saline solution (0.9% w/v NaCl) was used to wash the above collected pellets thrice. The cell pellets were dried in a hot oven at 105 °C until constant dry cell weight was obtained keeping in view the care that cells were not to be charred. The bacterial biomass involved in biosurfactant production was mathematically interpreted by the modified Eq. (2), as described by Rodrigues et al. (2006b):

$$X = \frac{X_o X_{max} e^{\mu_{max} t}}{X_{max} - X_o + X_o e^{\mu_{max} t}} \quad (2)$$

In Eq. (2), X represents the total biomass (bacterial), X_o represents the initial bacterial biomass, X_{max} shows the maximum bacterial biomass, and μ_{max} shows the ratio between *rp* and X_p (initial biomass).

The concentration of soybean oil (mL L^{-1}) as a substrate was calculated using the mathematical equation (Eq. 3):

$$S = S_o - \frac{1}{Y_{p/s}}(P - P_o) - \frac{1}{Y_{x/s}}(X - X_o) \quad (3)$$

Where S represents the substrate consumption, S_o shows the initial substrate concentration, P_o and P represent the initial and final biosurfactant concentration, X_o and X represent the initial and final biomass, and $Y_{x/s}$ and $Y_{p/s}$ represent the biomass and yield of biosurfactant. This information was used to find the relationship between experimental values and predicted results.

2.5. Measurement of biosurfactant stability

For investigating stability of the biosurfactant produced by *Klebsiella* sp. strain KOD36, reduction in surface tension activity and emulsification index (E24%) were used as criteria for stability of the biosurfactant, which were measured at various pH (4, 6, 8, 10, and 12), temperature (10, 20, 40, 60, 80, and 100 °C) and salinity levels (0%, 5%, 10%, 15%, 20%, and 25%). For evaluating the effect of pH, MSM (EC 2.1 dS m^{-1}) of different pH was prepared using 5 N NaOH or HCl, and freeze-dried powdered biosurfactant was dissolved to give a concentration of CMC (124 mg L^{-1}). After addition of biosurfactant samples were kept in an incubator at 40 °C. Then E24% and reduction of surface tension were measured using the emulsification and Du-Nouy-Ring method described by Gupta et al. (2020) and Ahmad et al. (2016), respectively. To investigate the influence of temperature on stability of the biosurfactant, MSM (EC 2.1 dS m^{-1}, pH 8) containing biosurfactant with CMC concentration was incubated at different temperatures. After 1 h E24% and reduction of surface tension were measured. Similarly, stability of the biosurfactant was estimated with different concentrations of salts using NaCl with the pH of the MSM adjusted to 8 and temperature maintained at 40 °C. All experiments were carried out in triplicate.

2.6. Efficacy of microbial surfactant (rhamnolipids) and synthetic surfactant for emulsifying various hydrocarbon substrates

The biosurfactant from strain KOD36 and synthetic surfactant (Triton X-100) were compared for emulsification of different hydrocarbons. Both biosurfactant and Triton X-100 were used at CMC level (124 mg L^{-1} and 150 mg L^{-1}) for biosurfactant and Triton X-100, respectively). E24% was recorded against each of toluene, xylene, light mineral oil, silicon, diesel, petrol, and diffusion pump oil according to Gaur et al. (2019) for its suitability as bioremediation agent for various hydrocarbons.

2.7. Phenanthrene mineralization studies

Phenanthrene (PHE) mineralization studies were conducted using ^{14}C-PHE (100 μCi in 2 mL methanol and the methanol was allowed to volatize before the degradation experiment) in three different systems i.e., soil, aqueous, and soil-slurry adopting modified method of Xia et al. (2011). The effect of various concentrations of rhamnolipid was investigated on PHE mineralization by *Klebsiella* sp. KOD36. The detail of each system is as follows.

2.7.1. Liquid culture

The experimental set up was run in triplicate to evaluate the effect of different concentration of biosurfactants, i.e., sub-CMC (62 mg L^{-1}), CMC (124 mg L^{-1}), and super-CMC (248 mg L^{-1}) by spiking 50 mL MSM solution with non-radiolabel PHE and 0.01 μCi L^{-1} ^{14}C PHE. The PHE was added with methanol and the methanol was allowed to volatize to give a final PHE concentration of 100 mg L^{-1} in the solution. The various conconcentrations of biosurfactant were prepared from the stock solution (500 mg L^{-1}) in PBS, while the working solutions (sub-CMC, CMC, super-CMC) were prepared from this stock solution for the desired volume of 50 mL. The pH of the medium was adjusted to 7.2. The cell density of the culture was done uniform as 2.3×10^7 CFU mL^{-1}. The flasks were placed on a gyratory shaker (New Brunswick Scientific, Edison, NJ, USA) and were continuously stirred at 125 RPM. All biodegradation experiments were conducted under aerobic conditions for seven days, and each experiment was conducted in triplicate. The evolved CO_2 was trapped in the vial (hanging from a rubber stopper) containing 2 mL of 1 M NaOH. The vials were replaced repeatedly after every 12 h. Liquid scintillation counter (Canberra Packard Tri-Carb 2250 CA, USA) was used to measure the ^{14}C activity with external source of calibration (Brune et al., 1995).

2.7.2. Soil system

For soil system mineralization studies, a loamy sand soil was taken from University of California, Riverside greenhouse experimental area was used which composed of sand (75%), clay (12%), silt (13%) and organic matter contents (0.72%). The soil was sieved through 2 mm sieve after air drying and spiked with radiolabeled and non-radio labeled PHE with a final concentration of 100 mg kg^{-1}, and ^{14}C-PHE concentration was 0.01 μCi g^{-1} soil following the method as described by Doick and Semple (2003). By using a sterilized spatula, 10 g of the soil was thoroughly mixed with a specific volume of biosurfactant solution (2 mL) at various concentrations (62 mg L^{-1}, 124 mg L^{-1}, and 248 mg L^{-1} as sub-CMC, CMC, super-CMC respectively) prepared from distilled water and the stock biosurfactant solution in PBS, and moisture content was a field capacity level (~17% soil water moisture content). A typical respirometer consists of a 250 mL conical flask with a stopper and sealed with Teflon. All biodegradation experiments were conducted under aerobic conditions for seven days, and each experiment was conducted in triplicate. 1 M solution of NaOH (2 mL) in 7 mL glass scintillation vials was placed inside the respirometer to trap the respired CO_2.

During the experiment, the experiment setup was placed at temperature-controlled incubator at 28 °C and 100 RPM. The CO_2 was quickly removed and replaced after selected time intervals (12 h). Liquid scintillation counter (Canberra Packard Tri-Carb 2250 CA, USA) was used to measure the ^{14}C activity with external source of calibration (Sun et al., 2015).

2.7.3. Soil-slurry system

For the soil-slurry experiment, 10 g soil and 50 mL MSM solution suspension (1:3 w/v) spiked with 100 mg L^{-1} non-radiolabel PHE, and 0.01 μCi L^{-1} ^{14}C PHE was used. 500 μL cell culture at mid-log phase and uniform cell density (2.3×10^7 CFU mL^{-1}) was added into each flask. Biosurfactants at various concentrations were added to the treatment. As mentioned above, the various conconcentrations of biosurfactant was prepared from the stock solution (500 mg L^{-1}) in PBS, while the working solutions were prepared from this stock solution for desired volume (50 mL) to investigate the varying effect of biosurfactant concentrations (62 mg L^{-1}, 124 mg L^{-1}, and 248 mg L^{-1} as sub-CMC, CMC, super-CMC, respectively) on a PHE degradation during the experiment. All biodegradation experiments were conducted under aerobic conditions i.e. the flasks were sealed with rubber stopper and placed in temperature-controlled shaking incubator at 28 °C and 100 RPM shaking speed for seven days ensuring volume ratio between air space and soil slurry 4:1. Each experiment was conducted in triplicate. At predetermined time intervals (12 h), the CO_2 traps were quickly removed and replaced. The ^{14}C activity was determined according to liquid scintillation counting the method described by Aryal and Liakopoulou-Kyriakides (2013).

2.8. Statistical analysis

Statistical analyses were performed using Statistix-10 and SPSS programs. Data regarding comparative efficacy of biosurfactant and Triton X-100 for emulsification of petroleum hydrocarbons were analyzed by one-way ANOVA. Least significance difference (LSD) test was used to compare the treatment means at 5% probability level (Steel and Torrie, 1990). Before applying ANOVA, data were tested for normality distribution and homogeneity of variances using Shapiro–Wilk's test and Levene's test, respectively. All the data were normally distributed and showed homogeneity of variances. Descriptive statistics including means and standard errors were performed by using Microsoft Excel 2013 (Microsoft Office).

3. Results and discussion

3.1. Identification of the biosurfactant

The results of TLC indicated that the biosurfactant samples developed spots at the same place where, mono-rhamnolipid standard visible as a dark brown spot [Retention factor (R_f) = 0.64] (Fig. S1). Previously, TLC has also been employed to identify rhamnolipids produced by different microorganisms (George and Jayachandran, 2009; Samadi et al., 2012). Priya and Usharani (2009) reported R_f values as 0.63–0.72 for rhamnolipids produced by *P. aeruginosa* using different substrates. Bhat et al. (2015) observed the presence of two different areas with R_f values of 0.73 and 0.35, corresponding to the spots developed for standard mono-rhamnolipids and di-rhamnolipids with R_f of 0.74 and 0.36 on TLC plates, respectively. Other researchers have also found a similar R_f of 0.7 for mono-rhamnolipids using the same solvent system and molisch reagent for color development (Luna et al., 2013). This result of biosurfactant identification was further examined using FTIR spectrum of the crude biosurfactant and comparing it with that of some known mono-rhamnolipid biosurfactants (Fig. S2). The strong 3280 cm^{-1} wide band indicates the occurrence of aliphatic –O–H fragments of hydrocarbon chains, the 1629 cm^{-1} bands may show the occurrence of the –C=O group belonging to the carboxylic acid, and the 1519 and 1237 cm^{-1} bands indicate C=O deformation oscillations. The transmission peak at 1054, indicated by C–O–C stretching, showed the presence of polysaccharide, and the peak (absorption) at 626 cm^{-1} showed occurrence of ethyl group. The observed peaks and corresponding bands are in line with the structural analysis of a rhamnolipid biosurfactant reported by Sood et al. (2020), who observed similar peaks in their freeze-dried sample of biosurfactants produced by *Pseudomonas aeruginosa* strain CR1 and later confirmed that the biosurfactant was mono-rhamnolipid by analysis using NMR, HPLC, LC-MS, and MALDI-TOF mass spectrometry. Based on these data and similarity of the identified FTIR bands of the biosurfactant in this study with those already reported in many other studies (Aparna et al., 2012; Moussa et al., 2014; Rodrigues et al., 2006a; Shavandi et al., 2011), it is concluded that the biosurfactant produced by *Klebsiella* sp. is a mono-rhamnolipid. Further physical characterization of biosurfactant through XRD and SEM showed biosurfactant sample was largely amorphous and cross-linked appearance (Fig. S3).

3.2. Numerical modeling of biosurfactant production

During the production of biosurfactant, soybean oil (2%) was used as the carbon source in the minimal salt culture medium. Fig. 1 shows the experimental data of biosurfactant production as well as the regression line based on Eqs. (1), (2) and (3). The parameters obtained from the regression are listed in Table 1. Model performance and parameters calculated in the present study were compared with the previous literature. P_{max} values for biosurfactant production by *Klebsiella* sp. KOD36 was 4.34 g L^{-1}. The P_{max} obtained in other studies using soluble carbon sources, i.e., glucose or lactose is lower than 1.74 g L^{-1}. The P_{max} is similar to that (4.68 g L^{-1}) of *Nocardia amarae* grown on olive oil, which is a more expensive carbon substrate than soybean oil. The overall higher value of P_{max} in the present study indicates higher activity for biosurfactant production of strain KOD36 compared to other bacterial strains reported in previous literature.

In this study, the parameters like yield of biosurfactant ($Y_{p/s}$), and bacterial biomass ($Y_{x/s}$) was calculated as 0.255 g g^{-1} and 0.310 g g^{-1}, respectively, which was higher than that calculated by Rodrigues et al. (2006b) (0.08–0.09 g g^{-1} and 0.34–1.38 g g^{-1} for $Y_{p/s}$ and $Y_{x/s}$ respectively) for four *Lactobacillus* bacteria in the presence of different substrates. In another study, Rodrigues et al. (2006a) reported $Y_{p/s}$ values for several fermentation media for L. *lactis* as 0.04–0.12 g g^{-1} and $Y_{x/s}$ as 0.22–0.43 g g^{-1}. The $Y_{p/s}$ of biosurfactant from *Klebsiella* sp. KOD36 higher than $Y_{p/s}$ of biosurfactants from the other bacteria strains (Table 1). This indicates a high efficiency of converting substrate to biosurfactant by *Klebsiella* sp. KOD36.

3.3. Biosurfactant stability

Application of biosurfactants in different areas depends on their stability at a different temperature, pH and salt concentrations (Jahan et al., 2020). Surface tension versus the biosurfactant concentrations produced by *Klebsiella* sp. strain KOD36 is shown in Fig. S4. The gradual increase in the concentration of biosurfactants to 124 mg L^{-1} led to a decrease in the surface tension to a specific value of 38 mN m^{-1}. This point on the curve was considered as CMC of the biosurfactant, which was calculated to be 124 mg L^{-1} with the method described by Zhong et al. (2007). Critical micelle concentration of the biosurfactant produced by *Klebsiella* sp. strain KOD36 was compared with CMCs of previously reported rhamnolipid biosurfactants produced by other bacterial strains (Table 2). It is worth mentioning that CMC value of rhamnolipid produced by strain KOD36 is lower than that of rhamnolipids produced by a number of other bacterial strains, which is advantageous as desired CMC value is achieved at a much lower rhamnolipid concentration.

3.3.1. Temperature

The successful use of microbial surfactants in various industries depends on the fact that their functionality should not be sensitive to temperature changes (Khopade et al., 2012). Therefore, the stability of biosurfactant produced by strain KOD36 in terms of E24% and surface tension consistency was investigated over a temperature range of 10–100 °C (Fig. 2A1 and A2). It was observed that E24% increased with an increase of temperature from 10 to 40 °C, however, further increase in temperature reduced E24% (Fig. 2A1). The E24% at 100 °C decreased by 39% compared to E24% of 40 °C; however, it remained at 40% at

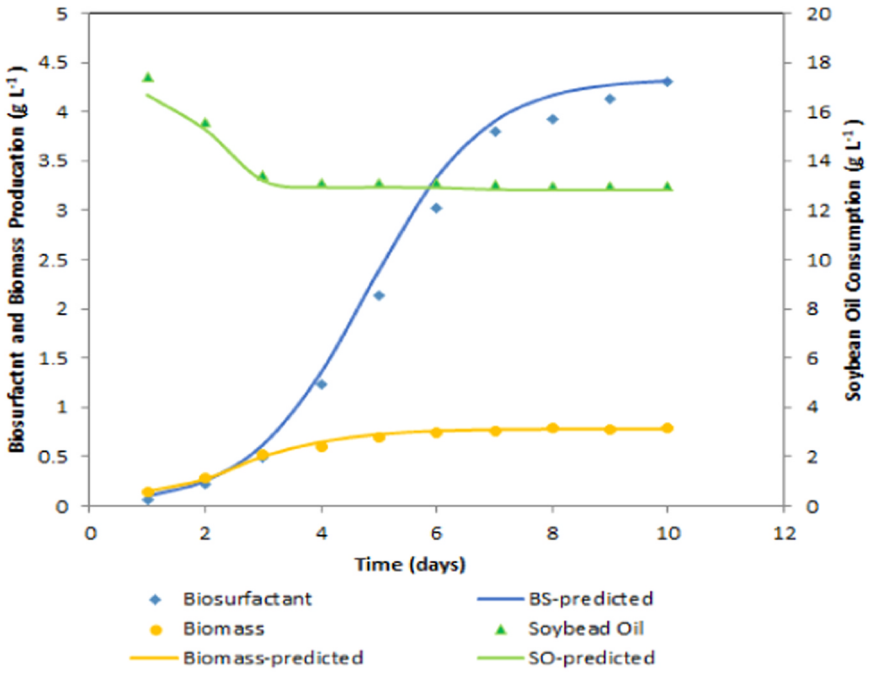

Fig. 1. Experimental and predicted data representation of biosurfactant and biomass production with time using *Klebsiella* sp. KOD36.

Table 1
Comparison of model parameters obtained in present study vs previous literature.

		Substrate consumption			Biomass production			Biosurfactant production			Reference
		Model parameters									
Bacteria	Substrate	S_o (mL L^{-1})	$Y_{p/s}$ (g g^{-1})	$Y_{x/s}$ (g g^{-1})	X_o (g L^{-1})	X_{max} (g L^{-1})	μ_{max} (h^{-1})	P_o (g L^{-1})	P_{max} (g L^{-1})	P_r (h^{-1})	
Klebsiella sp. KOD36	Soybean oil	18.8	0.255	0.310	0.25	3.13	0.08	0.035	4.34	0.083	Present study
Nocardia amarae	Olive oil	26.7	0.083	0.039	0.0285	1.77	0.0177	1.13	4.68		(Aronstein and Alexander, 1993)
L. *casei*	Glucose	26.7	0.08	0.34	0.11	5.5	0.324	0.8	1.6	0.612	(Rodrigues et al., 2006b)
L. *lactis 53*	Various medium	29–35*	0.04–0.12	0.22–0.43	0.068–0.064	4.24–5.99	0.40–0.20	0.03–0.11	0.693–1.735	0.64–0.29	(Rodrigues et al., 2006a)
L. *rhamnosus*	Glucose	28.4	0.09	0.25	0.12	4.6	0.299	0.8	1.7	1.215	(Rodrigues et al., 2006a)
L. *pentosus*	Glucose	24.5	0.09	0.41	0.10	6.4	0.409	0.9	1.7	0.506	(Rodrigues et al., 2006a)
L. *pentosus*	Lactose	55.6	0.09	3.1	0.2	1.5	0.05	0.4	1.4	0.353	(Rodrigues et al., 2006a)

Where
S_o, X_o and P_o are initial soybean oil concentration (mL L^{-1}); initial biomass concentration (g L^{-1}) and initial biosurfactant concentration (g L^{-1}) respectively.
$Y_{p/s}$, X_{max} and P_{max} are product yield (g g^{-1}), maximum concentration biomass (g L^{-1}) and maximum concentration of biosurfactant (g L^{-1}) respectively.
$Y_{x/s}$, μ_{max} and P_r are biomass yield (g g^{-1}), ratio between initial volumetric rate of biomass formation (*rp*) and initial biomass concentration.
X_o (h^{-1}), ratio between initial volumetric rate of biosurfactant formation (*rp*) and initial biosurfactant concentration P_o (h^{-1}) respectively.
* The quantity was in (g L^{-1}).

such a high temperature. Similar to our findings, biosurfactant produced by *Pseudomonas* sp. strain LP1 showed the highest E24% at 40 °C; however, E24% was significantly reduced (by 32%) at 100 °C (Obayori et al., 2009). The comparison of stability of biosurfactant produced by *Klebsiella* sp. KOD36, with the previously reported (Table 3) shows that the emulsification activity lost of biosurfactant produced by *Klebsiella* sp. FKOD36 at 100 °C (40%) was lower than that for *Candida lipolytica* (52%). This shows its applicability in bioremediation of contaminated soils in high temperature conditions.

Stability represented by surface tension reduction potential at different temperatures (10–100 °C) is presented in Fig. 2A2. The surface tension reduction was higher at elevated temperatures than lower temperatures, which shows that surface tension reduction property of the biosurfactant was not impaired by heating and indicates the thermostable nature of biosurfactant. These results are consistent with the results of our previous study showing that a higher temperature (100 °C) had no significant adverse effect on activity of surface tension (Table 3). The ability to reduce surface tension, however, was the highest for the biosurfactant produced by *Klebsiella* sp. KOD36 among these biosurfactants.

3.3.2. pH

Biosurfactant produced by *Klebsiella* sp. KOD36 showed high emulsifying activity over a broad pH range (4–12), with the highest E24% being observed at pH 8 (Fig. 2B1). Under highly alkaline conditions (pH 10), the emulsifying activity of the biosurfactant was reduced by 19%,

Table 2
Comparison of CMC of produced biosurfactant by *Klebsiella* sp. KOD36 with previous literature.

Biosurfactant (Rhamnolipid)	Carbon source	CMC (mg L^{-1})	Reference
Crude RL biosurfactant	Soybean oil	124	Present study
Crude RL biosurfactant	Palm oil	200	(Pornsunthorntawee et al., 2008)
RL mixtures	Passion fruit oil	163.44	(Costa et al., 2006)
RL supernatant	Babassu oil	210.77	(Nitschke et al., 2005b)
RL crude extract	Waste frying oil.	160	(Zhu et al., 2007)
RL biosurfactant	Palm oil	200	(Pornsunthorntawee et al., 2009)
Purified di-RL	Glycerol 3% (w/v)	150	(Singh and Cameotra, 2013)
RLs from *P. aeruginosa* NCAIM (P) B 001380	Sunflower oil	131	(Rikalovic et al., 2013)
Purified RLs extract from wild type *B. kururiensis* P23T	Glycerol	200	(Tavares et al., 2013)
RLs from *P. aeruginosa* MAT10	Waste free fatty acids from soybean oil production	150	(Benincasa et al., 2004)
Partially purified RLs from *P. aeruginosa* 57RP	Naphthalene	400	(Marcoux et al., 2000)
RL biosurfactant from	Sodium citrate	240	(Li et al., 2014)

which was quite low compared to the previously studied biosurfactants produced by *Rhodococcus* sp. strain TA6 (44%) and *Pseudomonas* sp. strain LP1 (100%) (Table 3). Furthermore, it has been observed that the emulsifying activity of the biosurfactant produced by *Klebsiella* sp. KOD36 (40%) at highly alkaline conditions was higher compared to the other biosurfactants produced by previously reported bacterial strains of *Candida lipolytica, Pseudomonas* sp. strain LP1, *Rhodococcus* sp. (Rufino et al., 2007; Obayori et al., 2009).

The pH over a broad range (4–12) had minimal influence on the surface tension reduction property of the crude biosurfactant, however, the most suitable pH found for functioning of the biosurfactant was observed at pH range between 6 and 10 (Fig. 2B2). The comparison of the stability of biosurfactant produced by *Klebsiella* sp. KOD36, with the previously reported biosurfactants indicates that the activity of biosurfactant produced by *Klebsiella* sp. KOD36 (29 mN m^{-1}) at higher pH was comparable with the previously reported biosurfactants, although a lost in the activity of 12% was observed at pH 10 (Table 3).

3.3.3. Salinity

Fig. 2C1 and C2 present the effects of NaCl induced salinity (0–25%) on E24% and surface tension reduction potential of biosurfactant produced by the strain KOD36, respectively. E24% of the biosurfactant was same as the control (no NaCl) at 5% NaCl and slightly decreased at 10% NaCl (10%). It had an apparent decrease with an increase of salinity higher than 10%; however, the biosurfactant did not completely lose emulsification capacity even at 25% NaCl. Stability in emulsification activity at a higher salt concentration (10%) shows that the biosurfactant produced by *Klebsiella* sp. KOD36 is more stable in maintaining emulsification activity compared to previously studied biosurfactants (Table 3). For instance, the biosurfactants produced by *Bacillus subtilis* strain JA-1 and *Pseudomonas* sp. strain LP1 almost completely lost emulsification activity (85% and 82% reduction, respectively) beyond 10% NaCl (Obayori et al., 2009). Likewise, biosurfactants produced by *Candida glabrata* and *Candida lipolytica* lost 30% and 22% emulsification activity, respectively, at a higher salt concentration (10% NaCl) (Rufino et al., 2007; Sarubbo et al., 2006). The presence of NaCl beyond 10% suppresses the surface tension reduction efficiency of the crude biosurfactant (Fig. 2C2), contrary to the previous findings which show that surface tension activity slightly affected/unaffected at 10% NaCl salt concentration (Amani et al., 2010; Rufino et al., 2008; Shavandi et al., 2011). However, the biosurfactant of the CMC concentration could still decrease the surface tension by ~20 mN m^{-1} at a salinity of 25% (Fig. 2C2). The superiority of the biosurfactant produced by the strain KOD36 over a wide range of temperature, pH, and salt concentrations compared to previously reported biosurfactants indicates its advantage in application to bioremediation of contaminated sites, where extreme in-*situ* halophytic condition usually hinders the activity of biosurfactants (Kumar et al., 2007; Mnif et al., 2009; Gaur et al., 2019; Karbalaei-Heidari et al., 2020).

3.4. Comparative between the biosurfactant and the synthetic surfactant (Triton X-100) for emulsification activity

The crude biosurfactant and Triton X-100 were compared for the emulsification of different petroleum hydrocarbons, i.e., n-hexane, mineral light oil, silicon oil, diesel oil, petrol, diffusion pump oil, toluene, and xylene (Fig. 3). The biosurfactant showed significant higher E24% values ($p < 0.05$) than Triton X-100 for most of the hydrocarbons tested, with only the exception for petrol and silicon oil. Similar to our findings, higher E24% of xylene, hexane and toluene was reported by using biosurfactant obtained from *Pseudomonas fluorescens* BD5 than using Triton-X100 (Janek et al., 2010). Sriram et al. (2011) observed statistically similar E24% of xylene, hexane and toluene hydrocarbons with biosurfactant collected from *Bacillus subtilis* SA9 and Tween-80 surfactant. Lower emulsification activity of biosurfactants than synthetic surfactants was also reported. For example, Pornsunthorntawee et al. (2008) observed lower E24% of hexane and toluene with biosurfactant produced by *Pseudomonas aeruginosa* SP4 than SDS. The results of the present study indicate a strong and superior emulsification activity against various hydrocarbons (diesel oil 83%, mineral light oil 52%, xyelen 63%) for the mono-rhamnolipid biosurfactant produced by *Klebsiella* sp. KOD36 compared to previously reported biosurfactants produced by *R. erythropolis* OSDS1, *Pseudomonas aeruginosa* NCIM 5514 and *P. aeruginosa* 99 for diesel oil (57%), mineral light oil (35%), xyelen (27%), respectively (Xia et al., 2019; Varjani and Upasani, 2016). Emulsification superiority of the biosurfactant produced by *Klebsiella* sp. KOD36 over synthetic surfactant like Triton X-100 indicates its potential application in biodegradation of such compounds where poor solubility is a big challenge for the successful bioremediation process.

3.5. Biodegradation studies

The PHE minerlization by bacterial strain *Klebsiella* sp. KOD36 in aqueous system was determined (Fig. 4A). After 168 h incubation, it was found that 56% of total PHE was mineralized in the system where external application of biosurfactant at all concentration was done, whereas 31.6% of PHE was mineralized in the system with no external application of biosurfactant. While in the sterile control no measurable fraction of PHE minerlization was observed. Such effect of the biosurfactant to enhance degradation, however, disappeared after 108 h, likely due to degradation of the biosurfactant itself. At 72 h, a maximum of 46% PHE mineralization occurred for biosurfactant concentrations of sub-CMC and CMC, while 40% was observed for the concentration of super CMC. Concentration above CMC at initial phase showed less PHE biodegradation compared to sub-CMC and CMC. Similar findings have been reported by Mehetre et al. (2019), they evaluated utility of thermophilic and thermo-tolerant bacteria for biodegradation of PHE under shake flask conditions using MSM as the culture medium. Moreover, biodegradation of PHE in our present study was higher than the phenanthrene biodegradation in aqueous system (31%) at enriched rhamnolipid (100 mg L^{-1}) after 72 h incubation reported by Mariaamalraj

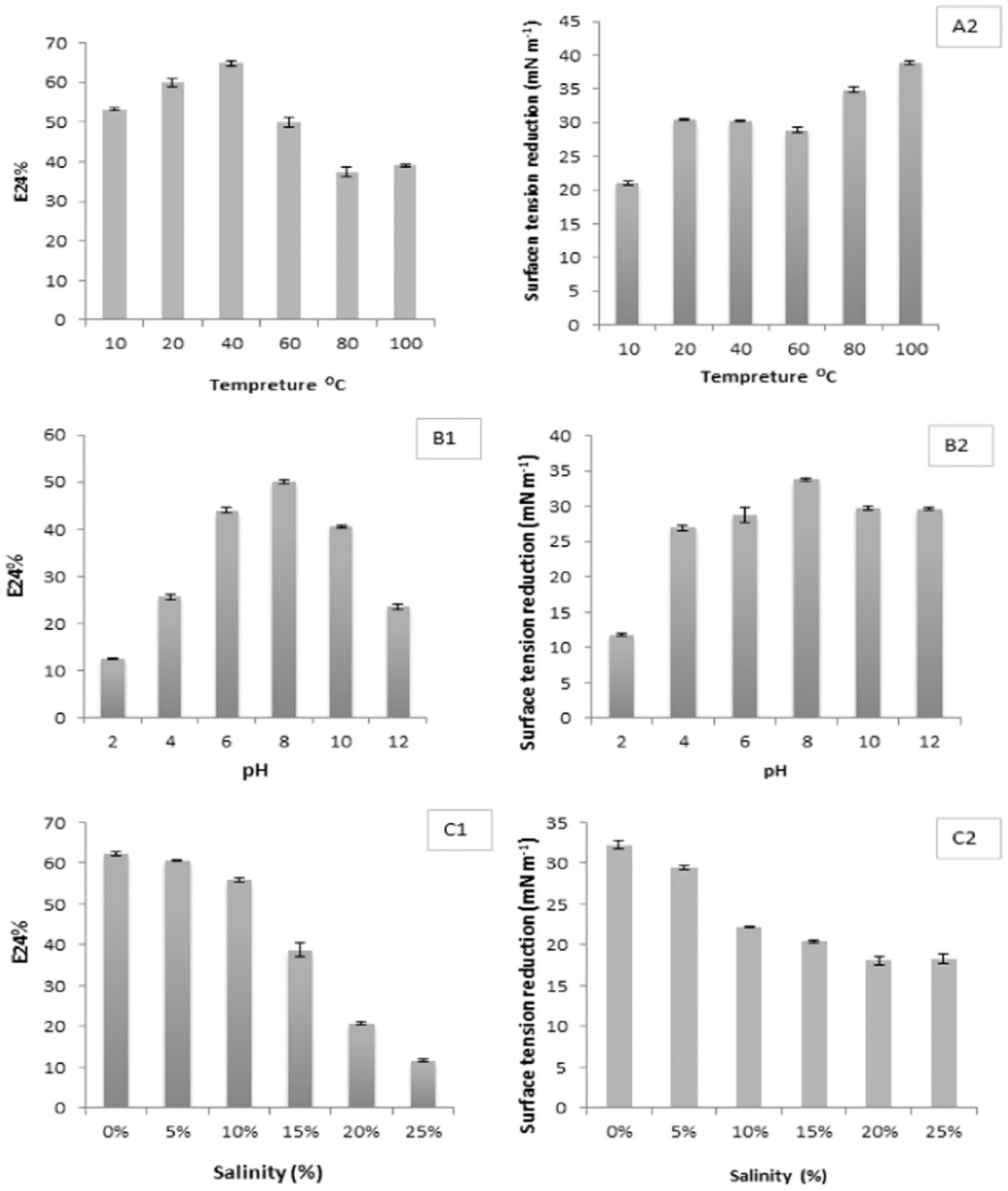

Fig. 2. Effect of temperature (A1, A2), pH (B1, B2) and salinity (C1, C2) on emulsification index and surface tension reduction properties of biosurfactants at CMC value (124 mg L^{-1}) produced by *Klebsiella* sp. KOD36. Date are shown as mean ± SE of three replicates.

et al. (2016). Several reasons explain the effective mineralization of PHE. In this study, the rapid mineralization of PHE by KOD36 was possibly due to the higher solubility of PHE. As has been observed in other mineralization studies, higher solubility increases the susceptibility of PAHs to initial microbial attack since they remain in solution (Koshlaf and Ball, 2017).

Contrary to the aqueous system, an initial lag phase (24 h) was observed in the soil system when no biosurfactant was used. A total of 23% of PHE was removed, which was lower than that in the aqueous system (30%). The reduction in biodegradation is likely due to the lowered availability of the PHE to the degraders caused by the adsorption of PHE to the soil matrix. The presence of biosurfactant resulted in immediate initiation of degradation (Fig. 4B), showing that the biosurfactant could enhance the bioavailability of PHE in the soil (Chang et al., 2020). The maximum PHE mineralization of 33% occurred at the end of the 168 h with the biosurfactant applied, which is 10% higher than in the treatment without the biosurfactant. Although the presence of the biosurfactant at all the three concentrations enhanced PHE biodegradation, however, similar to the aqueous system, sub-CMC concentration caused the highest degradation rate in the first 72 h.

The improvement in the biological degradation of PHE in aqueous and in soil systems through the use of biosurfactants at sub-CMC is mainly due to the enhanced partition of PHE to the aqueous phase and thus higher accessibility to the degrading microorganism, which in turn enhances their subsequent biodegradation and metabolism (Li et al., 2014). At super-CMC concentrations, the biosurfactant form micelles to enhance the solubility of PHE by incorporating PHE into micelle hydrophobic core in the aqueous phase. By such means, the partition of

Table 3
Comparison of stability of biosurfactant produced by *Klebsiella* sp. KOD36 with the previously reported.

Stability Parameter	Biosurfactant producing microorganism	Activity at pH 10 (% activity lost compared to pH 8)	Activity at 10% NaCl (% activity lost compared to 0% NaCl)	Activity 100 °C (% activity lost compared to 40 °C)	Reference
E24%	*Klebsiella* sp. KOD36	40 (19)	56 (10)	40 (39)	Present study
	Candida lipolytica	–	35 (22)	19 (52)	(Rufino et al., 2007)
	Pseudomonas sp. *strain LP1*	0 (100)	15 (82)	–	(Obayori et al., 2009)
	Rhodococcus sp. strain TA6	38 (44)	16 (20)	–	(Li et al., 2014)
	Bacillus subtilis strain JA-1	–	10 (85)	–	(Costa et al., 2006)
Surface tension reduction (mN m^{-1})	*Klebsiella* sp. KOD36	29 (12%)	22 (31%)	39 (NAL)	Present study
	Bacillus subtilis	27 (13%)	25 (NAL)	25 (NAL)	(Amani et al., 2010)
	Rhodococcus sp. strain TA6	30 (9%)	28 (3%)	31 (NAL)	(Li et al., 2014)
	Candida lipolytica	27 (NAL)	25 (3%)	27 (NAL)	(Rufino et al., 2007)

( ) shows % activity lost; NAL – No Activity Lost.

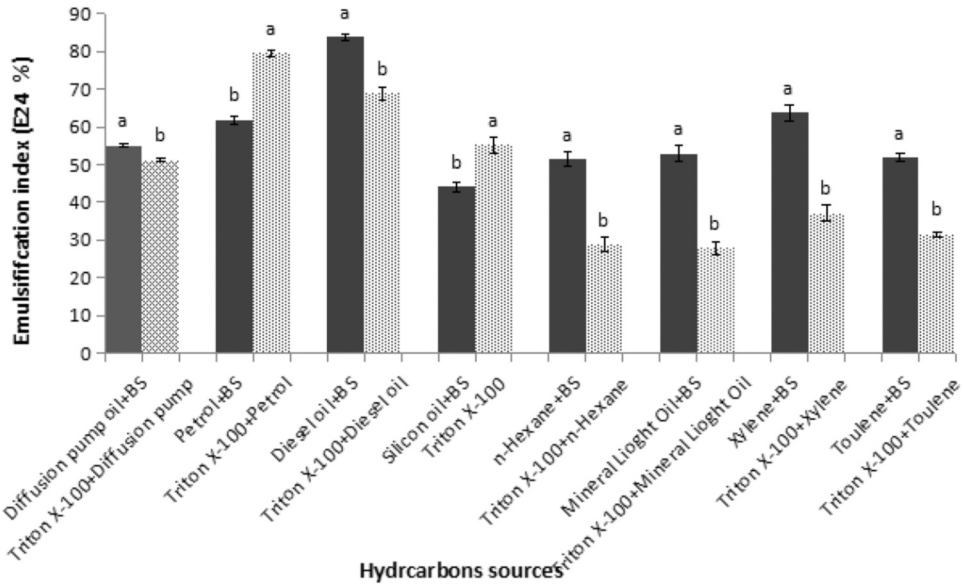

Fig. 3. Comparative efficacy of biosurfactants and Triton X-100 at CMC (124 mg L^{-1} and 150 mg L^{-1} for microbial surfactant and Triton X-100, respectively) for emulsification of petroleum hydrocarbons. Data are shown as mean ± SE of three replicates; BS: Biosurfactants.

biosurfactant into aqueous phase can be enhanced. However, sequestration of PHE into surfactant micelles may lower bioavailability of PHE compared to the freely dissolved molecules (Liu et al., 2017). Consequently, biosurfactant of the super-CMC concentration is less effective in enhancing PHE degradation than that of the sub-CMC concentration. Similar results have been reported previously by Liu et al. (2017), who described that the efficiency of PHE removal was maximum at CMC, which later decreased as the biosurfactant concentration increased to super-CMC. Moreover, the results were consistent with previous studies which showed that surfactants increased PAH release from the soil mainly by increasing matrix diffusivity, with an increase in solubility through partitioning of PAH into the micellar pseudophase being a secondary reason (Bezza and Nkhalambayausi-Chirwa, 2015; Wolf, 2019). Several studies showed that the addition of biosurfactant enhanced PHE desorption in PHE-contaminated soil systems, making the contaminants more bioavailable. Hou et al. (2018) used biosurfactant produced by *Achromobacter* sp. LH-1 to evaluate its effect on phenanthrene (PHE) mineralization and found that it enhanced the bioavailability and solubilization of PHE. LH-1 achieved a 40% mineralization rate with 100 mg L^{-1} PHE. Similarly, in another study, Chang et al. (2020) investigated the effect of biosurfactant produced from *Pseudomonas* strain R (PR) on phenanthrene (PHE) mineralization by two soil microorganisms were investigated. They observed a significant difference in the PHE mineralization capability in the systems. However, this is significant that the PHE mineralization in soil system by bacterial strain KOD36 in our present study was higher than biosurfactant produced by *Rhodococcus erythropolis* ATCC 4277, which increased PHE mineralization by *Sphingomonas sp*. strain P5-2 up to 25% (Chang et al., 2020).

It is important to understand the behavior of biosurfactants in soil slurry system for the design of ex situ soil biotreatment projects. The results of PHE mineralization under the effect of different biosurfactant concentrations in the soil-slurry system are shown in Fig. 4C. Different from the soil system, no lag phase was observed in the soil-slurry system. The maximum PHE mineralization of 43.7% occurred with the use of biosurfactants, whereas only 27.4% of PHE mineralization was observed in the absence of biosurfactants after 168 h. The findings were consistent with previous reported studies who described that apparent solubility and degradation of PHE increases with the amendment of biosurfactants (Kim et al., 2001; Yu et al., 2014; Chang et al., 2020). Phenanthrene

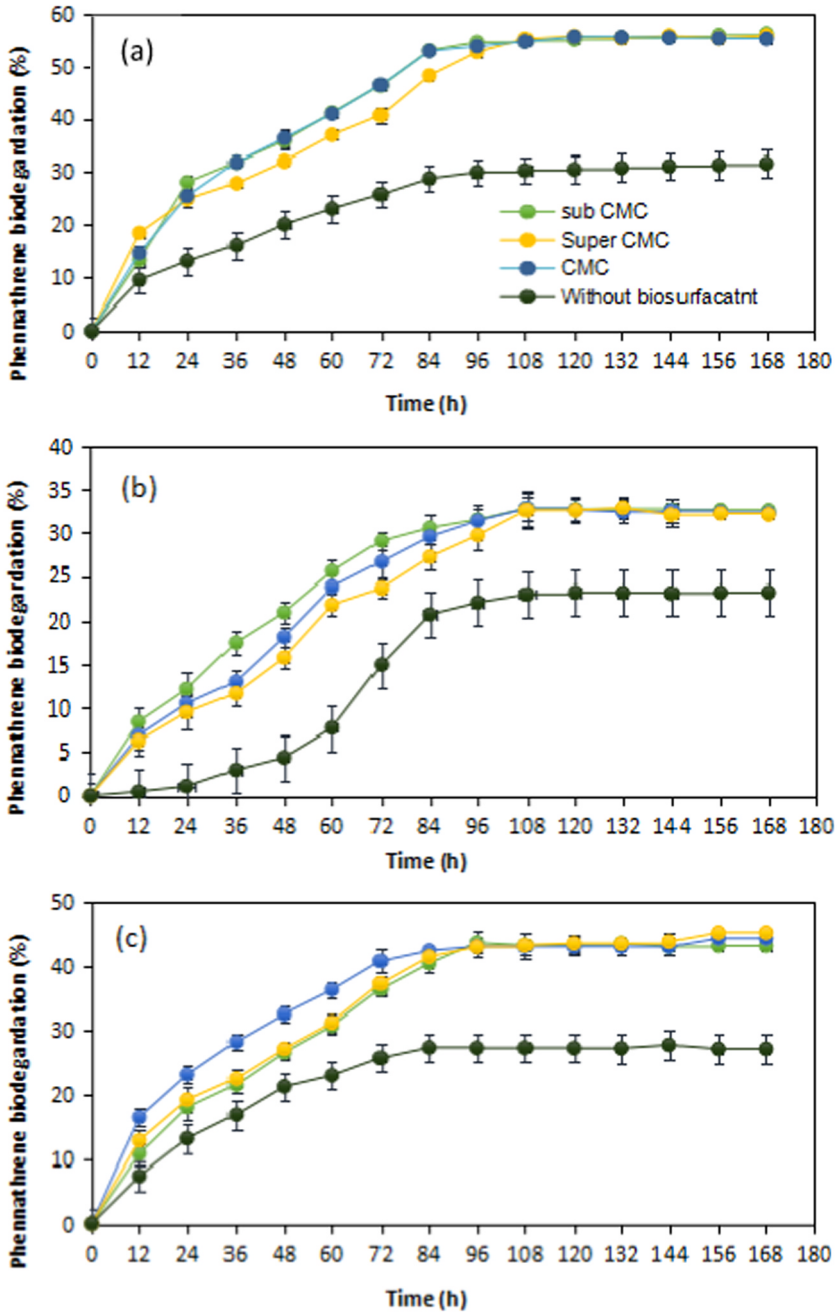

Fig. 4. Biosurfactant (at CMC 124 mg L^{-1}) facilitated phenanthrene mineralization in aqueous (A), soil (B) and soil-slurry system (C).

degradation by the effect of biosurfactant in present study was higher than the nonionic surfactants polyoxyethylene sorbitol hexaoleate (42%) under similar conditions as reported by Singleton et al. (2016), indicate the potential of biosurfactant produced by *Klebsiella* sp. KOD36.

The in situ production of biosurfactant, however, was not recorded. This is because the concentration of PHE in the degradation experiments was very low (100 mg L^{-1} or 100 mg kg^{-1}) and is much lower than the soybean oil concentration for biosurfactant production (20 g L^{-1}). Also PHE is relatively harder to use (recalcitrant) than the soybean oil by microbes. Therefore, the potential for the PHE to produce biosurfactant in the degradation experiment was very low. Furthermore, in the complex soil and soil slurry systems it is almost impossible to identify the part of biosurfactant produced from PHE degradation with the co-existence of the added biosurfactant. Therefore, it is assumed that the added biosurfactant that plays the major role for PHE degradation.

The higher PHE mineralization by KOD36 with the addition of monorhamnolipid biosurfactant may be primarily due to enhanced PHE dissolution. The biosurfactant has been reported to possess capacity to significantly increase aqueous solubility of hydrophobic organic contaminants (Chang et al., 2020; Cazals et al., 2020). The difference in PHE biodegradation in soil and soil-slurry systems by the external application of biosurfactant can be attributed to enhanced solubilization which was more significant in the soil-slurry system compared to the soil system due to better physical mixing conditions. Additionally, in soil-slurry system, better contact among the surfactant molecules, substrate (PHE), and degrading microorganism is also a possible mechanism (Woo et al., 2004a; Gottfried et al., 2010). The difference in biodegradation of PHE in the soil-slurry system at different biosurfactant concentrations also shows that super-CMC concentration is not the most effective. However, a higher concentration of biosurfactant is required in soil-slurry system compared to soil system to attain a better effect, which is probably due to adsorption of larger quantities of biosurfactant

molecules to solid particles in the soil-slurry system. Our findings are in line with the previous results (Li et al., 2014) which showed that PAH removal efficiency in the soil-slurry system depends on the surfactant concentrations.

4. Conclusions

Biosurfactant produced by *Klebsiella* sp. KOD36 is identified to be mono-rhamnolipid. High values of parameters for modeling the biosurfactant production, such as maximum biosurfactant concentration (P_{max}) and product yield ($Y_{p/s}$), indicates a higher efficiency of *Klebsiella* sp. KOD36 for the conversion of substrate to biosurfactant. Comparative stability analysis in terms of emulsification and surface tension reduction activity shows strong functional stability of the biosurfactant under extreme environmental conditions. Also, emulsification capability of the biosurfactant over a broad range of hydrocarbons indicates strong interfacial activity of the biosurfactant. Finally, the results of PHE degradation experiment demonstrate that the biosurfactant can improve the removal of hydrocarbons in soil systems. These results indicate great potential of application of the biosurfactant produced by *Klebsiella* sp. KOD36 in bioremediation of contaminated sites, especially under harsh environmental conditions.

CRediT authorship contribution statement

Zulfiqar Ahmad, Hua Zhong, Xuezhi Zhang, Muhammad Imran: Conceptualization; design of study.

Zulfiqar Ahmad, Shaista Andleeb, Rabail Zulekha, Guansheng Liu: Acquisition of data.

Guansheng Liu, Iftikhar Ahmad, Muhammad Imran, Frederic Coulon: Formal analysis; interpretation of data.

Zulfiqar Ahmad, Hua Zhong, Muhammad Imran: Drafting the manuscript.

Zulfiqar Ahmad, Hua Zhong, Xuezhi Zhang, Muhammad Imran, Rabail Zulekha, Shaista Andleeb, Frederic Coulon: Revising the manuscript critically for important intellectual content.

Zulfiqar Ahmad, Xuezhi Zhang, Hua Zhong, Muhammad Imran, Shaista Andleeb, Rabail Zulekha, Guansheng Liu, Iftikhar Ahmad, Frederic Coulon: Approval of the version of the manuscript to be published (the names of all authors must be listed).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111514.

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