J Am Oil Chem Soc (2011) 88:1935–1943
DOI 10.1007/s11746-011-1875-z

ORIGINAL PAPER

Non-traditional Oils as Newer Feedstock for Rhamnolipids Production by *Pseudomonas aeruginosa* (ATCC 10145)

Amit Pratap · Sushant Wadekar · Sandeep Kale · Arvind Lali · Dipfi Narayan Bhowmick

Received: 18 November 2010 / Revised: 3 May 2011 / Accepted: 27 May 2011 / Published online: 15 June 2011

Abstract Oils and fats serve as one of the most important renewable feedstocks for various chemicals such as lubricants, textiles auxiliaries, biodiesel and surfactants. The oils have also proved themselves to be better substrates than glucose for production of biosurfactants such as rhamnolipids. Cost is major hindrance in the commercialization of these biosurfactants and fresh refined oils cannot be used for rhamnolipid production. Non-traditional oils such as jatropha oil, karanja oil and neem oil can be used as newer feedstock for the synthesis of rhamnolipids. Jatropha oil gave the highest production of rhamnolipids, 4.55 g/L in non-traditional oils and the rhamnolipid concentration was comparable to that of most common oils, sunflower oil giving 5.08 g/L of rhamnolipids. The jatropha oil contained mainly linoleic acid that showed the highest consumption rate as compared to oleic and palmitic acid. Neem oil produced a lower concentration of rhamnolipids (2.63 g/L) than other oils. Both monorhamnolipids and dirhamnolipids were synthesized using these oils. The product obtained can find high value specialty applications such as biomedical drug delivery and cosmetics.

Keywords Biosurfactants · Rhamnolipids · *Pseudomonas aeruginosa* · Non-traditional oils · Mass spectra · NMR

A. Pratap (✉) · S. Wadekar · D. N. Bhowmick
Department of Oils, Institute of Chemical Technology, Mumbai, India
e-mail: amitpratap0101@rediffmail.com

S. Kale · A. Lali
DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai, India

Introduction

Oils and fats are one of the most promising renewable resources for various industrial derivatives including cosmetics, textiles, surfactants, etc. [1, 2]. Vegetable oils have been continuously researched as substrates for the production of rhamnolipids [3–5] this has indicated that vegetable oils are better carbon sources than water soluble substrates such as glucose.

Rhamnolipids are the simplest class of biosurfactants produced by various strains of *Pseudomonas aeruginosa.* They contain one or two molecules of rhamnose linked to β-hydroxy fatty acid (mainly C10) linked via a glycoside linkage [6, 7]. The two most commonly found structures [6] of rhamnolipids are L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (R1, monorhamnolipid) and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (R2, dirhamnolipid). In addition, the lipid moiety may also contain hydroxyoctanoic acid (C_8), hydroxydodecanoic acid (C_{12}) and hydroxydodecenoic acid ($C_{12:1}$) in place of hydroxydecanoic acid (C_{10}) [7]. Rhamnolipids can be used in the bioremediation of soil and aquatic media and as raw materials for the synthesis of fine chemicals such as pyrenacylester of rhamnolipids and rhamnose [8]. In addition rhamnolipids can find application in the biomedical field as emulsifying aids for the transport of drugs to the site of action, for a supplementing pulmonary surfactant or as an adjuvant for vaccines [9].

The cost of production of biosurfactants is relatively high and raw materials account for 10–30% of the overall cost [10]. The rhamnolipid production by *P. aeruginosa* was increased when vegetable oils were used as substrates in place of water soluble substrates such as glucose and glycerol [3–5]. Refined vegetable oils cannot be used for the production of rhamnolipids due to their high cost.

Various non-traditional oils such as jatropha oil, karanja oil and neem oil can be used as renewable and low cost substrates that have vast potential but yet unexploited. These oils cannot be used for edible purposes either due to a strong disagreeable odour or due to toxic components. Jatropha oil has been proposed as a low cost feedstock for biodiesel production. Other oils are used locally for soap-making and medicinal purpose. These non-traditional oils contain various nonglyceride minor constituents that may affect the metabolic activity of microbes and hence rhamnolipid production. Neem oil contains a number triterpenoids mainly azadirachtin (0.4–0.9%) and acts as an insecticide and thioketone as odor causing substances [11]. Similarly karanja oil contains flavanoids such as karanjin (2.2–4.5%) and pongamol (0.4–0.9%) etc. that possesses insect repelling properties [12]. Jatropha oil contains curcin, lectins and most toxic phorbol esters (~0.6%) that also act as a co-carcinogen [13]. These bioactive compounds are toxic to insects, but their effect on the metabolic activity of denitrifying bacteria especially on *P. aeruginosa* is not known. But recently, rhamnolipids have been produced on the deoiled cake of karanja, jatropha and neem [14] that indicates *P. aeruginosa* can produce rhamnolipids in the presence of these nonglyceride compounds.

In the present study, non-traditional oils such as neem oil, karanja oil and jatropha oil were used as newer feedstocks for rhamnolipids production. This paper briefs the effect of these non-traditional oils as substrates on rhamnolipid production by *P. aeruginosa* (ATCC 10145).

Materials and Methods

Raw Materials

Refined sunflower oil samples were received as a gift from M/s Cargill India Pvt. Ltd., New Delhi. The non-traditional oils (neem, karanja and jatropha) were procured from a local market. All other chemicals were procured from M/s Hi-Media, Mumbai and all of them were analytical reagent grade.

Microorganism

The natural strains of *P. aeruginosa* ATCC 10145 were procured from the National Collection of Industrial Microorganisms (NCIM) Pune, India as NCIM 2863.

Cultivation Conditions

P. aeruginosa ATCC 10145 strains were maintained on nutrient agar slants. A loopful of *P. aeruginosa* cells taken from the seed culture was placed in a 250-mL Erlenmeyer flask containing 50 mL nutrient broth (NB). *P. aeruginosa* was grown in a rotary shaker set at 30 °C and 200 rpm. The cells were separated by centrifugation and re-suspended in a sterile mineral salt medium without carbon source as mentioned below. A 2% (v/v) of this cell suspension was used as inoculums in a sterile mineral salt medium [7] with the following composition (g/L): $NaNO_3$ 4.0, K_2HPO_4 1.0, KH_2PO_4 0.5, $MgSO_4$ $7H_2O$ 0.01, KCl 0.1, $FeSO_4.7H_2O$ 0.01, $CaCl_2$ 0.01, yeast extract 0.01, HCl to pH 6.8 and 0.05 mL/L of the trace element solution containing (g/L) H_3BO_3 0.26, $CuSO_4$. $5H_2O$ 0.5, $MnSO_4$. H_2O 0.5, $MoNa_2O_4$. $2H_2O$ 0.06, $ZnSO_4$. $7H_2O$ 0.72. The oils were autoclaved separately and added to the medium just prior to inoculation. The carbon source (5% w/v) was varied with the types of oil from sunflower oil to karanja oil, jatropha oil and neem oil. pH of the medium is adjusted to 6.8 ± 0.1 using 1 N HCl before autoclaving.

Shake flask fermentations were carried out in a 250-mL Erlenmeyer flask containing 50 mL of the mineral salt medium along with the various oils as the carbon source, followed by cultivation at 30 ± 1 °C for 96 h at 200 rev/min.

Biomass Determination

The biomass was removed from 1 mL broth solution by centrifugation and re-suspended in 5 mL of 0.85% (w/v) NaCl solution to avoid cell lysis. The biomass was measured in term of absorbance at 610 nm [15]. The method was calibrated by using the dry weight of the samples measured after drying at 105 °C until constant weight.

Rhamnolipids Recovery

After completion of fermentation, the biomass was removed by centrifugation. The pH of the supernatant was adjusted to 2.0 using 1 N HCl solution. The broth solution (75 mL) was taken in the 250 mL separating funnel and extracted with 3 volumes of 75 mL *n*-hexane for removal of residual oil in *n*-hexane extract. Further, rhamnolipids were extracted from the raffinate i.e. aqueous layer with three volumes of ethyl acetate [6, 16]. Ethyl acetate extracts were combined and the solvent was evaporated under vacuum to give crude rhamnolipids.

The crude rhamnolipid samples were diluted with distilled water to obtain a concentration of 150 μg/mL. Total rhamnolipid concentration in the sample was determined by measuring the concentration of hydrolysis-released rhamnose by the orcinol method [17] after acid hydrolysis of the sample. Rhamnolipid content was determined by multiplying the rhamnose values by 3.4 [7].

The hexane washings were combined and hexane was evaporated to get the residual oil. Fatty acid compositions

of the residual oils and fresh oils were determined by A.O.C.S. method Ce-1-62 [18]. The final concentration of individual fatty acid in the broth was calculated based on the quantity of residual oils and its fatty acid composition. The % utilization of individual fatty acids was calculated by using its initial and final concentration in the broth.

TLC, HPLC, Mass Spectra and NMR

The formation of different components was checked by thin layer chromatography on silica gel plates (Merck DC Kieselgel 60 F_{254}) using chloroform/methanol/water (65:15:2, by vol.) as mobile phase [15, 19]. After development, the spots were visualized by spraying the plate with methanol/H_2SO_4 (50:50, v/v) and heating at 140 °C. The Rf values of the various spots were calculated as the ratio of distance run by the spot and solvent front.

Individual rhamnolipids in crude extract were separated and identified by HPLC (Agilent 1200 series) using a UV variable wavelength detector (Agilent G1314B). The samples (25 µL) were analyzed by HPLC on a Eurospher-100 C8 column, 5 µm, 4.6 × 250 mm (Dr. Ing. H. Knauer GmbH, Berlin, Germany). An acetonitrile water gradient was used starting with acetonitrile/water (30:70, v/v) for 5 min, increased to acetonitrile in 40 min and maintained there for the next 6 min. The solvent flow rate was kept at 1.0 mL/min and the elution was monitored at 225 nm [16].

Quantification of the HPLC method was done by the method mentioned previously for sophorolipids [20]. With repeated injections, fractions containing individual rhamnolipids corresponding to peaks were collected manually just downside of the UV detector. The purity of the individual fractions was checked by repeating the HPLC analysis of the fractions. The collected individual rhamnolipids were quantified by the orcinol method [21]. Calibration curves between the HPLC peak areas and the rhamnolipids concentrations were established accordingly.

Electrospray ionization (ESI) mass spectra were recorded on a Q-TOF LC–MS (Agilent 6520) mass spectrometer as per the method mentioned previously [22, 23]. The ESI mass spectrum in the negative ion mode was acquired using a capillary voltage of −3.5 kV, fragmentation voltage of 50 V and desolvation gas (nitrogen) was heated at 325 °C with flow rate of 7 L/min. ESI tandem mass spectra were acquired by mass-selecting the target ion using the quadrupole mass analyzer followed by 20 eV, collision induced dissociation using nitrogen in collision cell. The previously collected pure fractions from HPLC were dissolved in methanol/water (8:2, v/v), filtered (0.22 mm) and introduced into the source at 0.4 mL/min with a syringe pump. The gas nebulizer pressure

Table 1 Analysis of non-traditional oils

Test	A.O.C.S. method [18]	Sunflower oil	Jatropha oil	Karanja oil	Neem oil
Acid value (mg KOH/g)	Te-la-64	0.29	20.21	21.84	27.44
Iodine value (% iodine)	Tg-la-64	140.09	94.41	89.23	77.02
Saponification value (mg KOH/g)	TL-la-64	186.11	186.49	180.02	185.33
Fatty acid composition (% w/w) by A.O.C.S. method Ce-1-62 [18]					
Palmitic C16:0		6.13	14.65	12.47	9.70
Stearic C18:0		4.71	7.53	7.91	10.49
Oleic C18:1		24.00	40.12	59.18	54.93
Linoleic C18:2		63.65	34.52	13.53	17.07
C20 and above		1.13	2.18	6.92	4.62

Table 2 Rhamnolipid produced by *P. aeruginosa* (ATCC 10145) on sunflower oil and non-traditional oils (5% w/v) in a shake flask at 30 °C, 200 rpm after 96 h

	Carbon source (50 g/L w/v)			
	Sunflower oil	Jatropha oil	Karanja oil	Neem oil
Titre of rhamnolipids (g/L)	5.08 ± 0.09	4.55 ± 0.07	3.71 ± 0.17	2.63 ± 0.12
Residual oil (g/L)	22.17 ± 1.51	21.31 ± 0.80	24.75 ± 1.34	26.87 ± 1.16
Biomass (g/L)	1.33 ± 0.09	1.28 ± 0.08	1.17 ± 0.10	0.68 ± 0.08
Composition of rhamnolipids (% w/w)				
Rha_2C_{10}	5.79	<0.1	<0.1	<0.1
$Rha_2C_{10}C_{10}$	69.24	47.12	48.15	48.06
$Rha_2C_{10}C_{12}$	3.20	2.86	9.51	11.52
$RhaC_{10}C_{10}$	21.77	50.02	42.35	40.43
R2/R1 ratio	3.6	1.0	1.4	1.5

The values given are the means of three replicates ± standard deviations

Table 3 Rhamnolipids produced on various carbon sources by *P. aeruginosa*

Strain	Carbon source	Rhamnolipids (g/L)	Ref.
P. aeruginosa UW-1	Corn oil (6%)	**15.0**	[3]
	Soybean oil (6%)	**14.5**	
P. aeruginosa J4	Sunflower oil (6%)	**2–2.1**	[4]
P. aeruginosa 47T2 NCIB 40044	Used olive oil (4%)	**2.7**	[19]
P. aeruginosa AT10	Waste fatty acids (5%)	**9.5**	[26]
P. aeruginosa ATCC 10145	Glucose (6%)	**1.79**	[25]
	Glycerol (5%)	**2.75**	[25]
	Glycerol Residue (5%)	**2.50**	[21]
P. aeruginosa ATCC 10145	Sunflower oil	**5.08**[a]	
	Jatropha oil	**4.55**[a]	
	Karanja oil	**3.71**[a]	

Proton responsible for chemical shift in NMR are given in bold

[a] Rhamnolipids concentration obtained in the present work

was 45 psi. The fragmentation information from MS was used to assess the molecular structure of the rhamnolipids.

Structures of rhamnolipids were also confirmed by ^{1}H-NMR spectra obtained with a Mercury Plus 300 NMR Spectrometer (300 MHz, Varian, USA) at the Sophisticated Analytical Instrument Facility (SAIF), IIT-Bombay. The spectra were recorded in chemical shifts expressed as parts per million (ppm) downfield from tetramethylsilane (TMS) as an internal standard reference.

Results and Discussion

The synthesis of rhamnolipids have been reported using a variety of carbon sources such as glucose [24], glycerol [25], alkanes [6], vegetable oils [3, 4] and also waste streams such as waste free fatty acids [5, 26], used cooking oils [19], sweet water [25] and glycerol residue [21] from glycerin distillation plants, etc. In addition, *P. aeruginosa* can synthesize rhamnolipids on toxic polycyclic aromatic hydrocarbons (PAH) such as naphthalene [27]. Hence, *P. aeruginosa* can utilize a variety of chemicals as the carbon source. The concentration of rhamnolipids has been more on water insoluble substrates especially vegetable oils such as olive oil [28], canola oil [3] etc. These oils are rich in oleic acid and linoleic acid that may be one of the reasons for higher concentrations of rhamnolipids with these oils.

Analysis of Non-traditional Oils

Non-traditional oils were analyzed for various tests as shown in Table 1. These oils showed high free fatty acid (FFA) content. Karanja oil and neem oil contain oleic as the major fatty acid and jatropha oil contains both oleic and linoleic acid as the major component. Thus these oils are rich in oleic and linoleic acid and can be used as alternatives to vegetable oils as the substrate for rhamnolipid production.

Effect of Non-traditional Oils on Rhamnolipid Production

These non-tradition oils (50 g/L) were used as substrates for rhamnolipid production by *P. aeruginosa* (ATCC 10145) and the production was compared with that of refined sunflower oil (Table 2). Previous studies on rhamnolipid synthesis by *P. aeruginosa* indicated that rhamnolipids produced (g/L) changed with both type of strain and carbon source (Table 3). The rhamnolipid

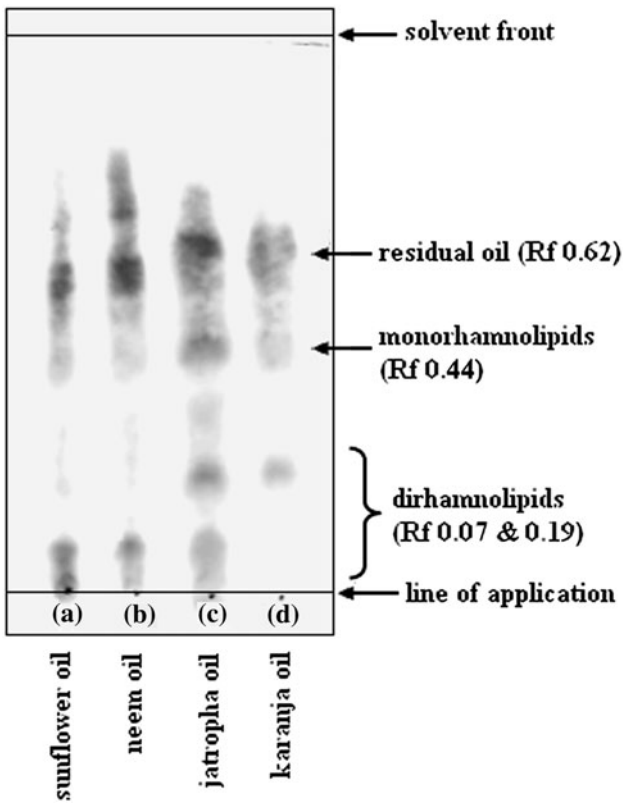

Fig. 1 Thin layer chromatography of rhamnolipids produced by *P. aeruginosa* (ATCC 10145)

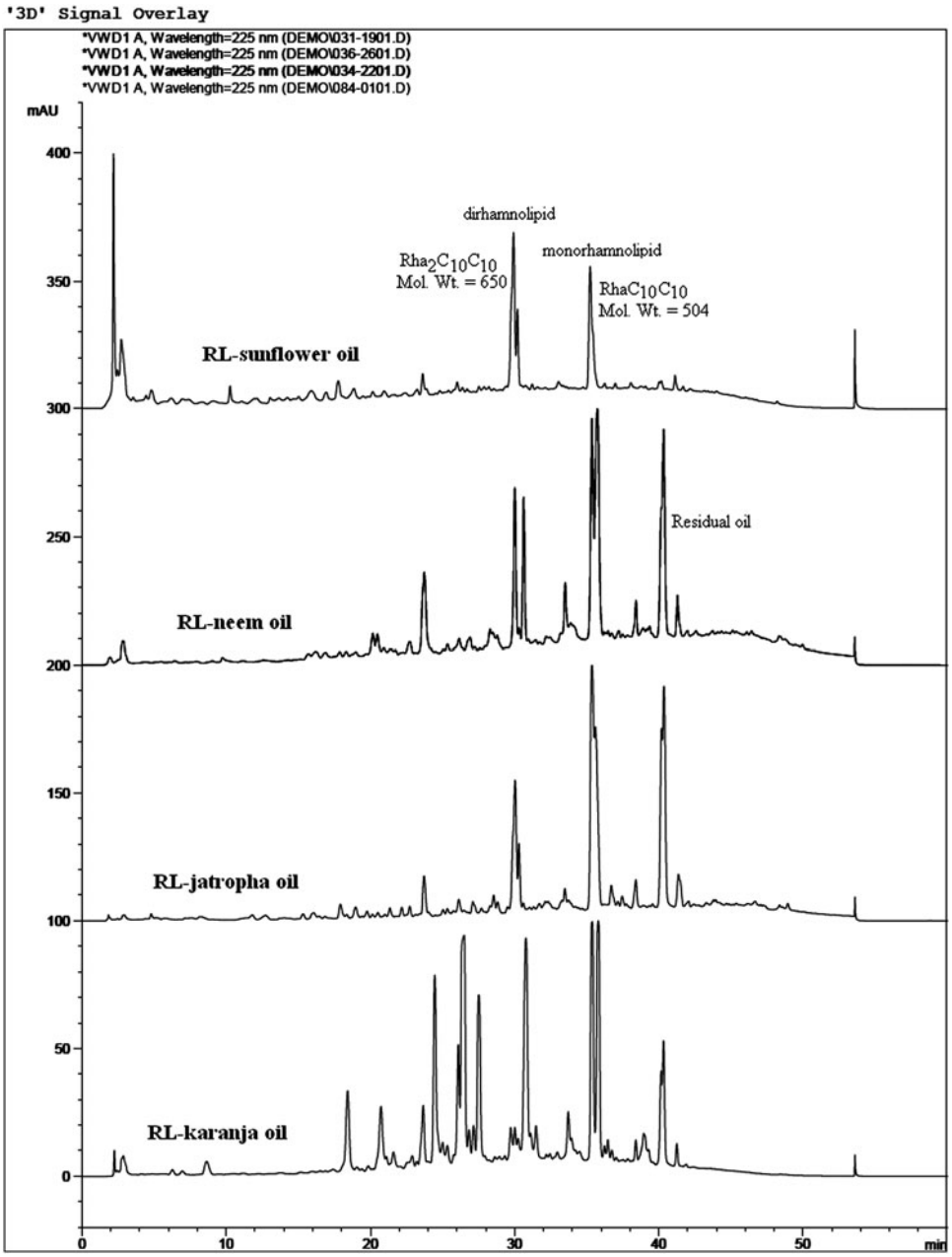

Fig. 2 Overlay of HPLC chromatogram crude rhamnolipids synthesized by *P. aeruginosa* (ATCC 10145) on 5% oils **a** sunflower oil, **b** neem oil, **c** jatropha oil, **d** karanja oil with the Agilent HPLC 1200 series

Table 4 Percentage utilization of fatty acid in various oils during rhamnolipid production by *P. aeruginosa* (ATCC 10145) after 96 h with 50 g/L of initial concentration of oils as carbon source

Fatty acids	Sunflower oil		Jatropha oil		Karanja oil	
	Residual oil composition (% w/w)	% utilization	Residual oil composition (% w/w)	% utilization	Residual oil composition (% w/w)	% utilization
C16	14.39 ± 0.31	3.62	27.30 ± 1.08	20.69	11.79 ± 0.41	53.20
C18:2	34.28 ± 2.39	74.62	5.97 ± 0.13	92.63	12.23 ± 0.32	55.26
C18:1	41.07 ± 1.22	35.77	45.64 ± 0.41	51.52	55.84 ± 1.13	53.29
C18:0	7.72 ± 0.29	12.45	16.12 ± 0.09	8.76	13.53 ± 0.46	15.33
C20	2.54 ± 0.10	7.69	4.97 ± 0.19	2.83	6.6 ± 0.20	52.79

The values given are means of three replicates ± standard deviations

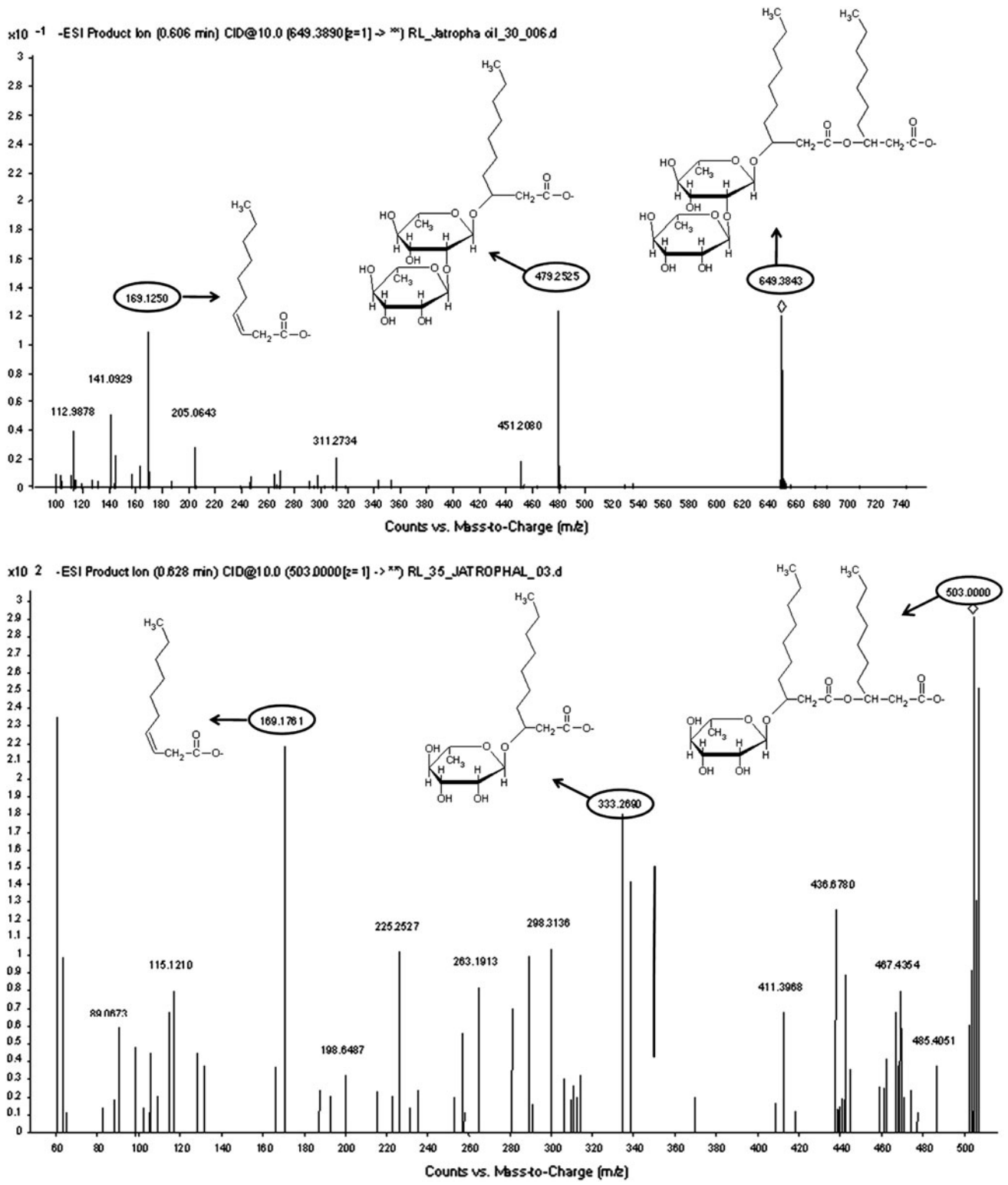

Fig. 3 ESI tandem mass spectra of **a** $(Rha_2C_{10}C_{10}\text{-}H)^-$ of *m/z* 649, **b** $(RhaC_{10}C_{10}\text{-}H)^-$ of *m/z* 503 using Q-TOF LC–MS Agilent 6520/Agilent 1200 system

produced (Table 3) also varies with the type of oil used as a carbon source [3–5]. Among the three oils studied, karanja oil and jatropha oil gave higher production of rhamnolipids (g/L) than neem oil (Table 2). The rhamnolipid concentrations were comparable to that of sunflower oil and higher than that of glucose and glycerol. The formation of

various rhamnolipids was checked by TLC and HPLC (Figs. 1, 2). The Rf values of the individual components were compared with purified components and also with Rf value mentioned previously [3]. The crude rhamnolipids contained both monorhamnolipid and dirhamnolipids. Both of them have surface activity [23, 29] and antimicrobial activity [30, 31].

The results indicated the potential of these non-traditional oils as low cost substrates for rhamnolipids production. Rhamnolipids synthesized on jatropha oil, karanja oil and neem oil showed lower dirhamnolipids to monorhamnolipids ratio as compared to sunflower oil. Rhamnolipids are synthesized from β-hydroxy fatty acids and the donor of the rhamnosyl moiety, TDP-L-rhamnose that is formed respectively by the fatty acid synthetic pathway and from glucose-1-phosphate [32]. The intracellular ratio of β-hydroxyacetyl-Co-A (C8–C12) and TDP-L-rhamnose determines the final ratio of monorhamnolipids and dirhamnolipids. First, monorhamnolipids are formed by transfer of one rhamnose moiety to β-hydroxyacetyl-Co-A and then additional rhamnose moiety is attached to it by TDP-L-rhamnose to form di-rhamnolipids. When formation of TDP-L-rhamnose is reduced, then overall formation of rhamnolipids is reduced and the ratio of mono-rhamnolipids to dirhamnolipids increases. This may be the case for jatropha oil and karanja oil.

Neem oil has produced a reduced amount of rhamnolipids with a lower dirhamnolipids to monorhamnolipid ratio. The formation of biomass was also affected. The lower concentration of rhamnolipids using neem oil can be due to inhibition of *P. aeruginosa* by terpenoids mainly azadirachtin along with sulfur-containing compounds [33].

The fatty acid compositions of residual oils (sunflower oil, jatropha oil, karanja oil and neem oil) are shown in Table 4. For sunflower oil, consumption of linoleic acid was highest as compared to oleic acid and palmitic acid. In the case of karanja oil, oleic acid is the major fatty acid whose extent of consumption is the same as that of other fatty acids. In the case of jatropha oil, consumption of linoleic acid was more like that of other fatty acids such as oleic acid and palmitic acid. It had been reported previously that linoleic acid was nearly exhausted (82% consumed) in the case of soybean oils as substrate [5] and the results are comparable with the present data. This also indicated that oils high in linoleic acids such as sunflower oil, soybean oil and jatropha oil produced more rhamnolipids as compared to the oils low in linoleic acid such as karanja oil and neem oil.

Structural Elucidation of Rhamnolipids Produced on Non-traditional Oils by HPLC, LC–MS and NMR

The HPLC analysis of the crude product revealed various peaks (Fig. 2) showing corresponding anions with different *m/z* ratio mass spectra. The two major peaks at 29 and 35 min confirmed the presence of anions with *m/z* ratios of 649 and 503 as major components in mass spectra [7, 26] These ions correspond to the deprotonated molecules $(M–H)^-$ of the dirhamnolipids and monorhamnolipids with molecular weights 650 ($Rha_2C_{10}C_{10}$) and 504 ($RhaC_{10}C_{10}$) respectively. Mass selection of these anions followed by collision-induced dissociation (CID) produced characteristic tandem mass spectra (Fig. 3) that confirmed the structural assignments [7, 22]. For instance $(RhaC_{10}C_{10})^-$ of *m/z* 503 dissociated to form two major fragment ions by the loss of a monounsaturated C_{10} carboxylic acid unit to form ($RhaC_{10}$–H) of *m/z* 333, and *m/z* 169 by the loss of $RhaC_{10}H$ to form (unsaturated C_{10}–H)$^-$. $(Rha_2C_{10}C_{12}–H)^-$ of *m/z* 649 dissociated similarly and showed fragments of *m/z* of 479 and 169 corresponding to structure (Rha_2C_{10}–H)$^-$ and (unsaturated C_{10}–H)$^-$. The structures of purified rhamnolipids were confirmed by ^{1}H NMR and the results are shown in Table 5. The chemicals shifts were comparable to previous reports [3, 4, 34] and the results were in accordance with the structures as shown by mass spectra (Fig. 3).

Table 5 ^{1}H-NMR chemical shift data for rhamnolipid components

^{1}H chemical shift (ppm)	Multiplicity	Assignment
0.880	Triplet	–C**H$_3$** (on β-hydroxyfatty acids)
1.185, 1.204	Doublet	–C**H$_3$** (on rhamnose moiety)
1.292	Multiplet	–(C**H$_2$**)$_5$– (on β-hydroxyfatty acids)
1.546	Multiplet	–(C**H$_2$**)–CH(O)–CH$_2$COO (on β-hydroxyfatty acids)
2.516	Multiplet	–CH(O)–C**H$_2$**COO (on β-hydroxyfatty acids)
3.371	Multiplet	–(CH$_2$)–C**H**(O-Rha)–CH$_2$COO (on β-hydroxyfatty acids)
3.634	Multiplet	–C**H**–OH (on rhamnose moiety)
4.109	Multiplet	–(CH$_2$)–C**H**(–O–C=O)–CH$_2$COO (on β-hydroxyfatty acids)
4.810	Singlet	–CH–O**H** (on rhamnose moiety)
5.275, 5.295	Doublet	–C**H**–O–C (on rhamnose moiety)

Conclusions

The non-traditional oils are rich in oleic and linoleic acids and hence, they can replace costly substrates such as sunflower oil without substantial effect on rhamnolipids concentration (g/L). The ratio of dirhamnolipids to monorhamnolipids was lower in the case of non-traditional oils. The % consumption of linoleic acid was more than other fatty acids (oleic acid and palmitic acid). This increased the concentration of rhamnolipids using jatropha oil and sunflower oil. The results of the present study showed that jatropha oil and karanja oil can be exploited as low cost substrates for a high value product, i.e., rhamnolipids to be used in specialty applications such as drug delivery and in cosmetics.

Acknowledgments This study was financially supported by the Department of Biotechnology (DBT), Ministry of Science & Technology, Government of India, under sanction order number BT/PR8481/PID/06/334/2006.

References

1. Johnson RW, Fritz E (1989) Fatty acids in industry, 1st edn. Marcel Dekker, New York
2. Matheson KL (1996) Surfactant raw materials, classification, synthesis and uses. In: Spitz L (ed) Soaps and detergents, a theoretical and practical review. AOCS Press, Illinois, pp 288–303
3. Sim L, Ward OP, Li Z-Y (1997) Production and characterization of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1. J Ind Microbiol Biotechnol 19:232–238
4. Wei YH, Chou CL, Chang JS (2005) Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater. Biochem Eng J 27:146–154
5. Nitschke M, Costa SGVAO, Haddad R, Gonçalves LAG, Eberlin MN, Contiero J (2005) Oil wastes as unconventional substrates for rhamnolipid biosurfactant production by *Pseudomonas aeruginosa* LBI. Biotechnol Prog 21:1562–1566
6. Itoh S, Honda H, Tomita F, Suzuki T (1971) Rhamnolipids produced by *Pseudomonas aeruginosa* grown on *n*-paraffin. J Antibiot (Tokyo) 24:855–859
7. Benincasa M, Abalos A, Oliveira I, Manresa A (2004) Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. Antonie van Leeuwenhoek 85:1–8
8. Maier RM, Chavez GS (2000) *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Appl Microbiol Biotechnol 54:625–633
9. Kosaric N (1996) Biosurfactants. In: Rehm HJ, Reed G, Pühler A, Stadler P (eds) Biotechnology, vol 6. VCH, Weinheim, New York, Basel, pp 659–717
10. Mulligan CN, Gibbs BF (1993) Factors influencing the economics of biosurfactants. In: Kosaric N (ed) Biosurfactants: production, properties, applications. Marcel Dekker, New York, pp 329–371
11. Boeke SJ, Boersma MG, Alink GM, van Loon JJA, van Huis A, Dicke M, Rietjens IMCM (2004) Review, safety evaluation of neem (*Azadirachta indica*) derived pesticides. J Ethnopharmacol 94:25–41
12. Gore VK, Satyamoorthy P (2000) Determination of pongamol and karanjin in karanja oil by reverse phase HPLC. Anal Lett 33:337–346
13. Ahmed WA, Salimon J (2009) Phorbol ester as toxic constituents of tropical *Jatropha curcas* seed oil. Eur J Sci Res 31:429–436
14. Hazra C, Kundu D, Ghosh P, Joshi S, Dandia N, Chaudharia A (2010) Screening and identification of *Pseudomonas aeruginosa* AB4 for improved production, characterization and application of a glycolipid biosurfactant using low-cost agro-based raw materials. J Chem Technol Biotechnol. doi:10.1002/jctb.2480
15. Babu PS, Vaidya AN, Bal AS, Kapur R, Juwarkar A, Khanna P (1996) Kinetics of biosurfactant production by *Pseudomonas aeruginosa* strain BS2 from industrial wastes. Biotechnol Lett 18:263–268
16. Chayabutra C, Wu J, Ju LK (2001) Rhamnolipid production by *Pseudomonas aeruginosa* under denitrification: effects of limiting nutrients and carbon substrates. Biotechnol Bioeng 72:25–33
17. Chandrasekaran EV, Bemiller JN (1980) Constituent analyses of glycosamino-glycans. In: Whistler RL (ed) Methods in carbohydrate chemistry, vol 8. Academic Press, New York, pp 89–96
18. Firestone D (1994) Official methods and recommended practices of the American Oil Chemists' Society, 4th edn. AOCS Press, Champaign
19. Haba E, Espuny MJ, Busquets M, Manresa A (2000) Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oil. J Appl Microbiol 88:379–387
20. Hu Y, Ju L (2001) Sophorolipids production from different lipid precursors observed with LC–MS. Enzyme Microb Technol 29:593–601
21. Wadekar SD, Patil SV, Kale SB, Lali AM, Bhowmick DN, Pratap AP (2011) Study of glycerol residue as a carbon source for production of rhamnolipids by *Pseudomonas aeruginosa* (ATCC 10145). Tenside Surfactants Deterg 48:16–22
22. Costa SGVAO, Nitschke M, Haddad R, Eberlin MN, Contiero J (2006) Production of *Pseudomonas aeruginosa* LBI rhamnolipids following growth on Brazilian native oils. Process Biochem 41:483–488
23. Wadekar SD, Patil SV, Kale SB, Lali AM, Bhowmick DN, Pratap AP (2011) Structural elucidation and surfactant properties of rhamnolipids synthesized by *Pseudomonas aeruginosa* (ATCC 10145) on sweet water as carbon source and stabilization effect on foam produced by sodium lauryl sulfate. Tenside Surfactants Deterg 48 (in press)
24. Guerra-Santos L, Kappeli O, Fiechter A (1984) *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl Environ Microbiol 48:301–305
25. Wadekar SD, Patil SV, Kale SB, Lali AM, Bhowmick DN, Pratap AP (2010) Study of glycerol and sweet water as a carbon source for production of rhamnolipids by naturally occurring strains of *Pseudomonas aeruginosa* (ATCC 10145 and ATCC 9027). Tenside Surfactants Deterg 47:238–242
26. Abalos A, Pinazo A, Infante MR, Casals M, Garcia F, Manresa A (2001) Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. Langmuir 17:1367–1371
27. Déziel É, Paquette G, Villemur R, Lépine F, Bisaillon J-G (1996) Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl Environ Microbiol 62:1908–1912
28. Robert M, Mercade ME, Bosch MP, Parra JL, Espuny MJ, Manresa MA, Guinea J (1989) Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. Biotechnol Lett 11:871–874

29. Özdemir G, Peker S, Helvaci SS (2004) Effect of pH on the surface and interfacial behavior of rhamnolipids R1 and R2. Colloids Surf A 234:135–143
30. Lang S, Wagner F (1993) Biological activities of biosurfactants. In: Kosaric N (ed) Biosurfactants: production, properties, applications. Marcel Dekker, New York, pp 251–268
31. Stanghellini ME, Miller RM (1997) Biosurfactants—their identity and potential efficacy in the biological control of zoosporic plant pathogens. Plant Dis 81:4–12
32. Zhu K, Rock CO (2008) RhlA converts β-hydroxyacyl-acyl carrier protein intermediates in fatty acid synthesis to the β-hydroxydecanoyl-β-hydroxydecanoate component of rhamnolipids in *Pseudomonas aeruginosa*. J Bacteriol 190:3147–3154
33. Bringi NV, Thakur MS (1987) Neem (*Azadirachta indica* Juss) seed oil. In: Bringi NV (ed) Non-traditional oilseeds and oils in India. Oxford & IBH Publishing, New Delhi, pp 118–142
34. Venkata Ramana K, Karanth NG (1989) Factors affecting biosurfactant production using *Pseudomonas aeruginosa* CFTR-6 under submerged conditions. J Chem Tech Biotechnol 45:249–257