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Rhamnolipid Production by *Pseudomonas Aeruginosa* GIM 32 Using Different Substrates Including Molasses Distillery Wastewater

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Abstract A rhamnolipid production strain newly isolated from oil-contaminated soil was identified as *Pseudomonas aeruginosa* GIM32 by its morphology and 16S rDNA sequence analysis. The effect of carbon source and carbon to nitrogen (C/N) ratio on rhamnolipids production was investigated. Palm oil was favorable as a carbon source for rhamnolipid production. The maximum biomass and rhamnolipid concentration were 8.24 g/L and 30.4 g/L, respectively, with an optimization medium containing 50 g/L palm oil and 5 g/L sodium nitrate. Molasses distillery wastewater as an unconventional substrate for rhamnolipid production was investigated. It was found that 2.6 g/L of rhamnolipids was produced; this amount was higher than that of past reports using wastewater as a substrate. In addition, 44% of the chemical oxygen demand of wastewater was removed at the same time under the optimization condition. Eleven kinds of different molecular weight rhamnolipid homologues were identified in the rhamnolipids obtained from molasses distillery wastewater by *P. aeruginosa* GIM32 by LC–MS analysis.

Keywords Rhamnolipids · *Pseudomonas aeruginosa* · Molasses distillery wastewater · Biosurfactant

Introduction

Biosurfactants produced by a variety of microorganisms such as bacteria, fungi, and yeasts are amphiphilic compounds which can reduce surface and interfacial tension. The molecular structures of these biosurfactants comprise a hydrophilic portion, which may consist of mono-, oligo- or polysaccharides, amino acids or peptides or phosphate groups,

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and a hydrophobic portion, which is composed of fatty acids or fatty alcohols. Those make up glycolipids, lipopeptides, fatty acids, polysaccharide–protein complexes, peptides, phospholipids, and neutral lipids. Biosurfactants have advantages over synthetic surfactants such as lower toxicity, higher biodegradability, ecological acceptability, and effectiveness at extremes of temperature, pH, and salinity. These properties make surfactants to be one of versatile class of chemical products, used on a variety of applications in pharmaceutical, cosmetic, detergent, and food industry [1–3].

Although biosurfactants have many advantages, their prices are not competitive with those of the chemical surfactants. This is ascribable to high production costs caused by low productivity and the expense of raw material. Hence, biosurfactants have not been commercialized extensively. However, the use of cheaper renewable substrates, optimal growth and production conditions, and the adoption of high-production microbial strains should reduce the costs of biosurfactant production [4].

The wastewater produced by the cane molasses-based distillery industry is characterized with extremely high chemical oxygen demand (COD) and biochemical oxygen demand [5]. The cost of the complicated molasses wastewater treatment is quite high. Furthermore, as this kind of wastewater includes different organic compounds such as acetic acid, lactic acid, glycerol, and various sugars and inorganic salts well suited to be good nutriments for microorganism growth, direct treatment accounts for an enormous waste of material resources [6, 7].

In this study, we described a *Pseudomonas aeruginosa* strain newly isolated from oil-contaminated soil that exhibits high production of rhamnolipids. Then the optimum carbon source and carbon to nitrogen (C/N) ratio conditions were achieved for high rhamnolipid production. Further, we successfully make use of molasses distillery wastewater as a culture medium to reduce the cost of production, and the COD of wastewater was cut down simultaneously. Finally, the composition and characteristics of rhamnolipids secreted by *P. aeruginosa* GIM32 were determined.

Materials and Methods

Medium Compositions

The mineral salts (MS) medium used for biosurfactant production was composed of (L^{-1}): 5 g $NaNO_3$, 2 g KH_2PO_4, 1.5 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCl_2$, and 0.024 g $FeSO_4{\cdot}7H_2O$ and supplemented with 1 ml trace element solution containing (L^{-1}): 0.75 g $ZnSO_4{\cdot}7H_2O$, 0.08 g $CoCl_2{\cdot}6H_2O$, 0.075 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MnSO_4{\cdot}H_2O$, 0.15 g H_3BO_3, and 0.06 g $NaMoO_4{\cdot}2H_2O$. For screening strain, the MS medium was supplied with 30 g/L glucose, 30 g/L soy oil, or 20 g/L diesel, respectively, as a sole carbon source.

For evaluating the effect of carbon source, MS medium was separately supplemented with 50 g/L of four kinds of vegetable oil as insoluble carbon sources. For evaluating appropriate C/N ratio, firstly, the concentration of palm oil was fixed at 50 g/L, while $NaNO_3$ was supplied at 2, 3, 4, 5, 6, 7 g/L, resulting in a C/N ratio of 138, 92, 69, 55, 46 and 39, respectively. Then, the concentration of $NaNO_3$ was maintained at 5 g/L, and the palm oil concentration was supplied at 20, 30, 40, 50, 60, and 70 g/L, resulting in a C/N ratio of 22, 33, 44, 55, and 66. The pH value of the media mentioned above was adjusted pH to 6.8 by 6.0 M HCl and was sterilized at 121 °C for 20 min.

Chemical properties of molasses distillery wastewater are shown in Table 1. The wastewater obtained from the manufacture of alcohol was stored at 4 °C until needed. For

Table 1 Composition of molasses distillery wastewater

Chemical components	Concentration
Total organic carbon (mg/L)	56,910±500
Total nitrogen (mg/L)	9,890±350
Calcium (mg/L)	7,360±200
Total phosphorus (mg/L)	111±5
Magnesium (mg/L)	6,920±430
Sulfate (mg/L)	7,520±610
pH	4.16±0.1
COD (mg/L)	171,200±2,600
Total sugar (mg/L)	132,700±2,800
Reducing sugar (mg/L)	16,170±400

exploring dilution rate and wastewater pH effect, the wastewater was prepared by adding the tap water to make series dilutions and pH value adjusted used $Ca(OH)_2$. The wastewater was autoclaved at 115 °C for 20 min.

Bacterial Strain Screening

The oil-contaminated soil samples utilized for microbial strain isolation were collected from Zhongyuan oilfield in Henan Province, China. A few grams of soil samples were added to the flask containing screening medium and incubated at 30 °C for 48 h. Then 0.1 ml of serial dilutions of fermented broth were spread on Luria-Bertani (LB) agar and cultivated at 30 °C for 48 h. The isolated colonies were picked and inoculated in the test tube containing screening medium and cultivated at 30 °C for 48 h. The cell suspension of each test tube was tested for the presence of biosurfactant using a drop-collapsing test [8]. The strains of producing biosurfactant were maintained in LB liquid with 20% glycerol at −30 °C. The critical micelle dilution (CMD) method, which was determined by measuring the surface tension of serial dilutions of supernatant from fermentation broth, was used as a criterion for selecting high production strain.

The high production strain GIM32 was identified using the Biolog plates system and 16S rDNA sequencing. The primers 27f (5′-AGAGTTTGATCCTGGCTCAG) and 1492r (5′-TACGGTTACCTTGTTACGACTT) were used to amplify the 16S rDNA. The strain GIM32 was identified by comparing its 16S rDNA sequence rDNA sequence with those in the National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) for their pairwise identities.

Fermentation Conditions

The same fermentation condition was used for culturing strain GIM32 with the MS media and wastewater medium. One loop of the strain GIM32 cell from LB agar slant was transferred into a 200-ml flask containing 40 ml LB liquid medium and cultivated at 35 °C on a shaker (220 rpm) for 18 h. The seed (3% *v*/*v* inoculum) was inoculated into a 500-ml flask containing 100 ml fermentation medium. Flask cultures were incubated for 120 h (MS medium) or 72 h (wastewater medium) on a reciprocal shaker (120 rpm) at 35 °C. During the course of batch fermentation, samples were taken at designated time intervals from the liquid culture to determine biomass, COD, and rhamnolipids yield.

Extraction of Crude Rhamnolipids

The supernatant was obtained from fermentation broth after centrifugation at 8,000×*g* for 20 min to remove cells. After the pH of supernatant was adjusted to 2.0 with 6 M HCl, the precipitate was harvested by centrifugation and extracted by a mixture of chloroform–ethanol (2:1). The organic phase was collected and concentrated by using a rotary evaporator at 50 °C. The obtained crude rhamnolipids was dissolved in distilled water and repeated acid precipitation, extraction, and concentration as mentioned above. Finally, the pure rhamnolipids were obtained for structure analysis.

Determination of Cell Concentration and Rhamnolipid Concentration

The precipitates were collected and washed three times with 0.9% NaCl solution after the culture samples were centrifuged at 8,000×*g* for 20 min. The pellets were dried at 80 °C until constant weight to determine the cell biomass. Cell concentration was determined by measuring absorbance of the samples at 600 nm which was converted to dry cell weight via a standard curve of dry cell weight vs. OD 600.

Rhamnolipid concentration of supernatant in the sugar-free media was determined directly by measuring the concentration of hydrolysis-released rhamnose by the phenol–sulfuric acid method [9]. But the rhamnolipid concentration in the medium containing wastewater was determined by the phenol–sulfuric acid method after the rhamnolipids were extracted two times from supernatant as the extraction method of crude rhamnolipids mentioned above. Rhamnolipid value was calculated by multiplying the rhamnose value by a coefficient of 2.7 for rhamnolipids from vegetable oil and a coefficient of 2.2 from wastewater. This coefficient was the ratio of the weight of the rhamnolipids to that of rhamnose from acid hydrolysis of the rhamnolipids, which was affected by the components in rhamnolipids.

Emulsification Activity and Surface Tension Assay

Two milliliters of supernatant was mixed with 3 ml of different compounds (palm oil, soybean oil, peanut oil, diesel, gasoline, toluene, and liquid paraffin) in a tube with gradations. The tube was shaken vigorously on a vortex for 2 min and left to stand for 24 h. The value emulsification index was determined as the height of the emulsified layer, expressed as a percentage the total height of the liquid column.

Structure Analysis of Rhamnolipids

About 2 mg of dried crude biosurfactant was mixed with 200 mg of KBr to form a powder and compressed into a thin pellet. The pellet was analyzed by Fourier transform infrared (FTIR) spectra measurement in wave range of 4,000–400 cm^{-1}.

The components of the crude biosurfactant were separated and identified by liquid chromatography-mass spectrometry (LC–MS)/MS. LC analytical conditions are as follows: chromatographic separation was achieved with a 250 mm×4.6 mm×5 μm Kromasil C18 reverse-phase column. An acetonitrile–water gradient containing 4 mM of ammonium acetate was used. The elution was started with 40% acetonitrile for 4 min, and the acetonitrile concentration was raised to 90% after 20 min. LC flow rate was 0.4 ml/min [10], and the eluate was directly detected with a mass spectrometer. Negative ion mode was employed, and mass spectra were recorded across the range of m/z 100–1,000.

Results and Discussion

Isolation and Identification of Bacterial Strain for Biosurfactant Production

Several biosurfactant production strains were isolated from soil samples, from which one strain, GIM32, was selected for further study as the highest producer by critical micelle dilution (CMD) method. The colony of strain GIM32 was circular, smooth, sticky, and white. The cell was Gram negative, non-spore forming, rod-shaped, and motile by means of a single polar flagellum. The alignment of the partial 16S rDNA of the GIM32 strain with sequences obtained from BLAST searching at NCBI server showed 99% similarity to *P. aeruginosa*. Therefore, the GIM32 strain was identified as a number of *P. aeruginosa*. The sequence of the 16S rRNA gene of the strain GIM32 is available under the GenBank accession number HM067869. The biosurfactant secreted by *P. aeruginosa* has been considered to be rhamnolipids in many reports [11] and also identified by subsequent FTIR and LC–MS analysis in this study.

Effects of Carbon Source on Rhamnolipid Production

Five vegetable oils were used to investigate their effects on rhamnolipid production by *P. aeruginosa* GIM32, as shown in Fig. 1. The result shows that palm oil was the most suitable carbon source for rhamnolipid production, by which the yield reached 30.4 g/L of rhamnolipids. The other vegetable oils also yielded more than 24 g/L of rhamnolipid production. About 8 g/L biomass was gained from vegetable oils as carbon source.

P. aeruginosa can utilize many substrates such as carbohydrates, hydrocarbon, lipid, alcohols, etc., for growth and rhamnolipid production [12]. The vegetable oils are the most available carbon sources in all substrates. Though vegetable oils are insoluble in water, they can easily be emulsified and utilized by *P. aeruginosa* coupling to rhamnolipid production. Insoluble vegetable oils in a high concentration, even up to 160 g/L, were harmless to

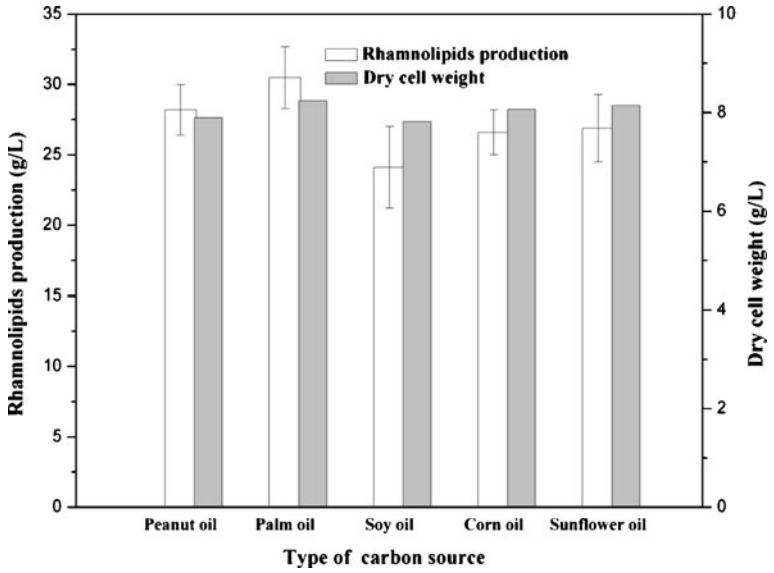

Fig. 1 The effect of carbon source on rhamnolipid production and dry cell weight

bacterial cells; this made it possible to use it for high-density fermentation [1]. Furthermore, at the same concentration, vegetable oil or soapstock can generate about twice as much energy as glucose by catabolism. In addition, vegetable oils can be easily hydrolyzed to produce long-chain fatty acids, which are important precursors for rhamnolipid synthesis [13]. Strains of *P. aeruginosa* could produce more than 15 g/L of rhamnolipids when utilizing vegetable oil, animal oil, or their byproducts as a carbon source in previous reports [14–17]. If glucose, the most common substrate, was used as the only carbon source, the rhamnolipid production did not exceed about 10 g/L in previous reports [18, 19]. Therefore, vegetable oils are the most appropriate carbon sources for rhamnolipid production.

Effect of Carbon to Nitrogen (C/N) Ratio on Rhamnolipid Production

The influence of C/N was investigated at a constant palm oil concentration (50 g/L). As shown in Fig. 2a, the rhamnolipid production reached the maximum values at 5 g/L of nitrate concentration (C/N ratio of 55). The production slightly declined as the nitrate content increased to 6 and 7 g/L (C/N ratio of 46 and 39). The biomass reached a maximum at 48 h and exhibited a positive relationship with the nitrate concentration. While the biomass exhibited a marked reduction at 120 h during culturing with a high nitrate concentration, under low nitrate concentration, it did not decrease with longer cultivation time.

The effect of C/N ratio with a constant nitrate concentration (50 g/L) was also investigated. The data from Fig. 2b suggested that palm oil concentration had a great influence on biomass concentration and rhamnolipid production. The maximum biomass was gained at a palm oil concentration of 40 g/L (C/N ratio of 44) and did not rise significantly with an increase in concentration. However, the maximum rhamnolipid production was reached by using 50 g/L palm oil (C/N ratio of 55). An insufficient carbon source would limit the cell growth and lead to lower biomass; the cell would then age and die in the later stages of cultivation. In contrast, an excess carbon source has no obvious effect on biomass or rhamnolipid production; the only effect is the increase in cost of the culture medium. Thus, a carbon source supply of 50 g/L is appropriate for rhamnolipid production (C/N ratio 55:1).

For an appropriate C/N ratio, some reports have considered that high-production rhamnolipids were achieved under a nitrogen-limiting condition [11, 16]. Other reports have found that the optimal C/N ratio varied with strains and carbon sources [17]. However, the biomass should also be taken into consideration. The nitrogen source supplied should satisfy the growth condition for a higher biomass, since high production of the strain must be accompanied by higher biomass. A carbon source not only provides energy for cell metabolism, but also is used as a raw material for rhamnolipid synthesis at the stable phase. Thus, a high biomass and sufficient carbon source supply conditions should be very helpful in enhancing rhamnolipid production.

Utilizing Molasses Distillery Wastewater as a Medium for Rhamnolipid Production

The optimal culture conditions, including appropriate pH (7.8) and dilution ratio (1:1), were determined by a series of experiments (data not shown). The wastewater pH was reduced from 7.8 to 6.5 after sterilization. The data acquired during cultivation of *P. aeruginosa* GIM32 in molasses distillery wastewater are presented in Fig. 3. The cell was able to grow well and secreted rhamnolipids using the half-strength of molasses distillery wastewater alone. The maximum biomass reached after culturing for 24 h was 2.7 g/L. The 2.6-g/L

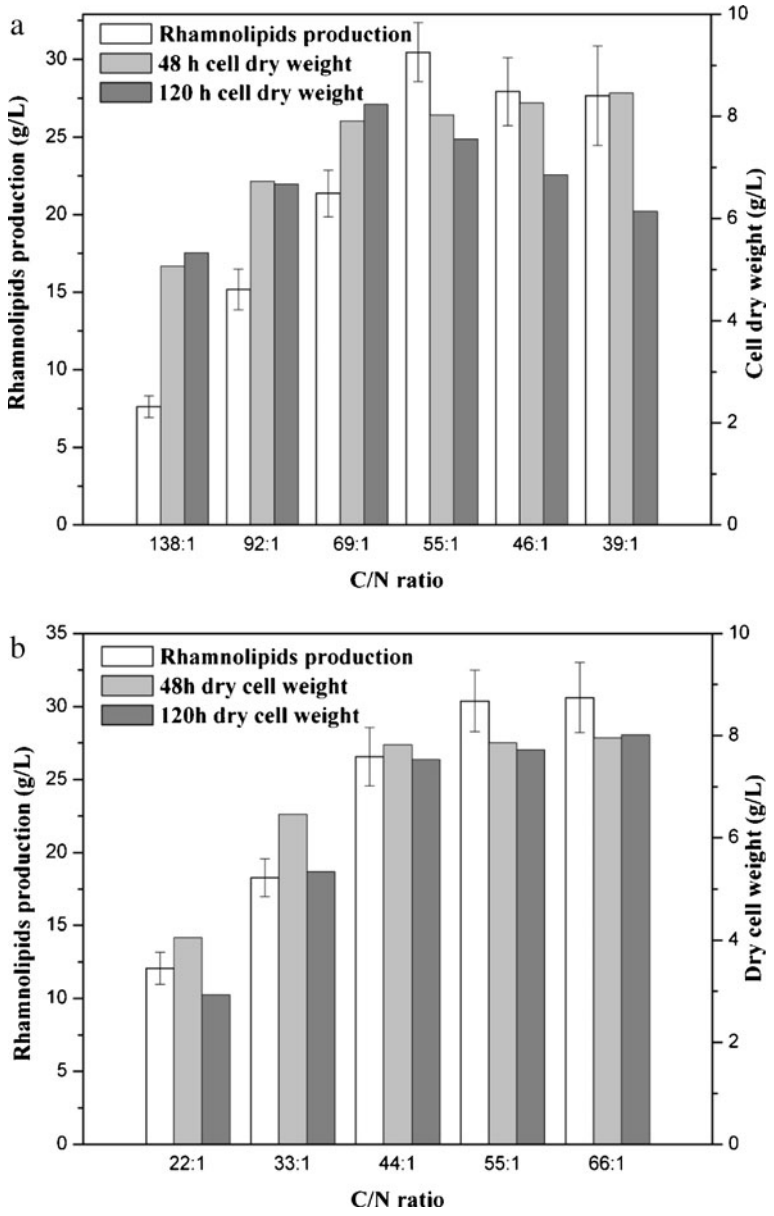

Fig. 2 The effect of carbon to nitrogen (C/N) ratio on rhamnolipid production by *P. aeruginosa* GIM32 with **a** constant 50 g/L of palm oil concentration and **b** constant 5 g/L of $NaNO_3$ concentration

rhamnolipids were obtained at 64 h, and the COD of the wastewater decreased from 86,900 mg/L to 48,600 mg/L. More than 52.5% of the rhamnolipids were produced during 36–48 h of culturing. After 48 h, the rhamnolipid production did not increase significantly, and the biomass obviously decreased. After culturing with *P. aeruginosa* GIM32, the COD of the wastewater decreased to 56%, and 2.6 g/L rhamnolipids were obtained from

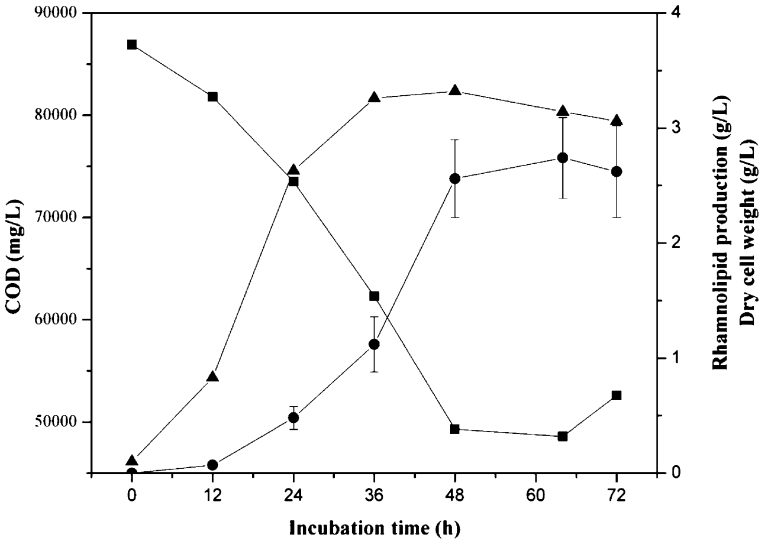

Fig. 3 The fermentation process course in half-strength molasses distillery wastewater. COD (*squares*), rhamnolipid production (*circles*), and dry cell weight (*triangles*) were measured every other 12 h during incubation time

wastewater at 60 h; thus, the complex, troublesome wastewater could be transformed into useful things.

Rhamnolipid production (2.6 g/L) using molasses distillery wastewater as an alternative medium production was lower than that by medium containing vegetable oils, but is 2.6 g/L much higher than in previous reports: 0.9 g/L rhamnolipid production by distillery and whey wastes [20], 0.24 g/L rhamnose production with molasses [21], and 0.206 g/L rhamnolipids with condensed molasses fermentation soluble [22]. The higher rhamnolipid production may be due to the excellent GIM32 strain and the suitable wastewater. Although the rhamnolipid production from wastewater was not higher than from vegetable oils, using wastewater as a growth substrate is a relatively inexpensive and economical process, which might make it more competitive with synthetics. What is more, 44% COD of wastewater was removed after rhamnolipid production, lowering the cost of wastewater treatment.

The utilization of the wastewater could reduce environmental pollution at processing industries while decreasing the cost of rhamnolipid production. However, it is uneconomical to extract pure rhamnolipids from this complicated wastewater culture broth. Rhamnolipids have many potential applications in the bioremediation environmental, including heavy-metal removal, hydrocarbon degradation, and pesticide degradation, which do not require pure rhamnolipids. Therefore, the low-cost acid precipitation method was an efficient and economical means for the recovery of crude rhamnolipids. Alternatively, the fermented liquid containing rhamnolipids might be directly injected into oil well for oil recovery [2]. Although the rhamnolipid production was low, the wastewater substrate was low cost, and there was no need to supply a carbon source or inorganic salts. Moreover, the yield may greatly increase using new processes such as solid-state culturing [20] or rest cell process [23] for further reducing of cost. More than half of the COD, including dark brown pigments, could not be utilized by *P. aeruginosa* GIM32. A similar result was reported of only 31% of COD reduction by *Hansenula* for single cell protein production [7]. Co-culturing *P. aeruginosa* and a decolorization microorganism strain may alleviate this problem by further enhancing COD

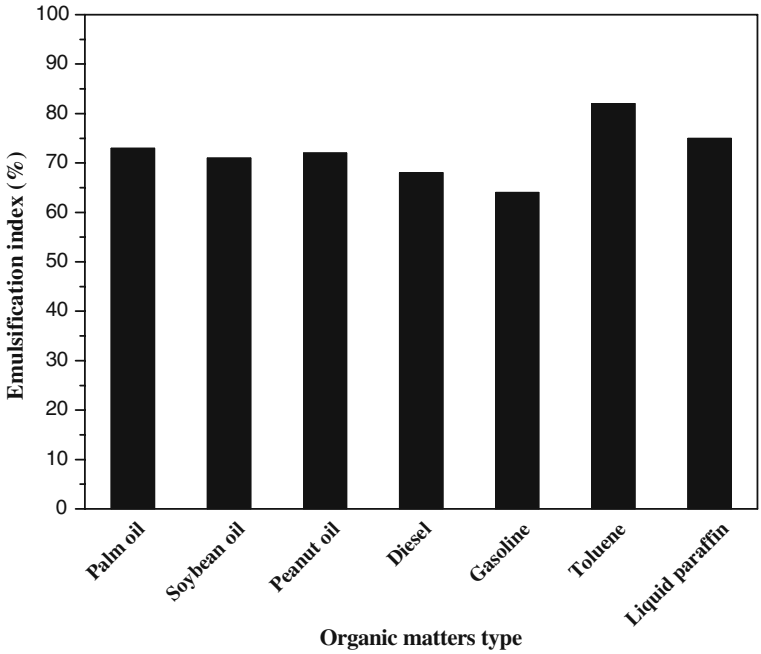

Fig. 4 Emulsification index of the rhamnolipids with various organic matters

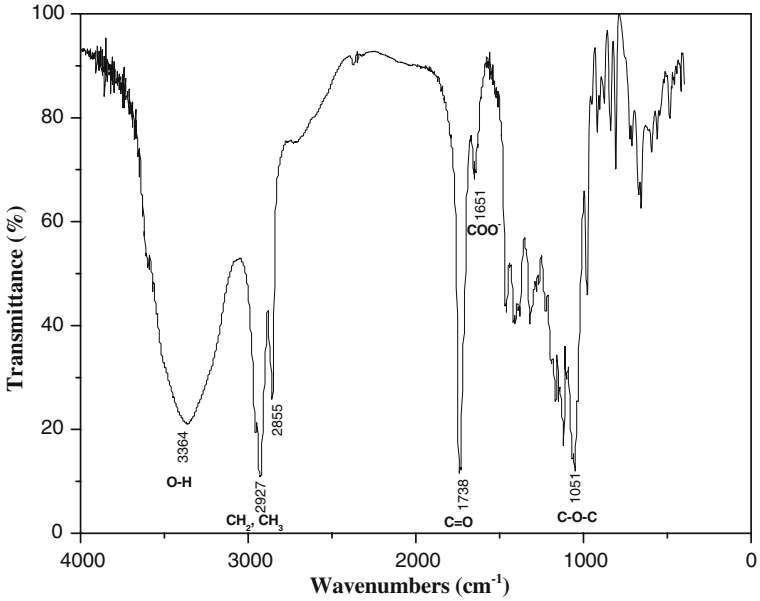

Fig. 5 Fourier transform infrared (FTIR) transmittance spectrum of rhamnolipids

Table 2 Structure and relative abundances of rhamnolipids homologues produced from molasses distillery wastewater by *P. aeruginosa* GIM32

Molecular weight	Possible chemical structure	Fragment ions	Relative abundance (%)
479	$RhaRhaC_{10}$	Rha,C_{10}	0.7
503	$RhaC_{10}C_{10}$	$RhaC_{10}$, $C_{10}C_{10}$, C_{10}	14.1
529	$RhaC_{10}C_{12:1}$	$RhaC_{10}$, $C_{10}C_{12:1}$, $C_{12:1}$, C_{10}	3.5
531	$RhaC_{10}C_{12,}RhaC_{12}C_{10}$	$RhaC_{12}$, $RhaC_{10}$, $C_{10}C_{12}$, C_{12}, C_{10}	5.8
593	$RhaRhaC_8C_8$ $RhaRhaC_{10}C_6$	$RhaRhaC_8$, $RhaRhaC_{10}$	0.5
621	$RhaRhaC_8C_{10,}$ $RhaRhaC_{10}C_8$	RhaRha C_8, $RhaRhaC_{10}$	13.0
647	$RhaRhaC_{10}C_{10:1,}$ $RhaRhaC_8C_{12:1}$	$RhaRhaC_{10}$,$RhaRhaC_8$	1.9
649	$RhaRhaC_{10}C_{10}$	$RhaRhaC_{10}$, $C_{10}C_{10}$	32.6
675	$RhaRhaC_{10}C_{12:1}$	$RhaRhaC_{10}$	12.0
677	$RhaRhaC_{10}C_{12,}$ $RhaRhaC_{12}C_{10}$	$RhaRhaC_{10}$, $RhaRhaC_{12}$, $C_{10}C_{12}$, C_{12}	13.3
705	$RhaRhaC_{12}C_{12,}$ $RhaRhaC_{14}C_{10}$	$RhaRhaC_{12}$, $RhaRhaC_{14}$, $C_{14}C_{10}$, $C_{12}C_{12}$	2.6

and the color removal rate at the same time; this would be similar to the combined *Saccharomyces cerevisiae* and *Coriolus versicolor* treatment process [24].

Characterization of Rhamnolipids Produced from Molasses Distillery Wastewater by *P Aeruginosa* GIM32

The emulsification index of the rhamnolipids for non-polar organic solvents was shown in Fig. 4. The emulsification indexes reached 71–73% with vegetable oils, suggesting a potential application as a cleaning and emulsifying agent in the food industry. Moreover, the emulsification indexes for diesel (68%) and gasoline (64%) suggested that this rhamnolipid product has the potential to be applied in bioremediation and oil recovery.

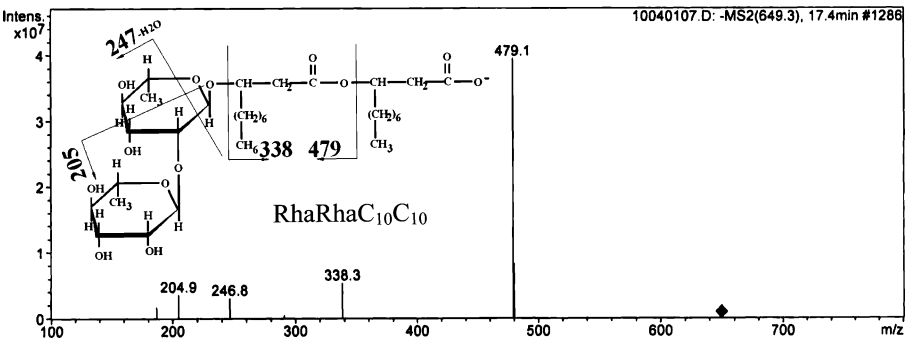

Fig. 6 Possible structure of rhamnolipids fragment ions at *m/z* 649 identified by tandem mass spectrometry

Chemical Structure of Biosurfactant Produced from Molasses Distillery Wastewater by *P. Aeruginosa* GIM32

As shown in Fig. 5, the important adsorption bands involved strong adsorption peaks at 3,364 cm^{-1} (O-H stretching vibrations), 2,928–2,957 cm^{-1} (CH_2, CH_3), 1,651 cm^{-1} (C=O stretching vibration of the carbonyl groups), and 1,150 cm^{-1} (C-O-C stretching in the sugar). These characteristic adsorption bands demonstrate that this biosurfactant is a glycolipid, containing sugar rings and long hydrocarbon chains. The sugar from the acid hydrolysis of biosurfactant has the same retention time as that of rhamnose, as determined by HPLC analysis (data not shown), which means that this glycolipid can be identified as rhamnolipids. Tandem mass spectrum analysis revealed the presence of five major components which were the molecular ions at *m/z* 503 (14.4%), *m/z* 621 (13.0%), *m/z* 649 (32.6%), *m/z* 675 (12.0%), and *m/z* 677 (13.3%) as shown in Table 2. Also, other six different molecular weight components in the biosurfactant extract sample were detected. The differences in rhamnolipids components depend on the bacterial strain and culture medium condition. It was reported that from two to 28 kinds of homologue were detected in rhamnolipids mixed samples [25, 26]. The ratio of monorhamnolipids and dirhamnolipids produced by the strain GIM32 was about 1:3.27. According to the tandem mass spectrum results, the chemical structure of each component can be illuminated as shown in Table 2. Figure 6 was the molecular structure of *m/z* 649 speculated by the tandem mass spectrum results. The daughter ions at *m/z* 479 ($RhaRhaC_{10}$) and *m/z* 338 ($C_{10}C_{10}$) indicated that the molecular structure of *m/z* 649 was $RhaRhaC_{10}C_{10}$.

Conclusions

In conclusion, *P. aeruginosa* GIM32 can secrete high-activity rhamnolipids. Palm oil as the carbon source and a high C/N ratio (55:1) were suitable for rhamnolipid production. Forty-four percent of the COD of the molasses distillery wastewater was removed, and 2.6 g/L rhamnolipid production was obtained from half-strength wastewater alone; this was much higher than the results of previous reports using wastewater as a substrate. The utilization of molasses distillery wastewater as a renewable resource with *P. aeruginosa* GIM32 would be an ideal biotechnological process for rhamnolipid production.

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