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ORIGINAL ARTICLE

Characterization of a biosurfactant-producing *Leclercia* sp. B45 with new transcriptional patterns of alkB gene

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Abstract

To investigate the hydrocarbon-degrading ability, biosurfactant-producing capacity, and alkane monooxygenase system of *Leclercia* spp. A bacterial strain classified as *Leclercia* sp. B45 was isolated, and its biosurfactant-producing capacity, hydrocarbon-degrading ability, and alkane hydroxylase (*alkB*) gene transcriptional patterns were evaluated by TLC, FTIR, GC-MS, and RT-qPCR, respectively. Strain B45 showed active biosurfactant-producing ability, which was preferentially induced by C16. The extracted biosurfactant tolerated a wide range of salinity, pH, and temperature. The degradation rate of *n*-decane (C10), *n*-hexadecane (C16), and octacosane (C28) by strain B45 could reach 92.6%, 94.1%, and 67.8%, respectively. Furthermore, the *alkB* transcription levels in the strain B45 with C10, C16, or C28 as a carbon source were distinctly higher than those of the control group during the late exponential and stationary phases. The relative *alkB* transcript copy number decreased with the increase in alkane chain length, which is consistent with B45 strain biodegradation kinetics. *Leclercia* sp. B45 showed excellent *n*-alkane degradation performance and biosurfactant-producing capacity. Meanwhile, the *alkB* gene in *Leclercia* sp. B45 is likely to represent a novel gene, whose transcription level was significantly upregulated when induced by *n*-alkane. These results provide new insights into alkane metabolism mechanism in *Leclercia* sp. B45.

Keywords *Leclercia* sp. · Alkane · alkB gene · Biosurfactant

Introduction

Petroleum hydrocarbons represent one of the most significant global pollution sources as well as one of the most complex organic mixtures, containing over 20,000 distinct compounds (Alan and Rodgers 2004). Petroleum hydrocarbons can be divided into four main groups: saturated hydrocarbons (20–50%), aromatic hydrocarbons (30%), resins, and asphaltenes (van Beilen et al. 2003; van Beilen et al. 2005). Since saturated hydrocarbons constitute the most abundant component of crude oil, their biodegradation serves as a crucial process for the removal of oil contaminants.

Hydrocarbon-degrading bacteria are ubiquitous in the environment. In addition, they tend to accumulate in petroleum-polluted areas as they can be triggered to bloom by regular inputs of petroleum hydrocarbon (Zhang et al. 2017). Therefore, a large number of bacteria that can metabolize saturated hydrocarbon as the sole carbon source have been isolated, such as *Oleispira* spp. (Yakimov et al. 2003), *Alcanivorax* spp. (Yakimov et al. 1998), and *Oleiphilus* spp. (Golyshin et al. 2002).

The uptake of alkane by microorganisms is a critical step for further metabolic processes. However, the uptake mechanism depends on the type of bacteria, environmental factors, and the molecular weight of the alkanes (Hayaishi et al. 1955).

Yiying Shuai and Hanghai Zhou contributed equally to this work.

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Specifically, it has been reported that biosurfactants increase the uptake of medium- and long-chain length *n*-alkanes in culture (Beal and Betts 2000; Noordman and Janssen 2002). However, the vast majority of alkanes are insoluble in water, and their solubility decreases with increasing molecular weight (Eastcott et al. 1988), which limits the microbial uptake of alkanes. For example, although microorganisms may directly use low molecular weight alkanes dissolved in water, it may be necessary to emulsify water-insoluble mid- and long-chain alkanes by surfactant production to increase microbial utilization (Rojo 2009). Biosurfactants are mainly used to form micelles to promote the cracking of hydrocarbon molecules, increase the surface area of hydrocarbons exposed to microorganisms, enhance the bioavailability of hydrocarbons, and thus contribute to biodegradation of hydrocarbons (Souza and Vessoni-Penna 2014). Accordingly, bacteria that are capable of oil degradation usually produce and store a variety of biosurfactants with different chemical characteristics to facilitate such emulsification (Hommel 1990; Ron and Rosenberg 2002; Qiao and Shao 2010; Shao 2011). It was found that the addition of surfactants (rhamnolipids and Tween 80), especially rhamnolipids, can effectively enhance the PHC degradation, and the degradation effect is equivalent to the promotion of petroleum hydrocarbon degradation through the addition of nutrients (Li et al. 2018). Over the past several decades, research on the mechanism of alkane degradation has focused on enzymes related to the first step in the use of short-, medium-, and long-chain alkanes by microorganisms (Throne-Holst et al. 2007; van Beilen and Funhoff 2007; Wang and Shao 2013). Depending on the length of the substrate chain, microorganisms require different enzyme systems to introduce oxygen to oxidize the substrate and promote its use. For simplicity, these systems are classified into three categories: C1–C4 (oxidized by methane monooxygenase-like enzymes) (van Beilen and Funhoff 2007), C5–C16 (oxidized by AlkB or cytochrome P450 enzymes), and ≥ C17 (longer alkanes, oxidized by long-chain alkane monooxygenase AlmA or LadA) (Feng et al. 2007; Throne-Holst et al. 2007; Wang and Shao 2013).

Moreover, the complexity of alkane monooxygenase systems is species specific. Some even include > 3 different enzyme systems, such as cytochrome P450 (CYP), alkane hydroxylases (AlkB), and a putative flavin-binding monooxygenase (AlmA) along with the long-chain monooxygenase (LadA) (Liu et al. 2011). However, the vast majority of cultured aerobic hydrocarbon-degrading bacteria contain the AlkB gene and can catalyze the terminal oxidation of medium-chain and long-chain alkanes to produce the corresponding alcohols. At present, more than 250 alkB homologs have been identified in at least 45 species (Whyte et al. 2002). A rubredoxin (AlkG) and a reductase (AlkT) were part of the AlkB system, which facilitates the flow of reducing agents from NADH. In addition, the majority of *alkB* genes specifically catalyze a relatively narrow range of substrates, i.e., C5–C22 (van Beilen and Witholt 2005).

In this study, a crude oil-degrading bacterium, *Leclercia* sp. B45, was isolated from an oil-field wastewater treatment plant. To the best of our knowledge, the biosurfactant-producing ability and alkane monooxygenase system of *Leclercia* spp. have not been previously reported. Therefore, the aims of this study were to (1) investigate the utilization spectrum (alkanes, cyclohexane, phenanthrene, crude oil) of petroleum hydrocarbons by *Leclercia* sp. B45; (2) assess the alkane hydroxylase (*alkB*) gene transcriptional patterns in *Leclercia* sp. B45, especially the *alkB* gene transcriptional patterns induced by *n*-alkanes of different chain lengths; and (3) evaluate the biosurfactant production ability.

Materials and methods

Isolation and identification of crude oil-degrading bacteria

The bacteria were isolated from the activated sludge of an oil-field wastewater treatment plant in the Weizhou Island at the Guangxi Province of China (109° 5′ 24″ N, 21° 2′ 24″ E) by using a minimal salt medium (MSM). The composition of the MSM was adopted and modified from Cai et al. (2014): 1.0 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g KCl, 0.01 g $FeSO_4$, 0.5 g $(NH_4)_2SO_4$, 1% of light crude oil (*v*/*v*), 10.0 g NaCl, 1 mL L^{-1} trace element solution, and 1 L distilled water. The pH value of the medium was adjusted to 6.5–7.0 and the culture was incubated at 30 °C with shaking (170 rpm) for 7 days. All chemicals and reagents used in the experiment were analytically pure reagents.

All isolated strains were identified using 16S rRNA gene analysis. The DNA was extracted by using the Easy Pure Bacteria Genomic DNA Kit (TransGen Biotechnology Co., Ltd., Beijing, China) and was used as template DNA for polymerase chain reaction (PCR) amplification of the 16S gene by using the universal primer pair of 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG CTT-3′). The 16S rRNA gene amplicons were sequenced (TSINGKE Biotechnology Co., Ltd., Hangzhou, China) and the results were compared in BLAST (https://blast.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed using the neighbor joining method and MEGA 7.0 software (Pennsylvania State University, State College, PA, USA).

Evaluation of the petroleum hydrocarbon utilization range by *Leclercia* sp. B45

The 2,6-dichlorophenolindophenol (2,6-DCPIP) test (Kubota et al. 2008; Habib et al. 2017) was modified as follows. *Leclercia* sp. B45 was pre-cultured in 20 mL LB broth at 30 °C and 170 rpm for 24 h. Then, the culture was centrifuged

at 4000*g* for 5 min followed by washing with 0.9% saline, after which, the cell density was adjusted to 1.0 according to the OD at 600 nm. Subsequently, 750 μL of modified MSM (Fe-free) medium, 200 μL 2,6-DCPIP solution (37.5 μg mL^{-1}), and 50 μL of $FeCl_3 \cdot 6 \cdot H_2O$ solution (150 μg mL^{-1}) were added to a 2-mL sterilized microtube. Octacosane and phenanthrene were both dissolved in petroleum ether to prepare a 10 mg/mL stock solution. Subsequently, 200 μL of cell suspension and 10 μL of sterilized substrate (*n*-decane, *n*-hexadecane, octacosane stock solution, cyclohexane, phenanthrene stock solution, light crude oil) were added to the medium, and the cells were cultivated at 30 °C and 170 rpm for 96 h. The color of the medium was monitored every 24 h, and the microbial hydrocarbon degradation ability was determined as positive when the medium became colorless or negative if the color of the medium remained blue.

Determination of the optimum *n*-alkane concentration for degradation

For the *n*-alkane utilization experiment, C10, C16, or C28 was used as the sole carbon source at different concentrations: 0.1%, 0.2%, 0.4%, 0.8%, and 1.6% (*v*/*v*). MSM without the addition of carbon source was used as a blank control group. *Leclercia* sp. B45 was inoculated into 30 mL freshly sterilized MSM containing C10, C16, or C28, and the cultures were incubated at 30 °C, 170 rpm, in the dark for 7 days. The cultures were extracted by petroleum ether with a ratio of 1:1 and the extracts were dehydrated using anhydrous sodium sulfate. The final extract as the residual concentration in the medium was analyzed by gas chromatography-mass spectrometry (GC-MS, Shimadzu QP 2020 Ultra/Plus, Kyoto, Japan) equipped with an SH-Rxi-5Sil MS capillary column (30 m × 0.25 mm × 0.25 μm). Parameters of the GC-MS: holding at 50 °C for 2 min, heating from 50 up to 100 °C at a rate of 15 °C min^{-1}, then heating at a rate of 10 °C min^{-1} to 280 °C and holding at 280 °C for 4 min (scan mode) (Abdel-Megeed 2013; Figueiredo et al. 2014). Standard curves ($R^2 > 0.99$) were made by using GC-MS to quantify five gradients dilution of substrate stock solution and were used for translating peak area of sample into concentration (mg/L), which in order to further calculate the biodegradation rate. The biodegradation rates were calculated as the following formula: degradation rate = (initial concentration of substrate − residual concentration of substrate) / initial concentration of substrate.

Amplification and sequencing of degradation-related genes

PCR amplification of the alkane monooxygenase gene was conducted by using different primers (Table 1). All the primers were synthesized by TSINGKE Biotechnology Co., Ltd. (Hangzhou, China).

The amplified *alkB* gene fragments were purified from 2% agarose gel using the Quick Gel Extraction kit (TransGen) and directly sequenced using the alkBWF or alkBWR primer (TSINGKE Biotechnology Co., Ltd., Hangzhou, China). The obtained sequence of the *alkB* gene from *Leclercia* sp. B45 was deposited in the GenBank nucleotide sequence database (NCBI) under accession number MH371472.

Transcriptional patterns of alkBs in *Leclercia* sp. B45 induced by *n*-alkane

The initial substrate concentrations corresponding to the highest degradation rates were used for the induction experiment, i.e., 0.4% C10, 0.1% C16, and 0.1% C28. The cultures were incubated at 30 °C, 170 rpm, in the dark for 10 days. MSM containing 1% NaAc was used as a blank control group. On the 4th, 7th, and 10th days, 10-mL sample was taken for RNA extraction. Three biological replicates were set up at each sampling time point.

The RNA was extracted using the TaKaRa Mini BEST Universal RNA Extraction Kit (Otsu, Shiga, Japan). Qualified total RNA was reverse transcribed into cDNA using the PrimeScript TM RT reagent kit (TaKaRa). Quantitative real-time RT-PCR (RT-qPCR) was performed by using the SYBR Fast qPCR MIX kit (TaKaRa). The 16S rRNA gene (housekeeping gene) of B45 was chosen as a reference gene, and real-time fluorescence quantification was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions for the real-time PCR were as follows: 95 °C for 5 min, 95 °C for 15 s, annealing at 60 °C for 40 s. The first two steps were repeated 40 times. The threshold cycle (C_T) for each reaction was obtained using Applied Biosystems 7500 software version 2.0, and the fold change values for the number of genes were obtained using $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). The $2^{-\Delta\Delta CT}$ was based on the change in the cycle numbers among the C10, C16, or C28 and NaAc samples.

The alkBwf and alkBwr primers were used for alkB, and 16SRT-F and 16SRT-R primers were used for the 16S rRNA gene. AlkB2-F and alkB2-R for alkB2, and 16SRT-F and 16SRT-R primers were used for the 16S rRNA gene (Table 1). Transcription levels of genes were determined using Student's *t* test with sigma plot. Relatively, copy number of alkB gene in strain B45 was analyzed with one-way ANOVA Tukey's HSD using PASW statistics Version 18.0 program (SPSS Inc., USA). $P < 0.05$ indicated statistically significant differences. For the amplification of the 16S rRNA gene and alkB gene, 16S-F/16S-R primers and alkBwf/alkBwr primers were used (Table 1).

Table 1 Primers used for alkane monooxygenase gene amplification

Number	Primer name	Oligonucleotide (5′ to 3′)	Ref.
1	alkBwf	AAYACNGCNCAYGARCTNGGVCAYAA	(Kloos et al. 2006)
2	alkBwr	GCRTGRTGRTCHGARTGNCGYTG	
3	monf	TCAAYACMGSNCAYGARCT	(Wang and Shao 2012)
4	monr	CCGTARTGYTCNAYRTARTT	
5	P450F	TGTCGGTTGAAATG TTCATYGCNMTGGAYCC	(Wang et al. 2010)
6	P450R	TGCAGTTCGGCAAGGCGGTT DCCSRYRCAVCKRTG	
7	CYP153-F1	ATGTTYATYGCNATGGAYCCN	(Kubota et al. 2005)
8	CYP153-R2	GCGRTTVCCCATRCARCGRTG	
9	Alm Adf	GGNGGNACNTGGGAYCTNTT	(Wang and Shao 2012)
10	Alm Adr	ATRTCNGCYTTNAGNGTCC	
11	16SRT-F	ACTCCTACGGGAGGCAGCAG	16S rRNA (*Leclercia* sp. B45)
12	16SRT-R	ATTACCGCGGCTGCTGG	16S rRNA (*Leclercia* sp. B45)

To further expand the RT-qPCR results, the incubation experiment was conducted to plotting the growth curve and degradation curve of *n*-alkanes by *Leclercia* sp. B45. The incubation condition and the parameters of the GC-MS were the same as detailed above, and in the "Determination of the optimum *n*-alkane concentration for degradation" section.

Extraction and characterization of the biosurfactant produced by *Leclercia* sp. B45

The oil spreading test was performed using a modified method of Chioma et al. (2013). The emulsification activity of the fermentation supernatant was measured as described by Dehghan et al. (2010) (detailed information is provided in the Supplementary Material).

The biosurfactant was extracted as follows (Monteiro et al. 2010; Joy et al. 2017). The strain was cultured for 7 days under optimum conditions, and the fermentation broth was centrifuged at 4 °C and 2151*g* for 5 min. The supernatant was adjusted to pH 2.0 with 6 M HCl (Zhou et al. 2015). After 24 h standing at 4 °C, the precipitate was collected by centrifugation and washed with 6 M HCl.

Critical micelle concentration (CMC) was also used to measure the efficiency of extracted biosurfactants (Asadollahi et al. 2016). To measure the CMC of the biosurfactants, solutions of different biosurfactant concentrations were prepared, and the surface tension of each solution was measured. With increasing surfactant concentration, the surface tension of the solution gradually decreases. The CMC reaches the lowest value when the surface tension reaches the minimum level (Abbasi et al. 2012; Luna et al. 2012; Dang et al. 2016).

The extracted biosurfactants were analyzed by thin-layer chromatography (TLC) and Fourier transform infrared (FTIR) spectroscopy (the detailed TLC analysis procedure is shown in the Supplementary material). For the FTIR analysis, the range of 4000 to 400 cm^{-1} was set to identify the functional groups.

The stability of biosurfactants against a wide range of temperature, pH, and NaCl concentration was evaluated. To examine the effect of pH, biosurfactant solutions (3 g L^{-1}) were prepared and treated with 6 mol L^{-1} HCl or 6 mol L^{-1} NaOH to adjust the pH values ranging from 1.5 to 13.0. Similarly, the biosurfactant solutions were treated at various temperatures (20–100 °C) and NaCl concentrations (0–90 g L^{-1}), and the surface tension of each solution (Csutak et al. 2012; Ayed et al. 2014).

Induction factors of biosurfactant production

Carbon sources (1%, *v*/*v*) including glucose, yeast extract, corn starch, or glycerol were selected as potential inducers and added in MSM medium for biosurfactant production. The induction effect of the nitrogen source on biosurfactant yield was evaluated by replacing $(NH_4)_2SO_4$ in MSM with several other nitrogen sources including beef extract, NH_4Cl, and peptone. All cultures were incubated in a rotary shaker at 30 °C with shaking (170 rpm) for 72 h. The resultant biosurfactant was evaluated according to surface tension. In this study, all surface tension measurements were conducted using the BZY-201 tension meter (Shanghai Fangrui Instrument Co. Ltd., China) at room temperature. Finally, to investigate the relationship between the surface tension and OD_{600} of *Leclercia* sp. B45 fermentation broth, *Leclercia* sp. B45 was incubated in MSM medium with optimum carbon and nitrogen sources for a period during which the surface tension and OD_{600} curve were measured and plotted.

Results

Isolation and identification of crude oil-degrading bacteria

Nine strains capable of degrading crude oil were isolated from activated sludge of an oil-field wastewater treatment plant. Among these, strain B45 (GenBank accession number LC170016) showed the highest degradation rate, which was closest to that of genus *Leclercia* (Table S1), and showed high homology with *Leclercia adecarboxylata* strain NBRC 102595 (Fig. S1) (information on other petroleum hydrocarbon-degrading strains is shown in Fig. S1, S2, and Table S1, S2).

The petroleum hydrocarbon utilization range of *Leclercia* sp. B45

The 2,6-DCPIP method was used to examine the petroleum hydrocarbon utilization range of *Leclercia* sp. B45. The results indicated that *Leclercia* sp. B45 prefer to use aliphatic hydrocarbons as sole carbon sources rather than naphthenes and aromatics (Table 2, Fig. S3) as the reaction systems containing C10, C16, C28, or crude oil become colorless after 48 h in the presence of *Leclercia* sp. B45. In contrast, the reaction systems with cyclohexane or phenanthrene as the sole carbon source remained blue after 72 h. Therefore, C10, C16, and C28 were considered as the preferred carbon sources for *Leclercia* sp. B45 and were used for further study.

Determination of the optimum *n*-alkane concentration for degradation

C10, C16, and C28 were selected as the representative of medium- and long-chain alkanes for the degradation experiment. GC-MS results showed that *Leclercia* sp. B45 could effectively degrade C10, C16, and C28. After 7 days of incubation, the degradation rate of C10 with an initial concentration of 0.4% and C16 with an initial concentration of 0.1% by *Leclercia* sp. B45 was over 90%. The degradation rate of C28 with an initial concentration of 0.1% reached 67%. The degradation rate decreased with increasing substrate concentration and chain length. The best biodegradation performance was observed with 0.4% of C10, 0.1% of C16, and 0.1% of C28 (Fig. 1).

Table 2 Results of the 2,6-DCPIP test at 24, 48, and 72 h of incubation

Substrate	24 h	48 h	72 h	Bacteria-free	Carbon source-free
n-Decane	−	+	+	−	−
n-Hexadecane	+	+	+	−	−
Octacosane	−	+	+	−	−
Cyclohexane	−	−	−	−	−
Phenanthrene	−	−	−	−	−
Light crude oil	+	+	+	−	−

"+" represents that the medium was colorless and positive, indicating that the microorganism could use the corresponding substrate as the sole carbon source. In contrast, "−" means that the medium has a blue color and reflects a negative reaction, indicating that the microorganism cannot use the corresponding substrate as the sole carbon source. The bacteria-free group and the carbon source-free group were used as blank controls, and the other reaction conditions were consistent

PCR amplification of degradation-related genes

To better understand the alkanes monooxygenase system contained in *Leclercia* sp. B45, two pairs of *alkB* primers, two pairs of *p450* primers, and one pair of *almA* degenerate primers were selected for PCR amplification. The *p450* and *almA* genes were not amplified using the selected primers, whereas one *alkB* gene was amplified (Fig. S4). The *alkB* gene has not previously been reported in the genus *Leclercia*. The PCR product was sequenced, and its nucleotide sequence was submitted to GenBank under accession number MH371472 (the BLAST results of the alkB gene in *Leclercia* sp. B45 are shown in Table S3). The phylogenetic tree (Fig. 2) illustrates that the *alkB* gene of *Leclercia* sp. B45 is closely related to that of *Pseudomonas* sp. CW-a2 alkane monooxygenase gene.

Transcriptional patterns of the alkB gene in *Leclercia* sp. B45 induced by *n*-alkane

To further understand the transcriptional patterns of the *alkB* gene in *Leclercia* sp. B45 induced by *n*-alkane, RT-qPCR was performed. The growth curve and residual substrate concentration curve were also plotted (the experimental grouping is shown in Table S4, the results of RNA quality testing are shown in Fig. S5, and the amplification and dissolved curves are shown in Fig. S6). As shown in Fig. 3, all conditions of 0.4% C10, 0.1% C16, or 0.1% C28 could upregulate the *alkB* gene in *Leclercia* sp. B45. On the 7th day of incubation, the transcription level of the *alkB* gene in the C10, C16, and C28 groups was at least > 40 times higher than that in the NaAc blank control, with the C10 group exhibiting a 150-fold upregulation. Furthermore, as the chain length of the alkane increased, the transcription level decreased (Fig. 3). The growth experiment showed that the strain reached late exponential phase after 4 days of incubation and was about to enter the stationary phase, whereas the substrate concentration decreased quickly from 25 to 1.0 mg L^{-1} (Fig. 4). Together, the above results indicated that C10, C16, and C28 induced *alkB* gene expression in *Leclercia* sp. B45 at the late exponential phase and the stationary phase, when the substrate concentration was low, and the cell density was high. The transcription patterns of *alkB* demonstrated that the transcription

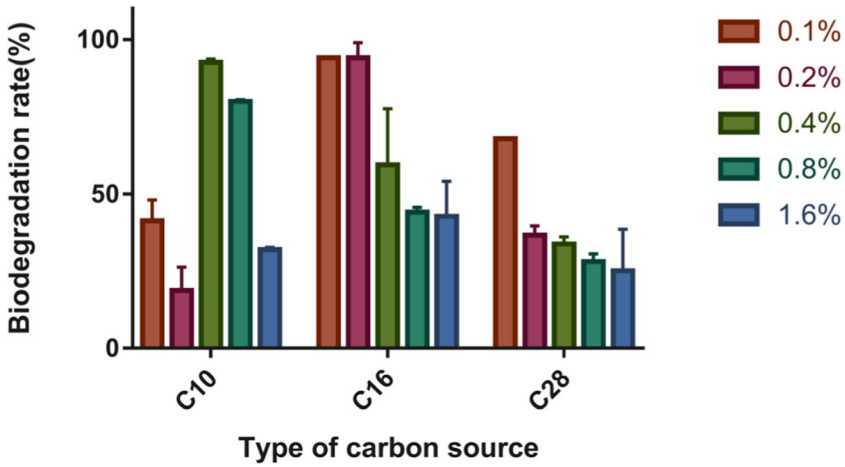

Fig. 1 Degradation performance of C10, C16, or C28 at different initial concentrations by *Leclercia* sp. B45 after 7 days of incubation. All data are shown as the mean with standard deviation (SD). The error bar represents the standard deviation of 3 biological replicates

level of *alkB* remained low on the 4th day, but then the level rose rapidly and reached its maximum on the 7th day of incubation.

Characterization of biosurfactants

Biosurfactant production capacity of *Leclercia* sp. B45

As shown in Fig. S7A, the fermentation supernatant of *Leclercia* sp. B45 showed high oil discharge performance (the result of the emulsification test is shown in Fig. S7B). The emulsification index was determined as 25.5%. Thus, the biosurfactant produced by *Leclercia* sp. B45 exhibited excellent activity.

Determination of CMC

As shown in Fig. S8, the CMC of the biosurfactant produced by B45 was determined as 1.2 mg mL^{-1}.

TLC and FTIR spectra

TLC spectra showed a light blue spot on the silica gel plate with an *Rf* value of 0.56. The surfactant could be characterized as a phospholipid based on the color of the spot (Fig. S9). FTIR analysis showed that a peak at around 1080 cm^{-1} was due to the absorption of P–O–C bonds and confirmed the phospholipid nature of the biosurfactant (Fig. 5). Combined with the TLC results, the biosurfactant produced by B45 was identified as a phospholipid.

Effects of salinity, pH, and temperature on biosurfactant stability

As shown in Fig. 6, the surface tension of the biosurfactant solution remained stable at about 35 mN m^{-1} when the pH value was adjusted in the range of 1.5–12. When the pH value increased further, the surface tension drastically increased. Overall, the surface tension of the solution was minimal at pH 7.0. Moreover, the surface tension of the biosurfactant

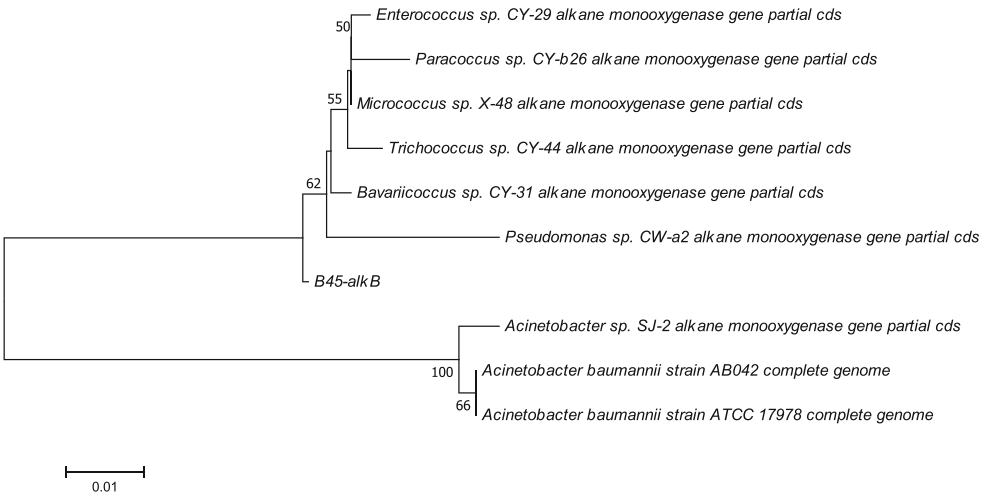

Fig. 2 Phylogenetic tree of the *alkB* gene sequence of *Leclercia* sp. B45 and other similar sequences. The tree was constructed using the neighbor joining algorithm. The numerals indicate the branching order determined by bootstrap analysis (values over 50% were considered significant)

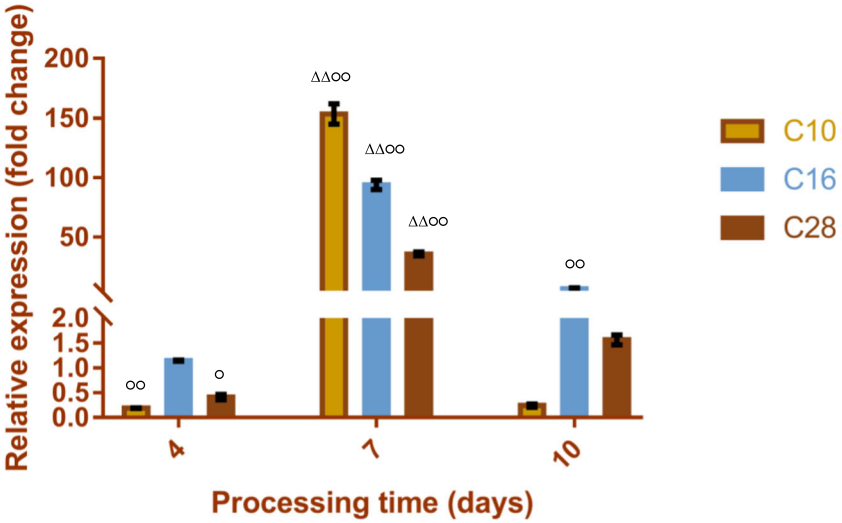

Fig. 3 Transcription levels of the alkane monooxygenase gene (*alkB*) in *Leclercia* sp. B45 induced by different alkanes and incubation times. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. $^{\Delta\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ (the transcription levels of the *alkB* gene at the 7th and 10th days in each group compared with those at the 4th day of each group); $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ (the transcription level of the *alkB* gene at days 4, 7, and 10 in the experimental group compared with that at days 4, 7, and 10 in the control (NaAc) group, respectively)

solution was maintained at about 35 mN m^{-1} even at a high salinity of 90 g L^{-1}, indicating a strong alkali resistance of the biosurfactant within the detection range.

To assess the effect of temperature on biosurfactant stability, the biosurfactant solution was treated in the range of 20–100 °C. The surface tension was maintained at about 35 mN m^{-1} within the range of 20–80 °C, indicating that the biosurfactant exhibited a strong high-temperature tolerance (Fig. 6).

Inducers of biosurfactant production

As shown in Fig. 7a, when C16 was selected as the carbon source, the surface tension of the fermentation broth exhibited the greatest reduction. Meanwhile, the highest OD_{600} value was observed in the culture with yeast extract as the sole carbon source, suggesting that the production of biosurfactant produced by *Leclercia* sp. is not growth associated. Generally, the C16-induced *Leclercia* sp. B45 exhibited 1.6-fold higher surfactant reduction than when induced by other carbon sources (e.g., glucose and glycerol), indicating that the produced surfactant as induced by C16 had better performance than when induced by other non-hydrocarbon sources. Similarly, NH_4Cl was more likely to act as a nitrogen source inducer for *Leclercia* sp. B45 surfactant production (Fig. 7b). Under optimum conditions, the surface tension of the fermentation broth reduced gradually from 74 to 35 mN/m within 48 h with the increase of OD_{600}. Then, the surface tension of the fermentation broth stabilized with a small fluctuation between 33 and 35 mN/m (Fig. 7c). By analyzing the range of orthogonal test results (Table S5), levels corresponding to the lowest *K* value were selected as the optimum condition. Therefore, the optimum temperature, salinity, and pH value of surfactant production were 30 °C, 10 g L^{-1}, and 7, respectively.

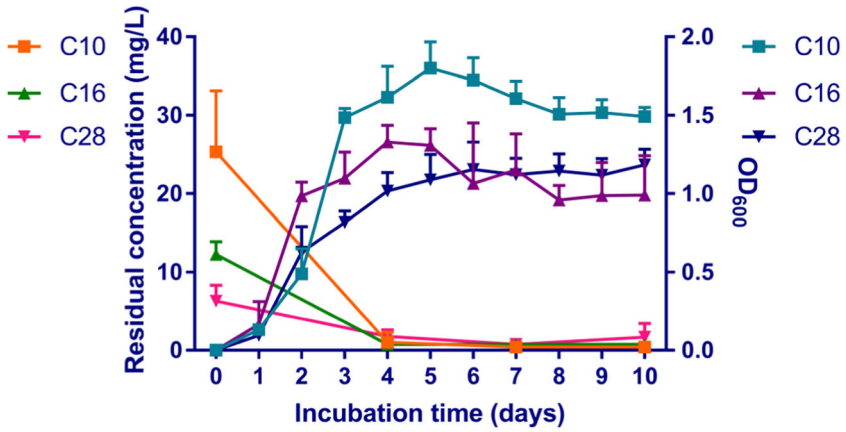

Fig. 4 The growth curve and the residual concentration curve. Right axis, growth curve of *Leclercia* sp. B45 cultured for 10 days in MSM with C10, C16, or C28 as the sole carbon source. Left axis, residual concentration curve of the substrates with the increase of incubation time. All data are shown as the mean with standard deviation (SD). The error bars represent the standard deviation of 3 biological replicates in each site

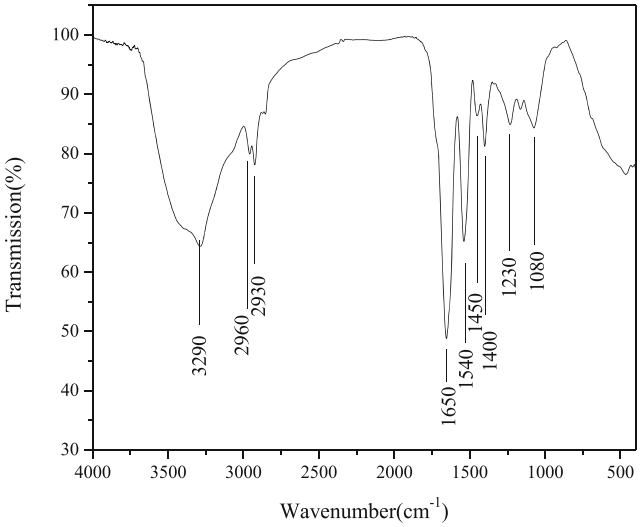

Fig. 5 Fourier transform infrared spectroscopy of biosurfactant

Discussion

In this study, a hydrocarbon-degrading bacterial strain classified as *Leclercia* sp. B45 was isolated. The hydrocarbon utilization experiment showed that *Leclercia* sp. B45 could utilize aliphatic hydrocarbons and light crude oil, but not naphthenes and aromatics. Further experiments showed that the degradation rate of C10, C16, and C28 by *Leclercia* sp. B45 could reach 92.6%, 94.1%, and 67.8%, respectively. Notably, the degradation of hydrocarbons by genus *Leclercia* has only previously been reported to involve aromatic compounds such as pyrene by *Leclercia adecarboxylata* PS4040 (Sarma et al. 2004; Sarma et al. 2010; Perchet et al. 2008). Although the alkane monooxygenase system contained in *Leclercia* sp. B45 was investigated using various primers for *alkB*, *p450*, and *almA* (degenerate), *p450* and *almA* genes were not amplified. This study identified a fragment with a high sequence similarity (80–95%) to the corresponding alkB region available from GenBank by BLAST comparisons, indicating that it belonged to the alkB gene family. To date, no study has reported an alkane monooxygenase in this genus, even an alkB gene as identified herein. Liu et al. (2011) reported that *Alcanivorax dieselolei* strain B-5 contains several alkane monooxygenase systems (AlkB, CYP, AlmA, LadA), while some other strains contain only one alkane monooxygenase system (AlkB) with more than one alkB homologues, such as *Pseudomonas aeruginosa* PAO1 (alkB1 and alkB2) (Holloway 1969; Smith et al. 2002), *Rhodococcus erythropolis* Q15 (alkB1–4) (Whyte et al. 1998), and *Acinetobacter* sp. M-1 (alkMa and alkMb) (Tani et al. 2001). The phylogenetic tree shows that the *alkB* gene detected in *Leclercia* sp. B45 is closely related to that of *Pseudomonas* sp. CW-a2 (Fig. 5). The sequence similarity of the two alkane monooxygenase genes is 97%; however, the query cover was only the 80%. The query covers with the other five *alkB* genes were also low (80–84%), suggesting that the *alkB* gene in *Leclercia* sp. B45 is likely to represent a novel gene.

We also showed that the transcription of the *alkB* gene induced by C10, C16, or C28 was significantly upregulated compared to that in the NaAc group. The relative copy number decreased with the increase of alkane chain length, which is consistent with the *n*-alkane biodegradation kinetics of the *Leclercia* sp. B45. Notably, some researchers have reported that AlkB contains a hydrophobic pocket composed of six transmembrane proteins, such that the terminal methyl group of the alkane molecule entering this pocket can be correctly positioned (van Beilen et al. 2005). It has also been reported that the AlkB alkane hydroxylase oxidizing substrate range is related to the size of the amino acid side chains in the hydrophobic pocket, with larger amino acid side chains allowing relatively shorter alkane chain lengths into the hydrophobic pocket (Rojo 2009). This detail may explain why the *alkB* gene could be induced by specific chain lengths of *n*-alkanes. Some researchers have reported that AlkB contains a hydrophobic pocket composed of six transmembrane proteins, such that the terminal methyl group of the alkane molecule entering this pocket can be correctly positioned (van Beilen et al. 2005).

Moreover, the results showed that the *alkB* gene of B45 was also upregulated under the induction of C28. Most researchers have considered that the AlkB-related alkane

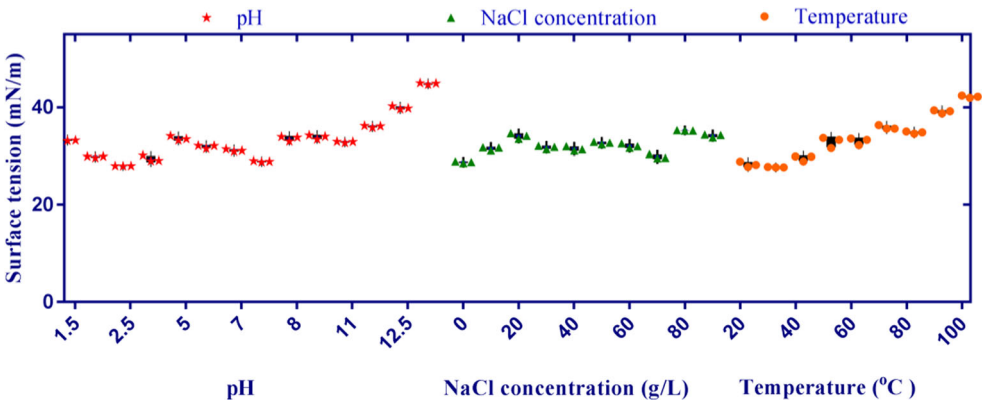

Fig. 6 Effects of pH, NaCl concentration, and temperature on biosurfactant stability

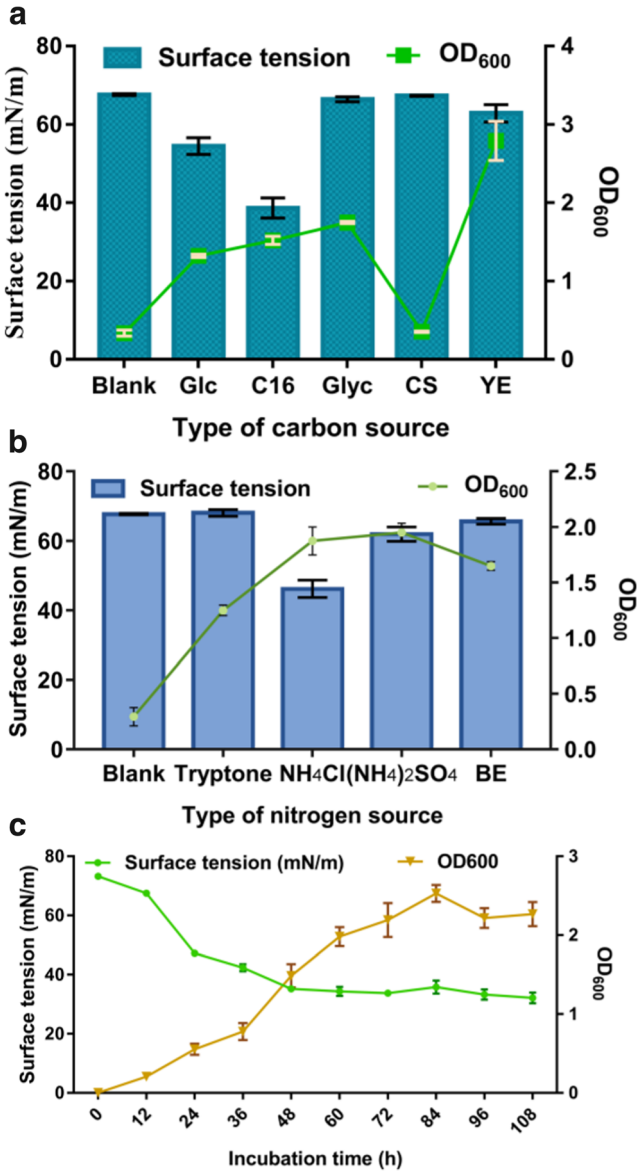

Fig. 7 Characterization of biosurfactant produced by *Leclercia* sp. B45. **a** Decrease in the surface tension of the fermentation broth induced by different carbon sources (Glc, glucose, C16; Glyc, glycerin; CS, corn starch; or YE, yeast extract) and the corresponding OD_{600} after 72 h of incubation. **b** Effect of different nitrogen sources (tryptone, NH_4Cl, $(NH_4)_2SO_4$, or BE, beef extract) on the surface tension reduction and OD_{600} after 72 h of incubation. **c** Surface tension change and growth curve during the 108-h incubation of B45 with C16 as the sole carbon source and NH_4CL as the sole nitrogen source

hydroxylases are associated with the degradation of a relatively narrow range of substrates (C5–C16) (van Beilen et al. 2005), e.g., the *Pseudomonas putida* GPo1 AlkB alkane hydroxylase could oxidize propane, *n*-butane (Johnson and Hyman 2006), and C5 to C13 alkanes (van Beilen et al. 2005), but not long-chain alkanes. Similarly, the recent report of Liu et al. showed that the *alkB1* and *alkB2* genes in *Alcanivorax dieselolei* strain B-5 responded in the presence of C12–C26 alkanes (Liu et al. 2011). Therefore, the present study sheds new light on the substrate regulation range of the *alkB* gene.

Under the induction of alkanes, we found that the transcription level of the *alkB* gene in *Leclercia* sp. B45 increased rapidly during the late exponential phase and reached the highest value at the stationary phase. Notably, before *Leclercia* sp. B45 entered the stationary phase, the substrate concentration had decreased to 1/20 of the initial concentration, and the transcription level of the *alkB* gene increased dramatically during the stationary phase. Therefore, it was speculated that the upregulation of *alkB* gene transcription was associated with the highly efficient utilization of the low concentration of carbon sources by strain B45. Similarly, Hassanshahian et al. (2014) confirmed that the copy number of alkane monooxygenase genes such as alk-B1 or alk-BT was positively related to the concentration of crude oil, since it was increased when the crude oil was present and decreased after the concentration of crude oil decreased. Other related studies have shown that the stage of cell growth in which the *alkB* gene is massively transcribed depends on the type of gene, the dissolved oxygen of the medium, and other unknown factors. For example, some studies on the molecular mechanisms of the *alk* operon in *P. putida* have shown that the positive regulator AlkS is expressed in a small amount in the exponential phase and is expressed in large quantity when cells enter the stationary phase (Canosa et al. 1999). Chen et al. (1996) considered that *alk* genes in the *P. putida* GPo1 strain are inactivated when they are not vital to the growth and impose detrimental effects on host cells. However, the expression of the *alk*BFGHJKL gene, which is associated with alkane utilization, can be upregulated by *n*-alkanes. In turn, Marín et al. (2003) reported the expression of the *alkB1* gene in *P. aeruginosa* during the late exponential phase, and the expression of the *alkB2* gene during the early exponential phase, which was considered to be related to gene type and substrate. They considered that *alkB1* may be more conducive to the use of oxygen, as *alkB1* is upregulated when the cell density is the highest and the oxygen concentration is limited.

In addition, no report on the production of biosurfactants by this genus of bacteria, or even by enteric bacteria, has been published. Our study presents *Leclercia* sp. B45 as a new biosurfactant producer, with promising results. In particular, the biosurfactants produced by *Leclercia* sp. B45 showed good oil discharge performance and surface activity, with the diameter of the clear zone reaching 6.2 cm and the surface tension of water being reduced to 27.24 mN m^{-1}, representing better results than those reported in other related studies (Velioglu and Ozturk Urek 2014; Soudi et al. 2010). Moreover, in the present study, the biosurfactants produced by *Leclercia* sp. B45 could reasonably tolerate various environmental conditions such as high temperature (20–80 °C), salinity (0–90 g L^{-1}), and strong acids and alkalis (pH range of 2–11). In addition, the yield of biosurfactants extracted was 3.72 g L^{-1}, which is higher than that reported in *Gordonia*

westfalica GY40 by Laorrattanasak et al. (2016) (1.85 g L^{-1}) and that reported in *Bacillus licheniformis* by Kumar et al. (2016) (1.796 g L^{-1}).

The biosurfactant-producing ability of *Leclercia* sp. B45 could be better induced by C16 than other non-petroleum hydrocarbon groups such as glucose and glycerinum (Fig. 4a). Similarly, in a previous study of biosurfactant production by *Dietzia maris* strain WR-3 upon culturing using several different carbon and nitrogen sources as growth substrates, the best biosurfactant performance was observed when the organism was incubated with *n*-hexadecane and nitrate ions (Nakano et al. 2011). Numerous reports also exist regarding the promoting effect of biosurfactants on the degradation ability of various strains. For example, Beal and Betts (2000) discovered that *Pseudomonas aeruginosa* transports alkanes into cells via emulsification. Noordman and Janssen (2002) speculated that *P. aeruginosa* strain UG2 has an energy-dependent system that correlates with the rapid uptake of hydrocarbons in the presence of biosurfactants. Hommel (1990) concluded that biosurfactants may play an essential role in the microbiological utilization of medium- and long-chain alkanes. Overall, it was considered that the majority of oil-decomposing bacteria could produce various biosurfactants to promote oil emulsification (Ron and Rosenberg 2002; Shao 2011; Qiao and Shao 2010), thereby facilitating the degradation of hydrocarbon pollutants.

Finally, the major strengths of this study were that (1) we isolated the bacteria *Leclercia* sp. B45 from an oil-field wastewater treatment plant that showed high C10, C16, and C28 alkane degradation rate; (2) the transcription of the alkB gene in B45 was induced by C10, C16, or C28 and its transcription decreased with increased alkane chain length while the alkB gene expression increased during late culture; (3) C16-induced B45 effectively produced biosurfactant with high stability; and (4) B45 biosurfactant, alkB gene, and the broad degradative spectrum are novel findings in the genus *Leclercia*.

It is possible, however, that considering the limitation of the selected primers used in this experiment, there may be other alkane degradation-related genes in *Leclercia* sp. B45 that have not yet been detected; thus, further study is required to explore other potential degradation genes.

Conclusion

Leclercia sp. B45 showed high-efficiency *n*-alkane degradation rate. The *alkB* gene in *Leclercia* sp. B45 is likely to represent a novel gene whose transcription level was significantly upregulated when induced by *n*-alkane. Notably, the *alkB* gene of B45 was also upregulated under the induction of C28, which shed new light on the substrate regulation range of the *alkB* gene. Furthermore, it was speculated that the upregulation of *alkB* gene transcription was associated with the highly efficient utilization of the low concentration of carbon sources by strain B45. Finally, the *Leclercia* sp. B45 could be better induced by C16 to produce more biosurfactant in order to promote oil emulsification, thereby facilitating the degradation of hydrocarbon pollutants.

Overall, the findings in this study are critical to better understand the mechanism of alkane degradation in *Leclercia* sp. B45 and point to the need for further research to explore the relationship between biosurfactant production and alkane degradation in *Leclercia* sp. B45. Regarding the excellent biosurfactant-producing performance of this strain and alkane degradation ability, we considered that this strain has great application potential in petroleum hydrocarbon pollution control.

Author Contribution Yiying Shuai and Hanghai Zhou conducted the experiments and the analysis, and drafted the initial version of the manuscript. Qinglin Mu helped write the molecular biology section. Dongdong Zhang helped modify the whole paper. Ning Zhang helped the analysis of molecular biology data. Jingchun Tang helped write the discussion section of the manuscript as well as constructing the phylogenetic tree for the bacterial strains. Chunfang Zhang conceived, designed, and supervised the research work. All authors read and approved the final manuscript. This manuscript has not been published or presented elsewhere in part or entirety and is not under consideration by another journal. The statements provided by all authors are true.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants performed by any of the authors.

Informed consent This study does not require informed consent.

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