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Rhamnolipid production from waste cooking oil using *Pseudomonas* SWP-4

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Graphical abstract

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Highlights

- Pseudomonas SWP-4 could produce rhamnolipid from waste cooking oil.
- Pseudomonas SWP-4 accumulated rhamnolipid steadily even in decline phase.
- Pseudomonas SWP-4 gave a maximum rhamnolipid yield of 13.93 g/L.
- The produced rhamnolipid showed excellent surface activity and stability.
- Biodegradation of waste cooking oil is a promising way of waste treatment.

Abstract

The present work aims to produce rhamnolipid from waste cooking oil (WCO) using a newly isolated bacterium named *Pseudomonas* SWP-4. SWP-4 was a high-yield strain that could accumulate rhamnolipid steadily even in decline phase and gave a maximum rhamnolipid yield of 13.93 g/L and WCO utilization percent around 88%. The critical micelle concentration of the produced rhamnolipid was only 27 mg/L and its emulsification index against n-hexadecane reached around 59%. Moreover, it reduced the surface tension of water from 71.8 mN/m to 24.1 mN/m and the interfacial tension against n-hexadecane from 29.4 mN/m to 0.9 mN/m. Results of biosurfactant stability show the rhamnolipid was effective when the salinity was lower than 8% and pH value ranged from 4 to 10, and it was quite thermostable based on thermal gravity analysis. Furthermore, it maintained high surface activity even after incubation under extreme conditions *i.e.* pH of 4.0, salinity of 8% and temperature of 80℃ for half a month. Based on free fatty acids metabolism analysis, *Pseudomonas* SWP-4 consumed palmitic acid, oleic acid and linoleic acid chiefly. All these characteristics demonstrate bioconversion and biodegradation of WCO by *Pseudomonas* SWP-4 is a promising and commercial way of rhamnolipid production and waste treatment.

Key words: Rhamnolipid; Waste cooking oil; Production kinetics; Bioconversion; Biodegradation; Waste treatment.

Abbreviations: WCO, waste cooking oil; CMC, critical micelle concentration; TGA, thermal gravity analysis; CTAB, cetyl trimethyl ammonium bromide; LB, Luria-Betrani; C/N, carbon nitrogen ratio; COF, cell-oil-free; TLC, thin layer chromatography; FT-IR, Fourier transform infrared spectroscopy; NMR, nuclear magnetic resonance; ESI-MS, Electrospray ionization mass spectra; E_{24},

emulsification index; $OD_{600,}$ optical density at 600 nm; GC-MS, gas chromatography-mass; SEM, scanning electron microscope; Rf, retardation factor; TG, thermal gravity; DSC, differential scanning calorimetry; DTG, derivative thermogravimetry.

1.Introduction

The continuous increase of waste cooking oil (WCO) from the household and catering industry becomes a growing problem all over the world. Thereinto, about seven million tons of WCO is generated per year in China. Besides fatty glyceride and salt, WCO mainly contains *cis* or *trans* free fatty acids together with a few polymers and putrefactions due to oxidation and hydrogenation [1]. However, this oil is often used for animal feeds, thus such unhealthy substances may move down the food chain towards human, which can result in food safety problems. What's worse, a large amount of WCO is poured into sewers directly without proper treatments, which negatively affects the downstream biological wastewater treatment process or clogging, especially in the cold winter. Therefore, the effective disposal of WCO has become an urgent problem to be solved.

In light of the present emphasis placed on the management and recycling of the wastes, developing the practical processes to transform WCO into products has been a great concern. WCO is a kind of cheap and available resource that can be used for biodiesel production, which is a very hot research topic in last decade. However, biodiesel production is quite heavily dependent on fossil fuel price. In terms of current volatile market for fossil fuel, it is worthwhile to develop alternative process to utilize WCO. Recently, production of biosurfactants from renewable resources has become increasingly popular due to their low toxicity, high biodegradability and ecological acceptability [2, 3]. Compared with the most of traditional chemosynthetic surfactants, biosurfactants demonstrate higher surface activity and lower critical micelle concentration (CMC) [4, 5]. Moreover, biosurfactants are usually effective even at extreme temperature, salinity and pH [6, 7]. However, large-scale biosurfactants production has not been applied due to high production cost, in which carbon source contributes significantly [7]. Thus, using a cheaper carbon source such as WCO to

produce biosurfactants can lower such expenses especially for industrial scale production [8].

Among various of biosurfactants, rhamnolipid is the most typical with excellent surface activity and wide application. Rhamnolipid was firstly reported in 1949 by Jarvis and Johnson using *Pseudomonas aeruginosa* as production strain [9], consisting of one (mono-rhamnolipid) or two (di-rhamnolipid) L-rhamnose moieties (hydrophilic group) linked to one up to four β-hydroxy aliphatic moieties (hydrophobic group) [10]. Afterwards, other bacteria such as genus *Burkholderia*, *Acinetobacter*, and *Renibacterium* were reported that could produce rhamnolipid as well, but the surface activity, homologues and productivity of rhamnolipid can be different from one species to another [11-13]. Nevertheless, the pathogenic nature of most of these bacteria was a major obstacle to commercial production of rhamnolipid, and WCO cannot be utilized by some of them. To the contrary, genus *Pseudomonas* was able to grow on waste oil as the sole carbon source and accumulate rhamnolipid [14-18], and that waste oil had positive effects on rhamnolipid synthesis and bacterial growth [14]. To date, most of the studies about rhamnolipid production from WCO were concentrated on using *Pseudomonas aeruginosa* as production strain, and other species of genus *Pseudomonas* were rarely reported.

In present study, we isolated a new bacterium of *Pseudomonas* SWP-4 from WCO-contaminated sludge samples, showing a homology of 99.8% with *Pseudomonas chlororaphis* (Z76673). Gunther *et al*. [19] has reported *Pseudomonas chlororaphis* was a strain of the nonpathogenic bacterial species that is capable of producing rhamnolipid. Since then, there is no report concerning rhamnolipid production from WCO using *Pseudomonas chlororaphis*. Thus this is the first time to particularly introduce the biodegradation and emulsification of waste cooking oil using *Pseudomonas* SWP-4, especially when considering that the substrate was mainly consisted of wastes that would otherwise constitute an environmental disposal problem. What's more, most of the other reports about rhamnolipid production from WCO using *Pseudomonas aeruginosa* were just focused on rhamnolipid production, and little work has been done on relevant kinetics and free fatty acids degradation, so the lack of detailed reports prompted us to investigate this study. Meanwhile, characterization, surface activity and stability of the purified product were also investigated in this work. Last but not least, the thermostability of rhamnolipid characterized by thermal gravity analysis (TGA) is not well understood, thus we have done this research.

2. Materials and methods

2.1 Materials

Tryptone and yeast extract were purchased from OXOID Company (Hampshire, England). The other chemicals (analytical reagent grade) were purchased from Kelong chemical regent factory (Chengdu, China), and deionized water (>18.25 MΩ cm) was used to make solutions. Standard rhamnolipid and WCO (a kind of vegetable oil; the major compositions were palmitic acid, oleic acid and linoleic acid) were provided by Biogas Institute of Ministry of Agriculture (Chengdu, China). The oil was initially precipitated for 24 h and then the upper foam and the bottom solid precipitate were discarded, and the rest of oil was collected as carbon source for microbial cultivation.

2.2 Isolation of biosurfactant-producing microorganisms

WCO-contaminated sludge samples used in this section were taken from the local sewer. Firstly, 20 g sludge samples were suspended in 100 mL sterile phosphate-buffered saline (pH 7.2), and then 0.5 mL of the supernatant was inoculated into the cetyl trimethyl ammonium bromide (CTAB) methylene blue agar plate with 2% (m/v) WCO. After being incubated at 25℃ for 3 days, the colonies on the plate with dark blue halos were selected as candidate biosurfactant producers [16, 20], and all the pure isolates were maintained and stored as described by Al-Wahaibi *et al*. [5]. Afterwards, the screened strains were cultivated in Luria-Bertani (LB) medium for 24 h at 30℃, 150 rpm on a rocking incubator (QYC-200, Shanghai, China). Surface tension of each medium was measured by a digital tensiometer (DT-102, Zibo, China) and oil spreading circle measurement was conducted in accordance to Youssef *et al*. [21]. Eventually, the most outstanding biosurfactant producer was selected as production strain, and then it was identified based on physiological and biochemical identification, 16S rDNA sequence analysis and phylogenetic analysis. Morphological character of the selected strain was studied by a JEOL scanning electron microscope (JSM-7500F, Japan). 16S rDNA sequence analysis was conducted according to Pasumarthi *et al*. [22], and phylogenetic analysis was conducted using Neighbor-Joining algorithim.

2.3 Optimization of culture conditions

Seed inoculum was prepared by cultivating of the microorganisms for 12 h in LB medium, while the original culture medium used for biosurfactant production contained the following components: 2 g/L NH_4NO_3, 5 g/L NaCl, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.3 g/L $MgSO_4·7H_20$, 0.1 g/L $FeSO_4·7H_20$, 0.1 g/L $CaCl_2$, and 20 g/L WCO. All media was autoclaved at 121℃ for 20 min before use. The experiments were conducted in Erlenmeyer flasks (250 ml) with 50 ml culture medium at 35℃ and 150 rpm. In this section, seven factors were investigated to obtain the optimum culture conditions, which included inoculum ratio (1–5%, v/v), nitrogen source (NH_4NO_3, NH_4Cl, $NaNO_3$, urea, yeast extract and tryptone), carbon nitrogen ratio

(C/N) of 8, 10, 13.3, 20 (used the optimum nitrogen source), concentration of the WCO (10–60 g/L, kept the optimum C/N unchanged), and concentration of the mineral elements (Ca^{2+} and Fe^{2+}: 0–0.4 g/L; Mg^{2+}: 0–1.2 g/L). SWP-4 was cultured in the original culture medium for 1 day, and then the culture broth was centrifuged for 10 min at 3000 rpm to remove bacterial cells and residual oil for the measurements of surface tension and product yield.

2.4 Biosurfactant recovery and purification

The collected cell-oil-free (COF) broth was acidified to pH 2.0 using HCl (6 M) and kept at 4℃ overnight for the precipitation of biosurfactant, which was harvested by centrifugation and washed three times with acidic water (pH 2.0). Subsequently, the precipitate was purified by n-hexane to remove residual oil and extracted with the same volume of ethyl acetate. Finally, the pure product was obtained by rotary evaporation [23, 24].

2.5 Chemical characterization of the purified biosurfactant

The type of the purified biosurfactant was identified by thin layer chromatography (TLC). In this part, chloroform was used as solvent, and silica gel plate (GF254, Qingdao, China) was used as TLC plate. This experiment was conducted in a sealed container with the developing solvent comprising of chloroform/methyl alcohol/acetic acid (65:15:2, v/v/v), using phenol-sulfuric acid (0.3 g phenol and 0.5 g sulfuric acid (98%) dissolved in 9.5 mL absolute ethyl alcohol) as developing agent [25, 26].

Then, the purified biosurfactant was subjected to further characterization with Fourier transform infrared spectroscopy (FT-IR), Nuclear magnetic resonance (NMR) and Electrospray ionization Mass spectra (ESI-MS). FT-IR spectra were collected from 500 to 4200 wave numbers (cm^{-1}) by averaging 12 scans at a resolution of 2 cm^{-1} on a FT-IR spectrometer (WQF-520, Beijing, China)[18, 23]. Both proton NMR (^{1}H NMR) and carbon NMR (^{13}C NMR) were carried out on a Bruker nuclear magnetic resonance (Ascend-400, Germany), using chloroform-d as the solvent. ESI-MS were recorded on a Thermo Fisher Scientific mass spectrometer (TSQ Quantum ultra, USA) in the negative ion mode, using methanol as the solvent.

2.6 Surface activity measurements

The surface activity of biosurfactant produced by isolated bacteria was evaluated by emulsification index (E_{24}), surface tension, interfacial tension and CMC, which were four most important parameters to evaluate biosurfactant. E_{24} was determined by adding 3 mL n-hexadecane to the same volume of COF broth (cultivation time of 48 h) in the test tube, and then the mixture was mixed by vortex for 3 min and left to stand at 25℃ for 24 h prior to measurement. E_{24} was expressed as the percentage of the height of the emulsified layer divided by the total height of the liquid column [27].

Surface tension and interfacial tension (against n-hexadecane) were measured by the digital tensiometer using the ring method [28]. CMC was determined from the inflection point of surface tension versus biosurfactant concentration (5–120 mg/L).

2.7 Biosurfactant stability studies

48 h-old culture broth (COF) was used in this part to evaluate the effects of pH, salinity, and temperature on biosurfactant stability (surface tension and E_{24} (against n-hexadecane)). To study the salinity stability, different concentration of NaCl (0–20%, w/v) was added to each sample. To study the pH stability, the pH value of each sample was adjusted to 2, 4, 6, 7, 8, 10 and 12 by using 1 M HCl and 1 M NaOH, respectively. To study the temperature stability, each sample was respectively incubated at different temperature (25℃, 40–100℃ in water bath and 150℃ in dryer) for 1 h, and the sample was also subjected to autoclave (121℃, 20 min). Meanwhile, we also investigated the effects that these three parameters acted on the biosurfactant simultaneously. In this section, the sample was left to incubate for half a month under the extreme conditions *i.e.* pH of 4.0, salinity of 8% and temperature of 80℃. In addition, to evaluate the thermal properties of the purified biosurfactant, TGA was conducted in a air flow of 20 cm^3/min at ramping rate of 10℃/min with a Netzsch simultaneous thermal analyzer (STA-449 F3, Germany), and the sample loading was typically 20-30 mg.

2.8 Kinetics of biosurfactant production

To study the kinetics of biosurfactant production, SWP-4 was cultivated in the optimum culture medium at 35℃, 150 rpm. Bacterial growth was studied by measuring the optical density at 600 nm (OD_{600}) of the oil-free broth (diluted by six times with the medium) every 2 h for 60 h by an ultraviolet spetrophotometer (V-1800, Kyoto, Japan). Surface tension, pH measurement, WCO utilization and biosurfactant concentration were measured every 12 h for 120 h. Surface tension was detected by the digital tensiometer and pH was measured by a Youke pH meter (PHS-3E, Shanghai, China). WCO utilization was determined by mass loss method, and the utilization was expressed as the percentage of the weight loss of WCO divided by the initial weight of WCO. Biosurfactant concentration was determined by anthrone-sulfuric acid colorimetric method [29, 30], and the rhamnose value was calculated from the standard curves prepared with L-rhamnose (10–90 mg/L). The concentration of rhamnolipid was determined by multiplying rhamnose value by a coefficient of 3.4, obtained from the correlation of pure rhamnolipid/rhamnose [31].

2.9 Analysis of free fatty acids degradation

Residual oil sample was respectively collected by centrifuging the culture broth at specific time intervals (36 h and 60 h) and extracting with n-hexane. Firstly, each

sample was preprocessed with methyl esterification by using sodium hydroxide-methyl alcohol (0.5 M) [32, 33]. Then, 1 μL organic phase was injected in an Agilent gas chromatography-mass (GC-MS) spectrometer (7890A/5975C, USA) with a split ratio of 20:1. Meanwhile, the original WCO was used as control. This experiment was conducted by the following temperature programming: initial temperature was 50℃, then raised at a rate of 20℃/min to 160℃, at which maintained for 5 min; a second ramp was then applied at 5℃/min to 200℃, at which maintained for 3 min; the last ramp was conducted at 3℃/min to 250℃, at which maintained for 1 min.

3. Results and discussion

3.1 Isolation and characterization of biosurfactant-producing microorganisms

An excellent biosurfactant-producing microorganism means that its metabolite must to exhibit high emulsifying ability as well as be able to reduce the surface tension of water to a low value. In this section, fifteen dark blue halos were observed on CTAB methylene blue agar plate after incubation at 35℃ for 3 days, and then all the colonies were purified. Afterwards, the screened strains were enriched in LB medium for 24 h. The results of oil spreading experiment showed that all the screened strains could form emulsification circle, but only five strains formed large circles that the diameters were larger than 0.8 cm (Table 1). It could be obviously found in Table 1 that the strain named SWP-4 formed the largest oil spreading circle and gave the maximum reduction of surface tension. Therefore, SWP-4 was selected as the present production strain and conducted to further characterization.

The result of Gram-stain test showed that SWP-4 was a Gram-negative bacterium. Physiological and biochemical identification showed that its growth temperature ranged from 25 to 42℃, with the optimum temperature at 35℃; adaptation pH value was 4-9, and the optimum value was found to be 7; salt tolerance was proven from 0 to 8% (m/v) while the optimum salinity was observed at 0.5%. The morphological character of SWP-4 indicated by scanning electron microscope (SEM) was shown in Fig. 1a, from which we can see that SWP-4 presented a short-rod shape without spores, and had a size of 0.4 μm ~ 0.6 μm × 1.0 μm ~ 1.2 μm (diameter×length). Eventually, based on 16S rDNA sequence analysis, the phylogenetic tree was constructed by Neighbor-Joining algorithim (Fig. 1b). Thus, it was identified as *Pseudomonas* SWP-4, showing a homology of 99.8% with *Pseudomonas chlororaphis* (Z76673).

Table 1

Diameter of the oil spreading circle and surface tension of the 24 h-old culture broth

produced by the screened strains, cultivated in LB medium at 30℃, 150 rpm[a].

Fig. 1. (a) Morphology of the isolated bacteria, SWP-4 shown by SEM. (b) Phylogenetic tree of the isolated bacteria, SWP-4, constructed by Neighbor-Joining algorithim.

3.2 Optimization of culture conditions for SWP-4

3.2.1 Effect of inoculum ratio on biosurfactant production

The effect of inoculum ratio on biosurfactant production was studied as shown in Fig. SM1a. It could be clearly seen that the optimum inoculums was 2% (v/v), and the inhibiting effect increased with the rise of inoculum ratio. Low inoculum ratio might extend lag phase of bacterial growth while high inoculum ratio could result in the limitation of nutrient substance and oxygen. The influence of inoculum ratio on the productivity and economics of the bioprocesses has also been shown in literature [34]. Thus, the optimum inoculum ratio was used in the following studies.

3.2.2 Effect of nitrogen sources on biosurfactant production

It seems from Fig. SM1b that all organic nitrogen sources produced similar results while inorganic nitrogen source resulted in big difference in terms of surface tension and biosurfactant concentration. The best nitrogen source for the present biosurfactant production was $NaNO_3$, followed by yeast extract. This result shows nitrate nitrogen was more effective than amino nitrogen for biosurfactant production. Moreover, it also had price advantage over organic nitrogen. Onwosi *et al*. [3] and Guerra-Santos *et al*. [35] have also verified that different kinds of nitrogen sources had a significant effect on the biosurfactant production.

3.3.3 Effect of C/N and concentration of WCO on biosurfactant production

Form Fig. SM1c, it was found that the ideal C/N for biosurfactant production was 10:1, which gave a highest reduction of surface tension to 27.5 mN/m and high biosurfactant productivity of almost 5 g/L when used $NaNO_3$ as nitrogen source. A low C/N culture condition might result in bacterial lysis in advance while excess C/N may result in bacteria metabolic disturbance. Benincasa *et al*. [31] have demonstrated that nitrogen-limiting condition was more suitable for biosurfactant production, and Xia *et al*. [17] also reported that C/N was one of the most vital factors affecting the performance of microorganisms in biosurfactant production.

Meanwhile, the effect of the concentration of WCO was studied as well with the optimum C/N of 10:1 (Fig. SM1d), and the optimum concentration of WCO was found to be 40 g/L. Das *et al*. [36] also proved that the concentration of carbon source played a decisive role in productivity, being in agreement with the present study.

3.3.3 Effect of mineral elements on biosurfactant production

Mineral elements are necessary substances for bacterial growth that can stimulate the accumulation of metabolite. As shown in Fig. SM1e to Fig. SM1g , the optimum concentrations of Ca^{2+}, Fe^{2+} and Mg^{2+} for biosurfactant production were all 0.2 g/L, which gave the maximum reduction of surface tension and high productivity of biosurfactant. The inhibiting effect of mineral elements was mainly caused by the osmotic pressure, namely mineral elements with high concentration might result in death or reduction in bacterial growth due to the cells dehydration [23, 37].

After a series of correlational studies, we obtained the optimum culture medium for the bacterial growth and biosurfactant production, containing flowing components: 4 g/L $NaNO_3$, 5 g/L NaCl, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_20$, 0.2 g/L $FeSO_4$, 0.2 g/L $CaCl_2$, and 40 g/L WCO. This optimum culture medium with an inoculum ratio of 2% was used in the following studies.

3.3 Chemical characterization of purified biosurfactant

It was found that our purified biosurfactant showed two spots on the TLC plate: mono-rhamnolipid and di-rhamnolipid with retardation factor (Rf) value about 0.48 and 0.66 respectively (Table 2), and standard rhamnolipid gave the similar Rf values. The results were in line with the previous reports [15, 16]. Therefore, it is quite reasonable to believe that the purified biosurfactant was rhamnolipid.

Table 2
Rf values of the the purified biosurfactant and standard sample based on TLC analysis[a].

As shown in Fig. 2, a broad stretching at 3410 cm^{-1} that was a characteristic stretching mode of –OH group can be clearly observed. Absorption bands around 2926 cm^{-1} , 2857 cm^{-1} and 1398 cm^{-1} represented the symmetric stretch (νC–H) of $–CH_2–$ and $–CH_3$ groups of aliphatic chains, and a absorption band at 618 cm^{-1} was assigned to $–(CH_2)_n–$ (n = 6) group. Also, an intense absorption band at 1619 cm^{-1} and 1147 cm^{-1} indicated the presence of –C=O group and –C–O–C– group respectively. Additionally, a weak absorption band at 3230 cm^{-1} and 1713 cm^{-1} was the unsaturated bond (–C=C–) of aliphatic chains. Compared with previous literatures [18, 38], these main chemical structure groups were in agreement with the characteristic of rhamnolipid.

Fig. 2. FT-IR spectra of the purified biosurfactant produced by *Pseudomonas* SWP-4.

The detail chemical structure groups of the biosurfactant were identified by NMR. Structure information is obtained from three parameters: chemical shifts of the absorption frequency, coupling (mutual influence of adjacent nuclei), and integral height [39].

^{1}H NMR spectra of the purified biosurfactant was presented in Fig. 3a. The hydrogen atoms of the two main compositions (L-rhamnose moiety and aliphatic moiety) of the biosurfactant were detected. Peaks at 0.881, 1.268 ppm and around 1.432 ppm were the $–CH_3$, $–(CH_2)_6–$ and $–CH–CH_2–$ groups of aliphatic chains, respectively. Chemical shifts of δ = 2.421–2.550 ppm and δ = 5.345–5.381 ppm demonstrated the presence of $–CH_2–COO–$ and $–COO–CH–$ groups [40]. Meanwhile, δ of 2.003–2.336 ppm and 5.396–5.412 ppm indicated the unsaturated bond (–C=C–) of aliphatic chains. Furthermore, the L-rhamnose moiety was also identified by ^{1}H NMR. Chemical shifts of δ = 3.381–4.905 ppm was the hydrogen atoms of pyranose ring [41], and the –OH group was confirmed by the peaks at 5.093 ppm. The sole methyl of pyranose ring was detected at the chemical shifts of δ = 1.432–1.450 ppm.

As shown in Fig. 3b, the carbon atoms of the two main compositions were also detcted by ^{13}C NMR. Peaks at 17.49 ppm was a methyl of the pyranose ring, and the other chemical shifts ranging from 14.13 ppm to 34.56 ppm were assigned to $–(CH_2)_6–CH_3$ of aliphatic chains. Groups of –C=O and –COOH were confirmed by the peaks at 171.49 ppm and 173.71 ppm respectively [42]. Meanwhile, peaks at 39.19 ppm and 39.56 ppm were the carbon atoms of methylene that adjoined the –C=O and –COOH respectively. Similarly, the unsaturated bond (–C=C–) of aliphatic chains was detected at δ = 127.93–130.05 ppm as well. Then, the carbon atoms of pyranose ring was identified by the peaks at δ = 68.17–102.53 ppm, among which δ of 94.65 ppm and 102.53 ppm demonstrated the presence of acetal [43].

Fig. 3. ^{1}H NMR spectra (a) and ^{13}C NMR spectra (b) of the purified biosurfactant produced by *Pseudomonas* SWP-4.

In addition, ESI-MS of the purified biosurfactant (Fig. 4) indicated the product gave major signals at m/z 480.78 and 651.23, corresponding to the RhaRhaC10 and RhaRhaC10C10 respectively, and the structural formulas were shown in the figure. The other intense signals at m/z 423.25 was the methanol adduct of RhaC14. These results *i.e.* TLC, FTIR, NMR and ESI-MS indicate clearly that the biosurfactant produced by *Pseudomonas* SWP-4 was rhamnolipid, a mixture of mono-rhamnolipid and di-rhamnolipid.

Fig. 4. ESI-MS of the purified biosurfactant produced by *Pseudomonas* SWP-4 in the negative ion mode.

3.4 Surface activity measurements

In the emulsification measurement, emulsified layer was found to be 2.7 cm (average value) while the total height of the liquid column was 4.6 cm after incubation for 24 h, thus E_{24} was calculated to be 58.7%. Similar value was reported by George and Jayachandran [25] that rhamnolipid showed high emulsification efficiency on kerosene, 62.4%.

As shown in Fig. SM2, the interfacial tension against n-hexadecane decreased from 29.4 mN/m to 0.9 mN/m as rhamnolipid concentration increased to 30 mg/L, and kept nearly unchanged above this concentration. Similar value was reported by Benincasa *et al*. [15] that rhamnolipid produced by *Pseudomonas aeruginosa* could decrease the interfacial tension against n-hexadecane to 1.3 mN/m when rhamnolipid concentration increased to 100 mg/L. However, this reported rhamnolipid exhibited less excellent than the present one.

From Fig. SM2, CMC of the purified rhamnolipid was found to be 27 mg/L, and the surface tension kept almost unchanged above this concentration. Meanwhile, the lowest value of surface tension was measured to be 24.1 mN/m at the CMC. To sum up, the present rhamnolipid exhibited excellent surface activity with a low CMC, which was much lower than most of other biosurfactants reported so far. For instance, Amani *et al*. [44] showed that CMC of the rhamnolipid produced by *Pseudomonas aeruginosa* was 120 mg/L while Zou *et al*. [23] found the biosurfactant obtained from *Acinetobacter baylyi* had a CMC of 90 mg/L. Furthermore, traditional chemosynthetic surfactants usually had much higher CMC too than the present rhamnolipid. Such as sodium dodecyl sulfate had a CMC of 2100 mg/L [17].

3.5 Stability of rhamnolipid

3.5.1 Effect of salinity on rhamnolipid stability

As shown in Fig. 5a, the rhamnolipid remained relatively stable when the NaCl concentration was lower than 8%. However, obvious changes occurred in rhamnolipid surface activity with the rising of NaCl concentration, and the surface tension increased to 27.5 mN/m and E_{24} decreased to 45.1% when the NaCl concentration reached 20%. Thus, the produced rhamnolipid could still maintain a low surface tension value even under high salinity condition, and the reduction of E_{24} was attributed to demulsification.

3.5.2 Effect of pH on rhamnolipid stability

The effects of pH on surface tension and E_{24} of the rhamnolipid were investigated as shown in Fig. 5b, from which we can see that the produced rhamnolipid had an effective surface activity from about pH = 4-10. When the pH value was acidized and adjusted to 2, the surface tension increased to 35.7 mN/m and E_{24} decreased to 44.1%, which was mainly because of the partial precipitation of rhamnolipid. However, E_{24} exhibited a high value of 59.2% but surface tension increased to 32.8 mN/m when the pH value was adjusted to 12. Therefore, E_{24} of the rhamnolipid was relatively stable but the surface tension was negatively affected under strong alkaline condition.

3.5.3 Effect of temperature on rhamnolipid stability

Results obtained from temperature stability showed that the rhamnolipid performance (both surface tension and E_{24}) was quite thermostable over a wide range of temperature from 25 to 100℃(Fig. 5c) as well as at 121℃ (during autoclaving) and 150℃ (in dryer).

Furthermore, after incubation for half a month under the extreme conditions *i.e.* pH of 4.0, salinity of 8% and temperature of 80, the surface tension of the produced rhamnolipid also kept a low value of 26.4 mN/m and E_{24} was found to be 51.1%. In conclusion, these results suggested that the rhamnolipid produced by *Pseudomonas* SWP-4 was a superior surfactant that can well adapt to diverse conditions. Similarly, Zou *et al.* [23] reported the stability of the biosurfactant produced by *Acinetobacter baylyi*, but its surface activity was significantly affected by pH and salinity. Amani *et al.* [44] reported the rhamnolipid produced by *Pseudomonas aeruginosa* was thermostable and had an effective surface activity at pH of 4–10, but it only resisted against salt concentration up to 2.5%.

3.5.4 TGA experiment

Thermal gravity (TG), differential scanning calorimetry (DSC) and derivative thermogravimetry (DTG) curves versus temperature were shown in Fig. 6. The sample of purified rhamnolipid showed an initial mass loss of 1.2% below temperature of 110℃, which was attributed to the loss of moisture content. From the DTG curve, a sharp peak centered at 243℃ with an end at 310℃, which indicated that the major mass loss of 9.8% appeared in this stage. Meanwhile, a weak endothermic peak was found in this stage as shown in the DSC curve, which was mainly because of a degradation process of depolymerization [45]. In addition, the third stage of mass loss was observed from 310℃ to 600℃, where around just 5.3% of mass was lost. All in all, the produced rhamnolipid was barely affected by the test temperature, being agreement with the result of temperature stability of rhamnolipid. Abbasi *et al.* [46] also conducted TGA experiment to investigate the thermostability

of rhamnolipid, but the reported rhamnolipid produced by *Pseudomonas aeruginosa* exhibited less thermostable compared with the present study, which showed almost 80% mass loss when temperature reached around 300℃ and degraded continuously as the temperature rose.

Fig. 5. Stability studies of rhamnolipid produced by *Pseudomonas* SWP-4 under different salinity (a), pH (b) and temperature (c). All measurements were done on 24 h-old culture broth and measured at room temperature. The error bars represents standard deviation values of three independent experiments (n = 3).

Fig. 6. TG, DSC, and DTG curves versus temperature of the purified rhamnolipid produced by *Pseudomonas* SWP-4.

3.6 Rhamnolipid production kinetics

Fig. 7A and Fig. 7B show the typical time course profile of rhamnolipid production. The maximum rhamnolipid concentration of 13.93 g/L was obtained by *Pseudomonas* SWP-4 at 60 h. At the same time, WCO utilization percent reached around 88% and the pH value of culture broth decreased to a minimum value of 6.02. After cultivation time of 42 h, the decrease of OD_{600} value indicated that the bacterial growth entered the decline phase. Thus, SWP-4 could accumulate rhamnolipid steadily even in decline phase. The surface tension of the culture broth decreased sharply during the lag phase (0–10 h) and exponential phase (10–22 h), and reached a minimum value of 24.4 mN/m at 36 h. However, a slight decrease of rhamnolipid concentration occurred in the bacterial late growth stage, which indicated that rhamnolipid might be consumed or degraded by bacteria. Meanwhile, the pH value of culture broth also had a slight increase, which could be assigned to bacterial autolysis and rhamnolipid consumption or biodegradation.

Meanwhile, surface view of the culture broth versus cultivation time was shown in Fig. 7a to Fig. 7f, from which we can see that WCO dispersed above the culture broth with a wine-yellow color initially. Obvious change was observed from Fig. 7b to Fig. 7c (cultivation time of 24–48 h), demonstrating WCO was mainly degraded and emulsified in this period (steady phase). With time, the color of the culture broth was lightened continuously due to the decrease of free fatty acids, and presented a milk-white color at the end of cultivation.

Silva *et al*. [47] have investigated the growth kinetics and rhamnolipid production of *Pseudomonas cepacia*, but their results showed a longer lag phase and lower rhamnolipid concentration compared with the present study. Moreover, they reported

the maximum concentration of rhamnolipid was obtained in the , but we found that the rhamnolipid could be steadily accumulated even after the steady phase. Abbasi *et al*. [46] reported that *Pseudomonas aeruginosa* gave a maximum rhamnolipid concentration of 12 g/L after 10 days of incubation, which exhibited a lower efficiency of rhamnolipid production than the present study. There were reports about high concentration of rhamnolipid as well. Partovi *et al*. [14] showed that *Pseudomonas aeruginosa* gave a maximum productivity around 14.55 g/L.

Fig. 7. Rhamnolipid production kinetics, obtained by cultivation of SWP-4 in the optimum culture medium at 35℃, 150 rpm: (A) growth curve; (B) surface tension and pH of culture broth, WCO utilization percent, rhamnolipid concentration curves; (a–f) surface view of the culture broth versus cultivation time, captured every 24 h for 120 h. The growth curve represents the OD_{600} values of the diluted (6-fold) culture broths, and the error bars represents standard deviation values of three independent experiments (n = 3).

3.7 Analysis of free fatty acids degradation

To obtain a more detailed analysis of the WCO degradation, the changes of WCO compositions were analyzed by GC-MS, and partial qualitative GC-MS profiles were illustrated in Fig. 8. The major compositions of the WCO were palmitic acid (C_{16}), oleic acid (E-$C_{18:1}$) and linoleic acid (Z-$C_{18:2}$), accounting for 12.80%, 32.06% and 41.91%, respectively. However, large quantities of *trans* fatty acids were detected from the WCO, including E-$C_{16:1}$, E-$C_{18:1}$ and E-$C_{18:2}$, which were potential causes of human health problem on condition that human ate this oil [48].

As Fig. 8 shown, most of the free fatty acids of carbon numbers below 20 were chiefly consumed during the first 36 h of cultivation, but stearic acid (C_{18}) was hard to utilize by bacteria. In this period, palmitic acid and oleic acid gave a degradation rate of 79.4% and 69.4% respectively while linoleic acid was degraded by 72.5%. During the cultivation time of 36–60 h, free fatty acids of carbon number below 20 were consumed sequentially, and the long chain saturation free fatty acid of even carbon numbers such as eicosanoic acid (C_{20}), docosanoic acid (C_{22}) and tetracosanoic acid (C_{24}) could also be consumed in this period. However, the abundance of free fatty acids shows that only a small number of oil was consumed by bacteria in the decline phase, corresponding to the results of rhamnolipid production kinetics. Palmitic acid, oleic acid and linoleic acid just gave a degradation rate of 5.3%, 8.3% and 7.1% respectively during 36–60 h.

Generally speaking, *Pseudomonas* SWP-4 mainly consumed palmitic acid, oleic

acid and linoleic acid for its growth, and it was also able to utilize saturation and unsaturation free fatty acids ranging from C_{12} to C_{19} except C_{18}. In addition, long chain saturation free fatty acids of even carbon numbers (C_{20}, C_{22} and C_{24}) could be consumed as well in the bacterial late growth stage. Benincasa *et al*. [32] have also reported that genus *Pseudomonas* mainly utilized oleic acid, linoleic acid and linolenic acid, but the detailed analysis of other free fatty acids metabolism was not covered in their report.

Fig. 8. Partial GC-MS profiles of the WCO: (a) retention time at 10–20 min; (b) retention time at 20–22 min; (c) retention time at 22–38 min. The black line represent the GC-MS profile of the original WCO. The red line and the blue line represent the GC-MS profile of the WCO obtained from 36 h-old and 60 h-old culture broth, respectively. Z and E represent *cis* and *trans* fatty acids, respectively.

4. Conclusions

In this work, a nonpathogenic biosurfactant-producing microorganism named *Pseudomonas* SWP-4 was isolated from WCO-contaminated sludge sample, which could produce rhamnolipid using WCO as sole carbon source. The produced rhamnolipid exhibited excellent physicochemical properties in terms of surface activity and stability measurements. Under the optimum culture conditions, *Pseudomonas* SWP-4 gave a maximum rhamnolipid concentration of 13.93 g/L and high WCO utilization percent around 88% during the bacterial decline phase. Based on GC-MS analysis, *Pseudomonas* SWP-4 could biodegrade most of the free fatty acids, and it was believed that there was a synergistic effect of biodegradation and emulsification. This work enriches the commercial ways of rhamnolipid production from WCO using a newly isolated bacterium, and presents an environmentally friendly method of waste treatment.

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References

[1] Y. Chen, B. Xiao, J. Chang, Y. Fu, P. Lv, X. Wang, Synthesis of biodiesel from waste cooking oil using immobilized lipase in fixed bed reactor, Energ. Convers. Manage 50 (2009) 668-673.

[2] M.E. Marti, W.J. Colonna, G. Reznik, M. Pynn, K. Jarrell, B. Lamsal, C.E. Glatz, Production of fatty-acyl-glutamate biosurfactant by *Bacillus subtilis* on soybean co-products, Biochem. Eng. J. 95 (2015) 48-55.

[3] C.O. Onwosi, F.J. Odibo, Effects of carbon and nitrogen sources on rhamnolipid biosurfactant production by *Pseudomonas nitroreducens* isolated from soil, World J. Microb. Biot. 28 (2012) 937-942.

[4] S.C. Lin, Biosurfactants: recent advances, J. Chem. Technol. Biotechnol. 66 (1996) 109-120.

[5] Y. Al-Wahaibi, S. Joshi, S. Al-Bahry, A. Elshafie, A. Al-Bemani, B. Shibulal, Biosurfactant production by *Bacillus subtilis* B30 and its application in enhancing oil recovery, Colloids Surf. B: Biointerfaces 114 (2014) 324-333.

[6] J.F.B. Pereira, E.J. Gudiña, R. Costa, R. Vitorino, J.A. Teixeira, J.A.P. Coutinho, L.R. Rodrigues, Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications, Fuel 111 (2013) 259-268.

[7] S. Mukherjee, P. Das, R. Sen, Towards commercial production of microbial surfactants, Trends Biotechnol. 24 (2006) 509-515.

[8] R. Thavasi, S. Jayalakshmi, T. Balasubramanian, I.M. Banat, Biosurfactant production by *Corynebacterium kutscheri* from waste motor lubricant oil and peanut oil cake, Lett. Appl. Microbiol. 45 (2007) 686-691.

[9] F.G. Jarvis, M.J. Johnson, A glyco-lipide produced by *Pseudomonas aeruginosa*, Am. Chem. Soc. 71 (1949) 4124-4126.

[10] J.F. Liu, G. Wu, S.Z. Yang, B.Z. Mu, Structural characterization of rhamnolipid produced by *Pseudomonas aeruginosa* strain FIN2 isolated from oil reservoir water, World J. Microb. Biot. 30 (2014) 1473-1484.

[11] B. Hörmann, M.M. Müller, C. Syldatk, R. Hausmann, Rhamnolipid production by *Burkholderia plantarii* DSM 9509T, Eur. J. Lipid Sci. Tech. 112 (2010) 674-680.

[12] A.P. Rooney, N.P. Price, K.J. Ray, T.M. Kuo, Isolation and characterization of rhamnolipid - producing bacterial strains from a biodiesel facility, FEMS Microbiol. Lett. 295 (2009) 82-87.

[13] N. Christova, B. Tuleva, Z. Lalchev, A. Jordanova, B. Jordanov, Rhamnolipid biosurfactants produced by *Renibacterium salmoninarum* 27BN during growth on n-hexadecane, Z. Naturforsch. C 59 (2004) 70-74.

[14] M. Partovi, T.B. Lotfabad, R. Roostaazad, M. Bahmaei, S. Tayyebi, Management of soybean oil refinery wastes through recycling them for producing biosurfactant using *Pseudomonas aeruginosa* MR01, World J. Microb. Biot. 29 (2013) 1039-1047.

[15] M. Benincasa, F.R. Accorsini, *Pseudomonas aeruginosa* LBI production as an integrated process using the wastes from sunflower-oil refining as a substrate, Bioresour. Technol. 99 (2008) 3843-3849.

[16] Y. Zhu, J.J. Gan, G.L. Zhang, B. Yao, W.J. Zhu, Q. Meng, Reuse of waste frying oil for production of rhamnolipids using *Pseudomonas aeruginosa* zju. u1M, J. Zhejiang Univ-Sc. A 8 (2007) 1514-1520.

[17] W.J. Xia, Z.B. Luo, H.P. Dong, L. Yu, Q.F. Cui, Y.Q. Bi, Synthesis, characterization, and oil recovery application of biosurfactant produced by indigenous *Pseudomonas aeruginosa* WJ-1 using waste vegetable oils, Appl. Biochem. Biotechnol. 166 (2012) 1148-1166.

[18] R. Thavasi, V.R. Subramanyam Nambaru, S. Jayalakshmi, T. Balasubramanian, I.M. Banat, Biosurfactant Production by *Pseudomonas aeruginosa* from Renewable Resources, Indian J. Microbiol. 51 (2011) 30-36.

[19] N.W. Gunther, A. Nunez, W. Fett, D.K. Solaiman, Production of rhamnolipids by *Pseudomonas chlororaphis*, a nonpathogenic bacterium, Appl. Environ. Microbiol. 71 (2005) 2288-2293.

[20] I. Siegmund, F. Wagner, New method for detecting rhamnolipids excreted by *Pseudomonas species* during growth on mineral agar, Biotechnol. Tech. 5 (1991) 265-268.

[21] N.H. Youssef, K.E. Duncan, D.P. Nagle, K.N. Savage, R.M. Knapp, M.J. McInerney, Comparison of methods to detect biosurfactant production by diverse microorganisms, J. Microbiol. Meth. 56 (2004) 339-347.

[22] R. Pasumarthi, S. Chandrasekaran, S. Mutnuri, Biodegradation of crude oil by *Pseudomonas aeruginosa* and *Escherichia fergusonii* isolated from the Goan coast, Mar. Pollut. Bull 76 (2013) 276-282.

[23] C.J. Zou, M. Wang, Y. Xing, G.H. Lan, T.T. Ge, X.L. Yan, T. Gu, Characterization and optimization of biosurfactants produced by *Acinetobacter baylyi* ZJ2 isolated from crude oil-contaminated soil sample toward microbial enhanced oil recovery applications, Biochem. Eng. J. 90 (2014) 49-58.

[24] J. Arutchelvi, C. Joseph, M. Doble, Process optimization for the production of rhamnolipid and formation of biofilm by *Pseudomonas aeruginosa* CPCL on polypropylene, Biochem. Eng. J. 56 (2011) 37-45.

[25] S. George, K. Jayachandran, Production and characterization of rhamnolipid biosurfactant from waste frying coconut oil using a novel *Pseudomonas aeruginosa* D, J. Appl. Microbiol. 114 (2013) 373-383.

[26] Y. Kryachko, S. Nathoo, P. Lai, J. Voordouw, E.J. Prenner, G. Voordouw, Prospects for using native and recombinant rhamnolipid producers for microbially enhanced oil recovery, Int. Biodete. Biodeg. 81 (2013) 133-140.

[27] Q. Liu, J. Lin, W. Wang, H. Huang, S. Li, Production of surfactin isoforms by *Bacillus subtilis* BS-37 and its applicability to enhanced oil recovery under laboratory conditions, Biochem. Eng. J. 93 (2015) 31-37.

[28] H. Khoshdast, H. Abbasi, A. Sam, K.A. Noghabi, Frothability and surface behavior of a rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* MA01, Biochem. Eng. J. 60 (2012) 127-134.

[29] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugar and related substances, Anal. Chem. 28 (1956) 350-356.

[30] A. Leyva, A. Quintana, M. Sanchez, E.N. Rodriguez, J. Cremata, J.C. Sanchez, Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: method development and validation, Biologicals, 36 (2008) 134-141.

[31] M. Benincasa, J. Contiero, M.A. Manresa, I.O. Moraes, Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source, J. Food Eng. 54 (2002) 283-288.

[32] M. Benincasa, A. Abalos , I. Oliveira, A. Manresa, Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock, Anton. Leeuw. Int. J. G. 85 (2004) 1-8.

[33] J. Folch, M. Lees, G. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. biol. Chem 226 (1957) 497-509.

[34] R. Sen, T. Swaminathan, Response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production, Biochem. Eng. J. 21 (2004) 141-148.

[35] L.H. Guerra-Santos, O. Käppeli, A. Fiechter, Dependence of *Pseudomonas aeruginosa* continous culture biosurfactant production on nutritional and environmental factors, J. Appl. Microbiol. 24 (1986) 443-448.

[36] P. Das, S. Mukherjee, R. Sen, Substrate dependent production of extracellular biosurfactant by a marine bacterium, Bioresour. Technol. 100 (2009) 1015-1019.

[37] D.S. Nichols, J. Olley, H. Garda, R.R. Brenner, T.A. McMeekin, Effect of temperature and salinity stress on growth and lipid composition of *Shewanella gelidimarina*, Appl. Environ. Microbiol. 66 (2000) 2422-2429.

[38] M.G. Rikalovic, G. Gojgic-Cvijovic, M.M. Vrvic, I. Karadzic, Production and characterization of rhamnolipids from *Pseudomonas aeruginosa* san ai, J. Serb. Chem. Soc. 77 (2012) 27-42.

[39] M. Heyd, A. Kohnert, T.H. Tan, M. Nusser, F. Kirschhofer, G. Brenner-Weiss, M. Franzreb, S. Berensmeier, Development and trends of biosurfactant analysis and purification using rhamnolipids as an example, Anal. Bioanal. Chem. 391 (2008) 1579-1590.

[40] Y.H. Wei, C.L. Chou, J.S. Chang, Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater, Biochem. Eng. J. 27 (2005) 146-154.

[41] T. Morita, T. Fukuoka, M. Konishi, T. Imura, S. Yamamoto, M. Kitagawa, A. Sogabe, D. Kitamoto, Production of a novel glycolipid biosurfactant, mannosylmannitol lipid, by *Pseudozyma parantarctica* and its interfacial properties, Appl. Microbiol. Biotechnol. 83 (2009) 1017-1025.

[42] L. Sim, O. Ward, Z.Y. Li, Production and characterisation of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1, J. Ind. Microbiol. Biot. 19 (1997) 232-238.

[43] S.A. Monteiro, G.L. Sassaki, L.M. de Souza, J.A. Meira, J.M. de Araujo, D.A. Mitchell, L.P. Ramos, N. Krieger, Molecular and structural characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614, Chem. Phys. Lipids 147 (2007) 1-13.

[44] H. Amani, M.M. Muller, C. Syldatk, R. Hausmann, Production of microbial rhamnolipid by *Pseudomonas aeruginosa* MM1011 for ex situ enhanced oil recovery, Appl. Biochem. Biotechnol. 170 (2013) 1080-1093.

[45] T.B. Lotfabad, M. Shourian, R. Roostaazad, A.R. Najafabadi, M.R. Adelzadeh, K.A. Noghabi, An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in south of Iran, Colloids Surf. B Biointerfaces 69 (2009) 183-193.

[46] H. Abbasi, M.M. Hamedi, T.B. Lotfabad, H.S. Zahiri, H. Sharafi, F. Masoomi, A.A. Moosavi-Movahedi, A. Ortiz, M. Amanlou, K.A. Noghabi, Biosurfactant-producing bacterium, *Pseudomonas aeruginosa* MA01 isolated from spoiled apples: physicochemical and structural characteristics of isolated biosurfactant, J. Biosci. Bioeng. 113 (2012) 211-219.

[47] M.M.P.R. Silva, R.D. Rufino, J.M. Luna, V.A. Santos, L.A. Sarubbo, Screening of *Pseudomonas* species for biosurfactan production using low-cost substrates, Biocatal. Agr. Biotechnol. 3 (2014) 132-139.

[48] C.M. Oomen, M.C. Ocké, E.J.M. Feskens, M.A.J. van Erp-Baart, F.J. Kok, D. Kromhout, Association between *trans* fatty acid intake and 10-year risk of coronary heart disease in the Zutphen Elderly Study: a prospective population-based study, The Lancet 357 (2001) 746-751.

Table 1

Diameter of the oil spreading circle and surface tension of the culture broth produced by the screened strains[a].

Strain number	SWP-1	SWP-2	SWP-3	SWP-4	SWP-5
Diameter (cm)	0.8 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	1.6 ± 0.2	1.2 ± 0.1
Surface tension (mN/m)	53.0 ± 0.1	39.6 ± 0.3	48.2 ± 0.5	33.1 ± 0.2	44.7 ± 0.5

[a] Results represent the average of three independent experiments ± standard deviation.

Table 2

Rf values of the the purified biosurfactant and standard sample based on TLC analysis[a].

Rhamnolipid sample	Rf1	Rf2
purified biosurfactant	0.48 ± 0.01	0.66 ± 0.01
standard sample	0.47 ± 0.02	0.66 ± 0.01

[a] Results represent the average of three independent experiments ± standard deviation.

Figure captions:

Fig. 1. (a) Morphology of the isolated bacteria, SWP-4 shown by SEM. (b) Phylogenetic tree of the isolated bacteria, SWP-4, constructed by Neighbor-Joining algorithim.

Fig. 2. FT-IR spectra of the purified biosurfactant produced by *Pseudomonas* SWP-4.

Fig. 3. ^{1}H NMR spectra (a) and ^{13}C NMR spectra (b) of the purified biosurfactant produced by *Pseudomonas* SWP-4.

Fig. 4. ESI-MS of the purified biosurfactant produced by *Pseudomonas* SWP-4 in the negative ion mode.

Fig. 5. Stability studies of rhamnolipid produced by *Pseudomonas* SWP-4 under different salinity (a), pH (b) and temperature (c). All measurements were done on 24 h-old culture broth and measured at room temperature. The error bars represents standard deviation values of three independent experiments (n = 3).

Fig. 6. TG, DSC, and DTG curves versus temperature of the purified rhamnolipid produced by *Pseudomonas* SWP-4.

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and the error bars represents standard deviation values of three independent experiments (n = 3).

Fig. 8. Partial GC-MS profiles of the WCO: (a) retention time at 10–20 min; (b) retention time at 20–22 min; (c) retention time at 22–38 min. The black line represent the GC-MS profile of the original WCO. The red line and the blue line represent the GC-MS profile of the WCO obtained from 36 h-old and 60 h-old culture broth, respectively. Z and E represent *cis* and *trans* fatty acids, respectively.

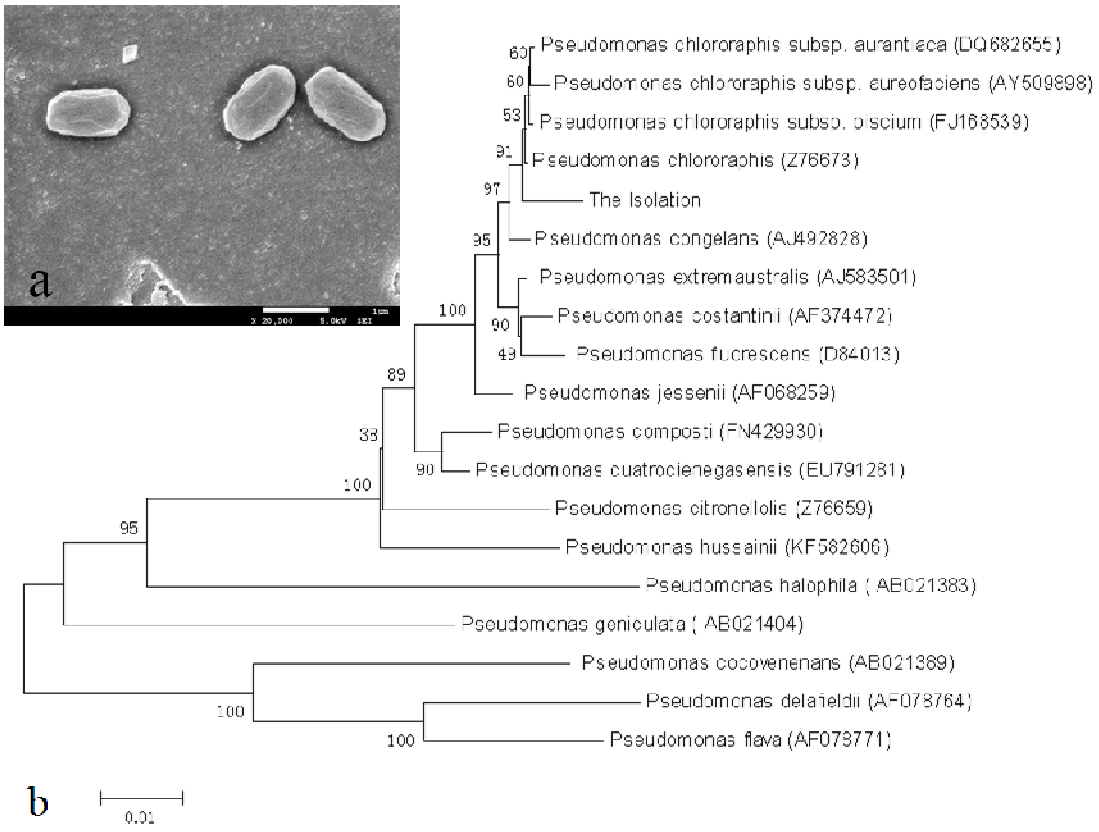

Fig. 1. Morphology of the isolated bacteria, SWP-4 shown by SEM (a); phylogenetic tree of the isolated bacteria, SWP-4, constructed by Neighbor-Joining algorithim (b).

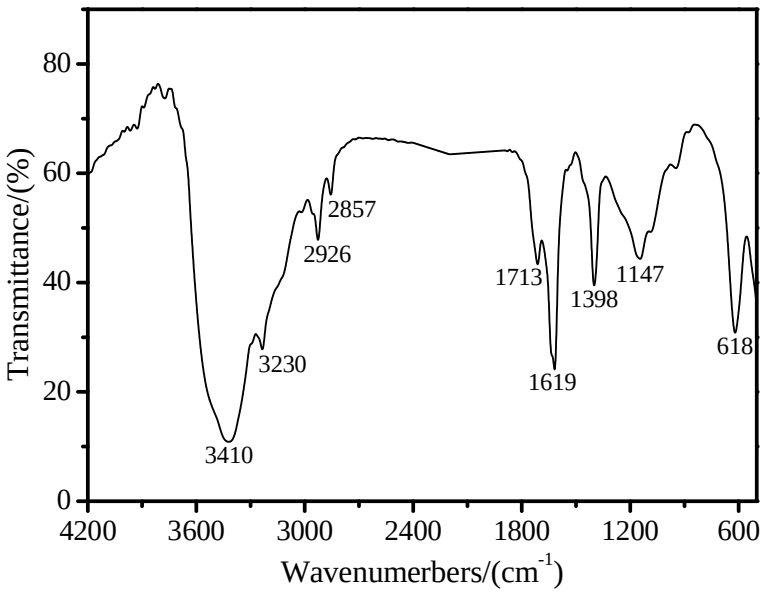

Fig. 2. FT-IR spectra of the purified biosurfactant produced by *Pseudomonas* SWP-4.

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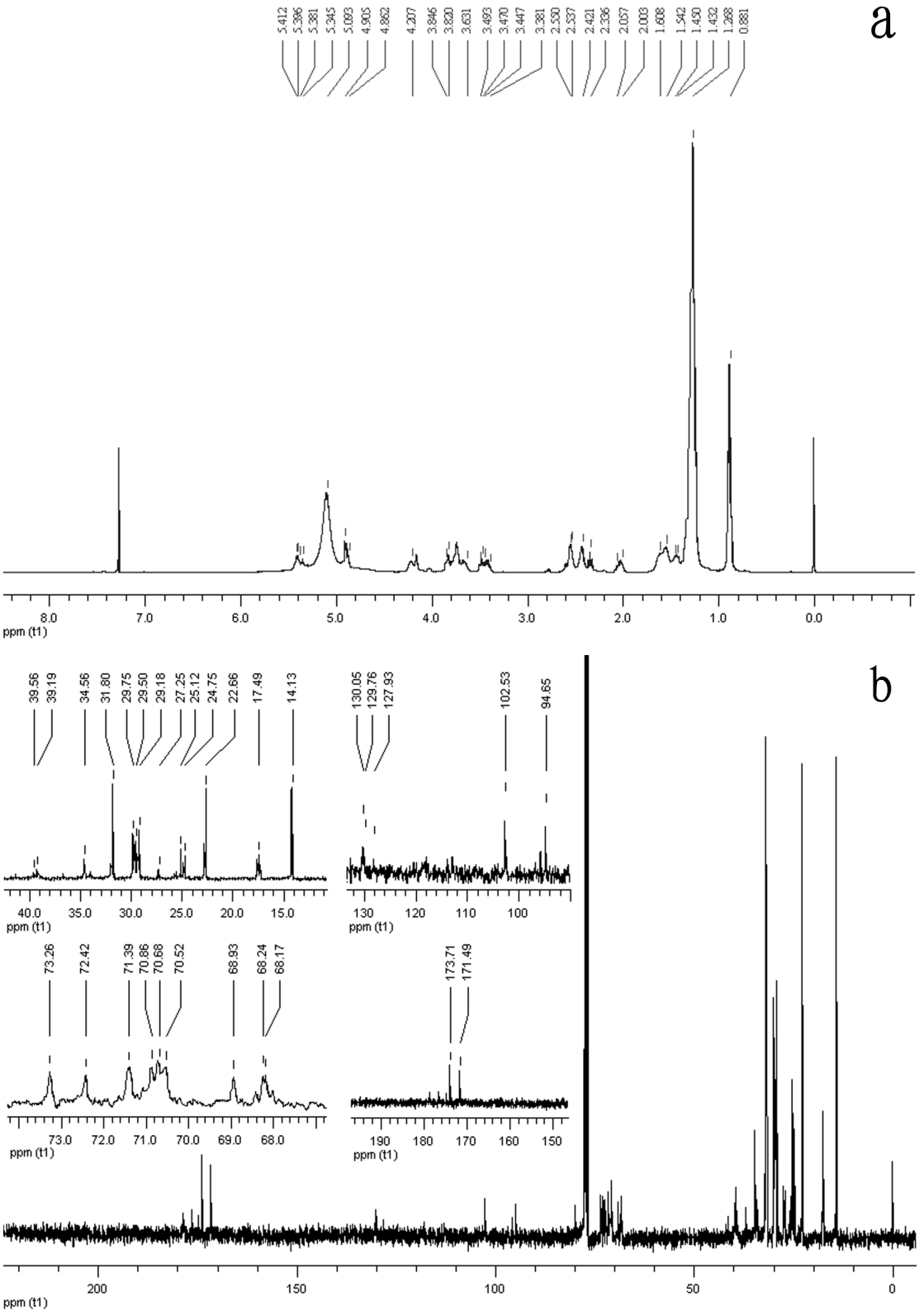


Fig. 3. ^{1}H NMR spectra (a) and ^{13}C NMR spectra (b) of the purified biosurfactant produced by *Pseudomonas* SWP-4.

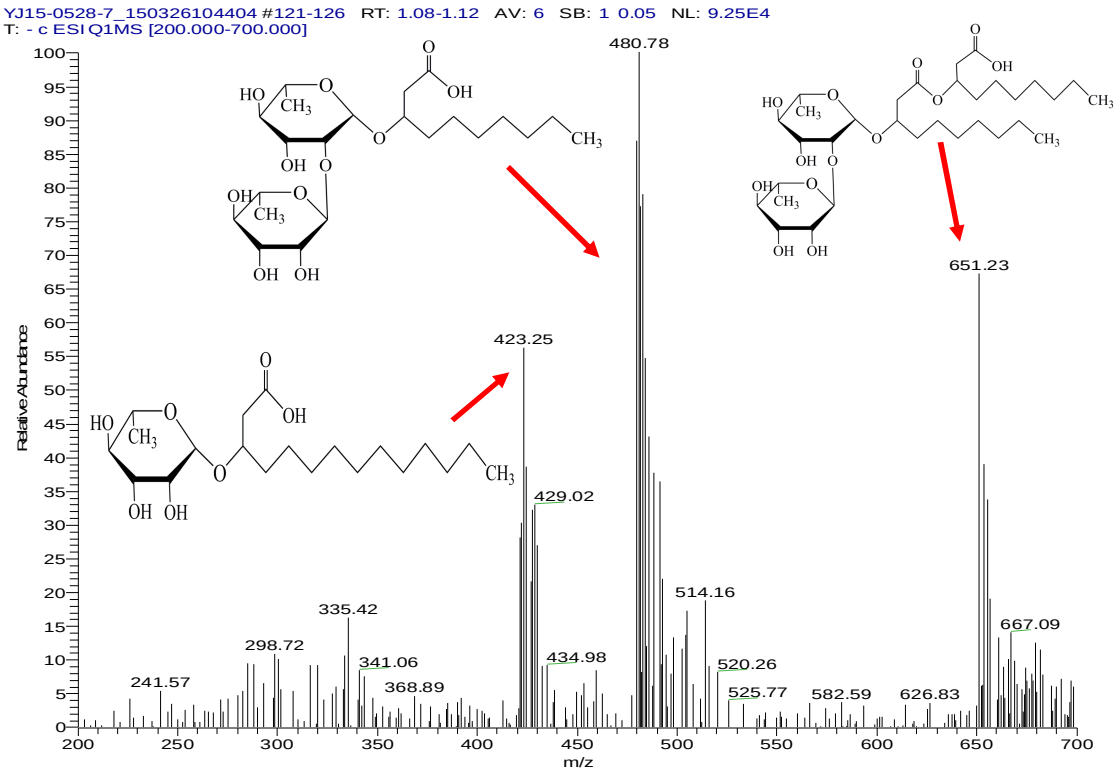
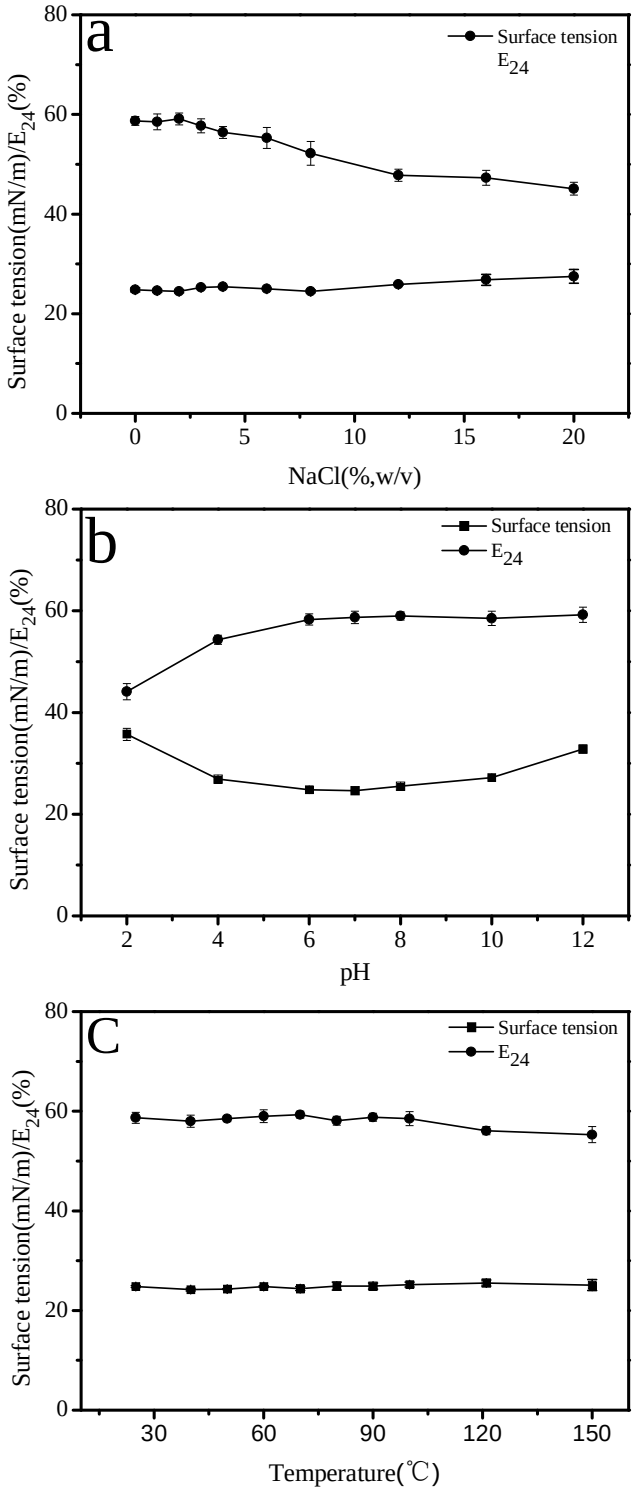


Fig. 4. ESI-MS of the purified biosurfactant produced by *Pseudomonas* SWP-4 in the negative ion mode.



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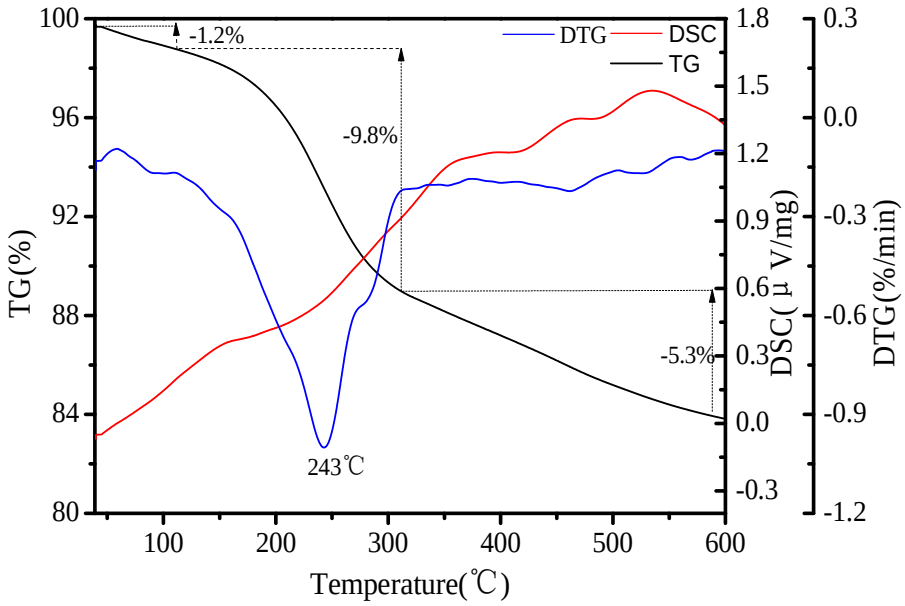

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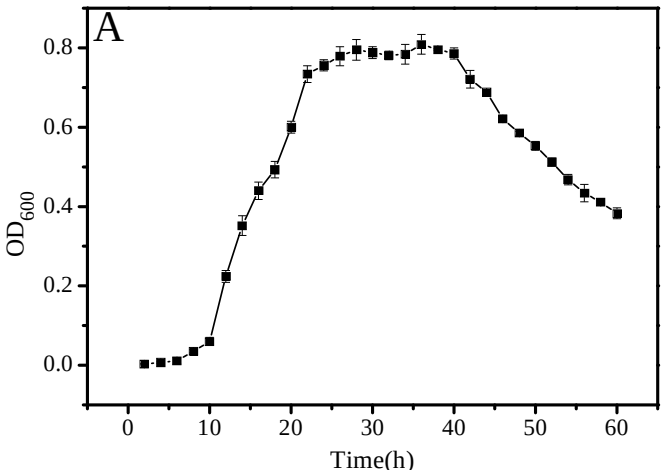

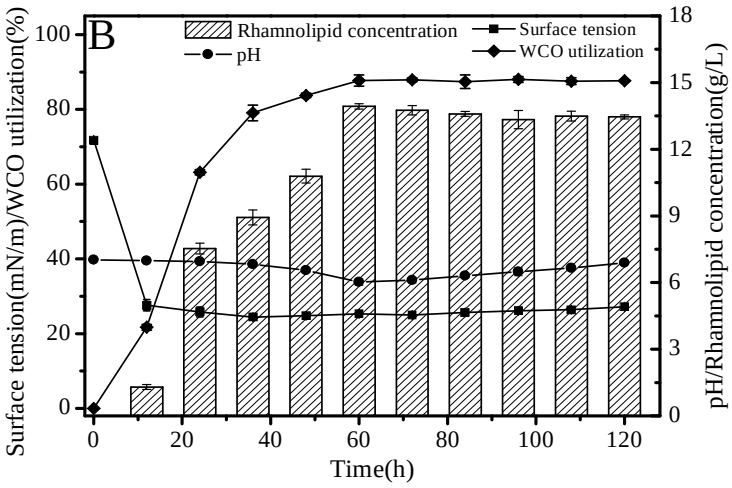

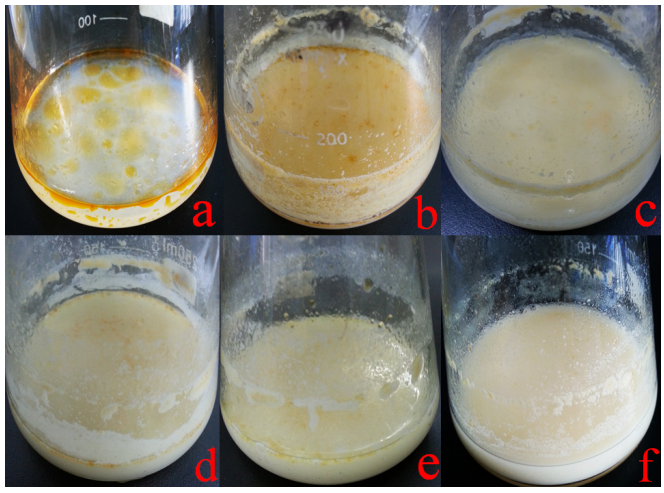

Fig. 7. Rhamnolipid production kinetics, obtained by cultivation of SWP-4 in the optimum culture medium at 35℃, 150 rpm: (A) growth curve; (B) surface tension and pH of culture broth, WCO utilization percent, rhamnolipid concentration curves; (a–f) surface view of the culture broth versus cultivation time, captured every 24 h for 120 h. The growth curve represents the OD_{600} values of the diluted (6-fold) culture broths, and the error bars represents standard deviation values of three independent experiments (n = 3).

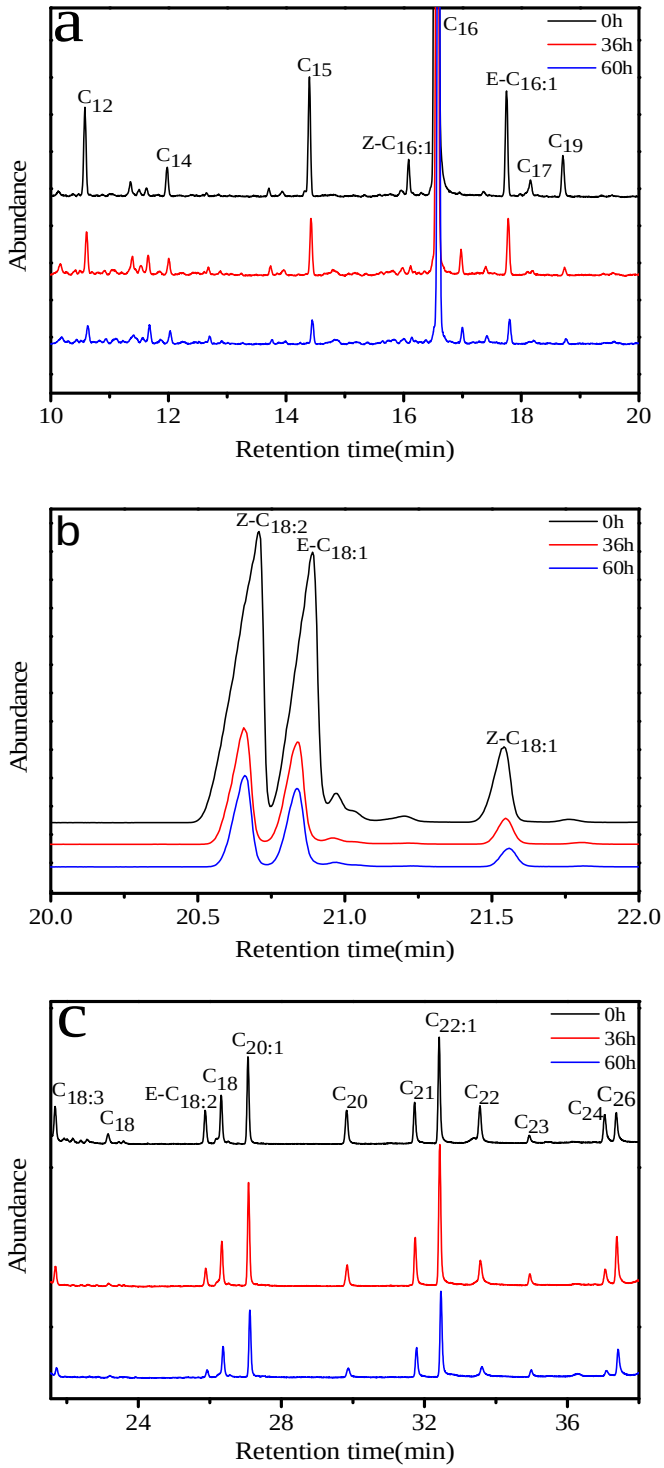

Fig. 8. Partial GC-MS profiles of the WCO: (a) retention time at 10–20 min; (b) retention time at 20–22 min; (c) retention time at 22–38 min. The black line represent the GC-MS profile of the original WCO. The red line and the blue line represent the GC-MS profile of the WCO obtained from 36 h-old and 60 h-old culture broth, respectively. Z and E represent *cis* and *trans* fatty acids, respectively.

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