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Analysis of Rhamnolipid Biosurfactants Produced Through Submerged Fermentation Using Orange Fruit Peelings as Sole Carbon Source

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Abstract The fermentative production of rhamnolipid biosurfactant from *Pseudomonas aeruginosa* MTCC 2297 was carried out by submerged fermentation using various cost-effective waste materials such as orange peelings, carrot peel waste, lime peelings, coconut oil cake, and banana waste. The orange peel was found to be the best substrate generating 9.18 g/l of rhamnolipid biosurfactant with a surface tension reduction up to 31.3 mN/m. The production was growth independent, and optimum conditions were standardized. The emulsifying activity was highest against kerosene (73.3%). Rhamnolipid components were purified and separated by ethyl acetate extraction, preparative silica gel column chromatography, high-performance liquid chromatography and thin-layer chromatography. The major rhamnolipid components were characterized, by fast atom bombardment mass spectrometry, as a mixture of dirhamnolipids and monorhamnolipids.

Keywords Biosurfactant · Fermentation · Orange peel · *Pseudomonas aeruginosa* · Rhamnolipid

Introduction

Surfactants are surface-active compounds capable of reducing surface and interfacial tension between liquids, solids, and gases. They have established their position as some of the most versatile process chemicals used in industry [1]. But many synthetic surfactants cause environmental problems due to their resistance to biodegradation and toxicity to ecosystems. Increasing environmental awareness has led to serious consideration of biological surfactants as possible alternatives to synthetic surfactants [2]. Expensive traditional carbon sources for surfactant production could be replaced with cheaply available natural raw materials. Agro-industrial wastes with high contents of lipids and carbohydrates meet the requirements for carbon substrates for biosurfactant production [3].

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A variety of substrates have been suggested for biosurfactants production, which include molasses, whey milk, distillery waste, olive oil mill effluent, soap stock [4], cassava waste [5], and potato substrates [6]. The objective of the present study was to produce rhamnolipid biosurfactant from *Pseudomonas aeruginosa* using cheap orange fruit peeling as the sole carbon source. Citrus fruits are the most important value added fruit crop in international trade. There are two clearly differentiated markets in the citrus sector: fresh citrus fruit market, with a predominance of oranges, and processed citrus fruit market, mainly orange juice. The projected orange production in 2010 is 66.4 million metric tonnes, approximately 14% greater than that realized over the 1997–1999 period. India is the fifth largest producer of oranges, and more than 80% of its orange processing is for orange juice production. The increasing demand and consumption of oranges generates large quantities of waste [7]. This waste is often an economic liability to the processor, and waste disposal is a growing problem, which explains the increasing interest in the utilization of this waste for microbial transformation [8]. The exploitation of these types of waste materials for biotechnological processes will mitigate the waste management problem and surfactant production costs. To the best of our knowledge, no reports have been published, yet, with orange fruit peelings as a potential alternative substrate for the biosurfactant production.

Materials and Methods

Microorganism, Growth, and Maintenance Conditions

P. aeruginosa MTCC 2297 was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on nutrient agar slants and was stored at 4 °C. A 5% cell suspension of 1 OD concentration in 0.9% saline solution prepared from a 24-hour culture in nutrient broth was used as inoculum.

Screening and Selection of Biowaste for Biosurfactant Production

Aliquots of liquid media were prepared from a basal solution having the following compositions per liter: KH_2PO4 0.68 g, Na_2HPO_4 4.5 g, $MgSO_4.7H_2O$ 0.1 g, glycerol 30 g, $NaNO_3$ 6.5 g, and yeast extract 0.5 g. The pH was maintained at 6.5 [9]. The natural waste materials, such as (1) orange peelings, (2) carrot peel waste, (3) lime peelings, (4) coconut oil cake, and (5) banana waste, were evaluated as carbon sources in the place of glycerol in the above medium at the same concentration of 3% (weight/volume) for the maximum yield of biosurfactant. All these waste materials were dried and powdered before use. Experiments were carried out in 250-ml Erlenmeyer flasks containing 100 ml of the medium inoculated with 5% (*v*/*v*) inoculum and incubated at 37 °C for 96 h. Cells were separated from the culture broth by centrifugation at 10,000 rpm for 20 min. The cell-free supernatant was used for analytical measurements. The most efficient carbon source was selected on the basis of maximum biosurfactant yield and the emulsification efficiency of the biosurfactant on kerosene.

Optimization of the Process Parameters for the Biosurfactant Production Using Orange Peel as Carbon Source

The effects of incubation time, substrate concentration, and pH were optimized. For all these experiments, 100 ml of the medium in 250-ml flask was inoculated with 5% (*v*/*v*) of inoculum at 37 °C.

The effect of incubation time on biosurfactant production was studied by incubating the medium between 1 and 10 days. The pH was kept at 6.5 [9], and the orange peel concentration was 3%. The yield of biosurfactant was calculated at regular intervals of 24 h, and the minimum period for maximum biosurfactant production was selected as the optimum incubation time. The concentration of the processed orange peel was optimized between 1% and 5% at pH 6.5 and 5% inoculum size. The biosurfactant production was also monitored at variations of the pH of the medium. The pH range used was 5.0 to 7.5 at an interval of 0.5.

Effect of Seasonal Variation of Orange Peel in the Production of Biosurfactant

Three batches of orange fruit peelings were collected during three different seasons. The production of biosurfactant and its surface-active properties were evaluated using each sample. The experiments were carried out under the optimized process parameters. For all these experiments, 100 ml of the medium, in a 250-ml Erlenmeyer flask, was inoculated with 5% (*v*/*v*) of inoculum and was incubated at 37 °C for 7 days. The pH was kept at 6.5, and the orange peel concentration was 4%. The cell-free supernatant was used for analytical measurements.

Isolation and Purification of Rhamnolipid

Rhamnolipid Recovery

Cells were removed from the culture by centrifugation at 10,000 rpm for 20 min. Cell-free culture broth was then deproteinized by heating at 110°C for 10 min. After cooling, it was acidified to pH 3.0 by the addition of 2 N HCl. Rhamnolipids were extracted continuously with ethyl acetate at room temperature. The mixture was shaken vigorously and then left static for phase separation. The organic phase was then transferred to a rotary evaporator and recovered a viscous honey-colored rhamnolipid product after solvent evaporation at 40 °C under reduced pressure.

Rhamnolipid Purification

Liquid column chromatography was used for the separation of rhamnolipids [10]. The polar lipids were separated in a 26-×3.3-cm column containing 50 g of activated silica gel 60-chloroform slurry. The column was loaded with a 5-g sample of crude rhamnolipid prepared in 10 ml $CHCl_3$ and washed with chloroform until the neutral lipids were completely eluted. Then, chloroform/methanol mobile phases were applied in sequence; 50:3 *v*/*v* (1000 ml), 50:5 *v*/*v* (200 ml), and 50:50 *v*/*v* (100 ml) at a flow rate of 1 ml min^{-1} and 20 ml fractions were collected. A final wash with 50:50 chloroform/methanol removed any remaining rhamnolipid from the column. Fractions were combined and dried under vaccum with a rotor evaporator at 40 °C under reduced pressure.

These purified rhamnolipids were then subjected to preparative thin-layer chromatography (TLC). The samples were dissolved in 1 ml $CHCl_3$, and 100 μl of each sample was applied to a 20×20 silica gel TLC plate and developed in a chloroform/methanol/acetic acid (65:15:2 *v*/*v*/*v*) solvent system. The silica gel scrapings of the separated spots were collected and the rhamnolipids extracted thrice with 8 ml of $CHCl_3/CH_3OH$ (1:2 *v*/*v*).The solvent scraping mixture was vortexed for 1 min, centrifuged down the silica gel for 10 min, and pipetted off the solvent.

Analytical Methods

Rhamnolipid Quantification

Rhamnolipid concentration in the cell-free culture broth was estimated by the determination of rhamnose concentration [11]. One milliliter of the cell-free culture broth was mixed with 4.5 ml of dilute sulfuric acid and heated at 100 °C for 10 min. It was cooled to room temperature, mixed with 0.1 ml of freshly prepared 3% thioglycolic acid and was incubated in darkness for 3 h. Absorbance was measured at 420 nm and the rhamnolipid concentration was calculated using a standard curve prepared using different concentrations of L-rhamnose [4, 12]. Rhamnolipid values were determined by multiplying rhamnose values by a coefficient of 3.4 obtained from the correlation [$y=(0.0139x-0.0058)\times 0.68$] of pure rhamnolipids/rhamnose [4].All experiments were conducted in three independent replicates, and controls were kept under similar conditions.

Measurement of Emulsification Activity

Emulsification index (EI) was determined by vortexing 1 ml of cell-free culture broth with 4 ml of water and 6 ml of kerosene at a high speed for 2 min. After 48 h, the percentage of emulsification was calculated, and the stability of the emulsions was monitored for 1 month [13].

Surface Tension Measurements

The culture samples were centrifuged at 10,000 rpm for 20 min, and the surface tension of the supernatant was measured with a KSV Sigma 701 tensiometer using the Du Nouy ring method. The density of each sample was calculated using Hares apparatus [14].

Characterization of Orange Peel

Ten grams of dried orange peel powder was suspended in 100 ml distilled water and was filtered after autoclaving. The filtrate was analyzed for carbon, hydrogen, and nitrogen using the CHN analyzer (VarioEL III CHNS). The level of chemical oxygen demand (COD) [15], total protein [16], and total carbohydrate [17] of the orange peel were also analyzed. The orange peels at different seasons were characterized, and the stability in biosurfactant production was checked. The surface-active properties of the produced biosurfactant along with its substrate yield were calculated.

TLC Analysis

The quantitative analysis of glycolipids was done by two-dimensional TLC using silica gel plates [9]. The solvent system used was chloroform/methanol/acetic acid (65:15:2 *v*/*v*/*v*), and the detection was done by three methods: (1) exposure to iodine vapor [9], (2) exposure to anisaldehyde solution [18], and (3) exposure to diphenylamine solution [13]. The reagents were sprayed, and the plates were heated for 30 to 40 min at 110 °C until the appearance of the respective color. The TLC plates were developed in hexane/diethyl ether/acetic acid (80:20:2 *v*/*v*/*v*) for the separation of neutral lipids. After development, the plates were charred with sulfuric acid [19].

HPLC Analysis

The purity of the separated components were tested by gradient elution high-performance liquid chromatography (HPLC; Laboratory for polymer analysis, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram) using a Waters C 18 column (4.6×250 mm) with a Waters 717 plus auto sampler and 2487 refractive index detector. The flow rate was 0.4 ml min^{-1}, and the mobile phase used was acetone/acetonitrile (30:70 *v*/*v*). The injected sample volume was 20.0 µl.

Characterization of Purified Rhamnolipid

Fast atom bombardment mass spectra (FAB-MS) [18, 20] were recorded on a JEOL JMS 600 H mass spectrometer (Mass Spectrum Laboratory, NIST, Thiruvananthapuram). FAB positive ion mode was used and the matrix used was 4-nitro benzyl alcohol.

Statistical Analysis

The data represent the arithmetical averages of at least three replicates, and the error bars indicate the standard deviations. One-way analysis of variance with post-test ($P<0.05$) was performed with all the experimental results to test the significance of the mean values.

Results and Discussion

Biosurfactant production was studied using a mineral salt medium with different low-cost materials as carbon sources (Table 1). Among the five substrates tested, the highest percentage of emulsification was obtained on using orange peelings as carbon source, and the lowest emulsification index was found on banana waste. Qualitative analysis of crude biosurfactant extracts was done by thin-layer chromatography. On spraying with anisaldehyde reagent, blue spots indicative of carbohydrate units were detected in silica plates. When exposing the similar plates with iodine vapor, yellow spots indicative of lipids giving same R_f value as that of glycosyl units were obtained on the same region. The presence of both glycosyl units and lipid moieties on the same spots indicated that the sample was a glycolipid and the R_f value obtained was 0.6, corresponding to that of a rhamnolipid [21]. All these results showed that *P. aeruginosa* MTCC 2297 was able to grow and produce biosurfactant when cultivated in the waste materials tested. Considering

Table 1 Rhamnolipid concentration and emulsification Index of the biosurfactant produced from *Pseudomonas aeruginosa* MTCC 2297 with different natural waste substrates as the sole carbon source.

Substrates	Rhamnolipid concentration[a] (g/l)	Emulsification index[a] (EI) (%)
Orange peelings	9.180±0.004[b]	73.3
Carrot waste	5.712±0.570	30.12
Lime peelings	4.352±0.016	9.64
Coconut oil cake	2.174±0.413	50.60
Banana waste	2.108±0.386	48.19

[a] Estimated after 192 h of incubation

[b] Mean ± SD, n=4

the results shown in Table 1, the orange peel was selected for further studies as a potential alternative carbon source for biosurfactant production.

The optimum incubation time for rhamnolipid production using orange peel was found to be 7 days (Fig. 1).

The diauxic profile of surfactant accumulation observed in this study had been reported elsewhere [22, 23]. The rhamnolipid production was studied with varying concentrations of orange peel as the sole carbon source in the medium. The rhamnolipid production showed significant increase with the increase in the orange peel concentration and attained the maximum yield when 4% orange peel was used as the carbon source. The optimum pH of the medium for maximum biosurfactant yield (9.18 g/l) was found to be 6.5. A similar optimum pH range was observed for *Pseudomonas* species in the fermentative production of biosurfactant [22]. The production decreased with the increase in pH beyond 6.5.

The chemical composition of the orange peel may slightly vary with the seasonal variation. The COD, total carbohydrate, and total protein analysis of orange fruit waste from three different seasons revealed the difference in the core composition of the fruit waste (Table 2). Lack of differences in biosurfactant yield among seasonal orange peels suggested that different batches of orange peels could be combined and used for biosurfactant production without significant reduction in product yield and surface-active properties (Table 3). The CHN analysis of orange fruit peelings showed its carbon concentration as 2.51%, nitrogen as 0.26%, and hydrogen concentration as 12.57%.

The percentage of emulsification was checked with kerosene, sunflower oil, and rubber seed oil. For sunflower oil, the emulsification index was 50.3%, and for rubber seed oil, it was 6.02%. The kerosene-emulsifying ability of rhamnolipid produced from orange peel was higher (73.3%) than that of the other substrates studied (Fig. 1). Above all, the culture broths containing the rhamnolipid biosurfactants were able to form stable emulsions for up to 1 month, suggesting great potential for pharmaceutical and cosmetic industrial applications. The ability to emulsify hydrocarbons may also be useful for the effective biodegradation of hydrocarbons [24]. From Table 1, it was clear that the emulsification index was not proportional to rhamnolipid concentration. Especially for lime peelings, a rhamnolipid concentration of 4.352 g/l was measured but the EI value was only 9.64%, much lower than those of coconut oil and banana waste, which produced only half of the rhamnolipid concentration from lime peelings. The same was found by Deziel et al. [25], Turkovskaya et al. [26], and Nitschke et al. [27]. The rhamnolipids produced by *P. aeruginosa* 57 RP were different both in quantity and in structure depending upon the

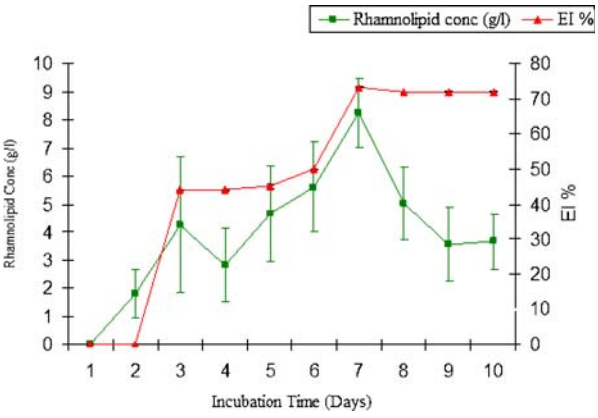

Fig. 1 Effect of incubation time on rhamnolipid production and emulsification index (%) with kerosene by *Pseudomonas aeruginosa* using orange peel (4%) as sole carbon source

Table 2 Chemical oxygen demand, total protein, and total carbohydrate analysis of the orange peel collected during three different seasons.

Parameters	Sample 1[a] ORP	Sample 2[a] ORP	Sample 3[a] ORP
COD (mg/l)	97,333.6	61,583.2	79,456
Total protein (weight in g per 1 g orange peel)	0.169±0.25 g	0.165±0.75 g	0.127±0.94 g
Total carbohydrate (weight in g per 1 g orange peel)	0.189±0.45 g	0.238±1.172 g	0.228±0.75 g

ORP Orange peel

[a] Sample 1, 2, and 3—Samples collected during three different seasons

carbon sources used [25]. The emulsification index varied with the carbon substrate, nitrogen source, and the extracting solvent. The same *P. aeruginosa* strain showed an emulsification index of 40% when glucose was used as the substrate, 0% when sucrose was used, 60% with glycerol, 30% with sorghum syrup, 7% with vegetable oil, and 0% with ethanol. When KNO_3 was used as nitrogen source, this strain showed the EI as 68%, where it was 59% when $NH_4H_2PO_4$ was used as the nitrogen source [26].

Rhamnolipids comprise a mixture of homologous species RL 1, RL 2, RL 3, and RL 4. It is known that the properties of rhamnolipids depend on the distribution of these homologues. The biosurfactant properties of the rhamnolipid depend on their composition and distribution of the homologues that vary according to bacterial strain, culture condition, and medium composition [27]. The variations observed in the surface-active properties such, as emulsifying ability of biosurfactants, were probably due to differences in the individual homologue concentration. Another possibility was the presence of impurities such as extracted non-metabolized fatty acids from the culture broth that could influence the surface-active properties [27]. It was evident that the emulsification index was not at all proportional to rhamnolipid concentration. There could be emulsification index of 10.1% for 10.55 g/l rhamnolipid while 71.4% for 8.62 g/l rhamnolipid [27].

Surface tension measurements were used as an indirect measure of surfactant production and to evaluate the efficiency of the produced biosurfactant. The biosurfactant produced by *P. aeruginosa* MTCC 2297 using orange peel as the carbon source reduced the surface tension of culture broth, and the final surface tension reached from a value of 57 mN/m to a level up to 31.3 mN/m. Reduction of surface tension observed in all these tests indicated the production of surface-active compounds.

Purification of the extracted product was done by different chromatographical methods and characterized by mass spectrometry. The rhamnolipid components were separated by column chromatography. Twenty-milliliter fractions were collected from the column, concentrated and was tested by TLC in order to monitor the separation of the components. The column was first washed out with 100% $CHCl_3$ to remove all impurities. Neutral lipids

Table 3 Biosurfactant production, surface-active parameters, and yield of the orange peel collected during three different seasons.

ORP samples from three different seasons	Rhamnolipid concentration (g/l)	Surface tension (mN/m)	Emulsification index (%)	Yield of biosurfactant per 1 g ORP (g/l)
Sample 1	7.496±0.296	31.496±0.0179	73.3	1.9255
Sample 2	7.178±0.453	31.269±0.0523	73.3	2.9141
Sample 3	6.975±0.104	31.249±0.00785	73.3	2.1946

ORP Orange peel

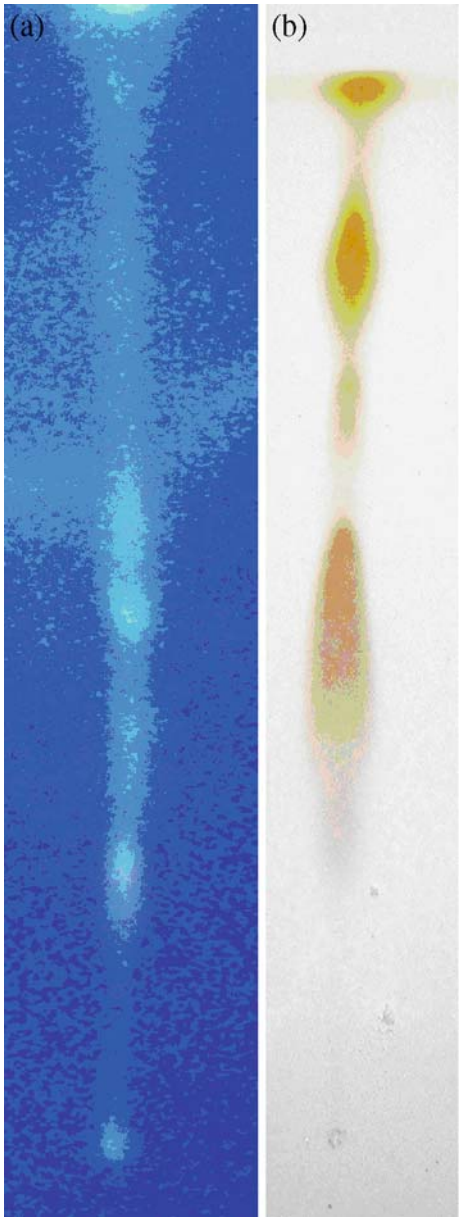

Fig. 2 **a** Thin-layer chromatogram of monorhamnolipid and dirhamnolipid produced by *Pseudomonas aeruginosa* MTCC 2297 using orange peel. **b** TLC plate showing spots having R_f values 0.36, 0.59, 0.71, 0.82, and 0.98 on iodine detection of the rhamnolipid mixture produced by *Pseudomonas aeruginosa* MTCC 2297 using orange peel

were first eluted in fractions 26–56. These fractions showed TLC spots at R_f values 0.82 and 0.98 [10]. When the fractions from 50:3 $CHCl_3/CH_3OH$ elutions were combined and tested by TLC, four different spots were isolated on R_f values 0.19, 0.36, 0.59 and 0.71 (Fig. 2a,b).

The lower spot having 0.19 R_f value consisted of dirhamnolipids, while the higher spot (R_f 0.36) consisted of monorhamnolipid. The spot of R_f 0.59 and 0.71 consisted of various rhamnolipid forms. These results exhibited similarities in the separation profiles with previously reported TLC results of commercially available purified rhamnolipids from

P. aeruginosa [28]. The other fractions of 50:5, 50:25, and 50:50 elutions did not give prominent spots on TLC detection. The column loaded with extracted sample was compared with another column loaded with a blank sample. The fractions with blank sample gave only neutral lipid spots on TLC analysis.

The mixture of two distinct rhamnolipid spots (R_f 0.19 and 0.36) were then checked further for purity by HPLC. Two components were observed in retention times of 3.3 and 4.28 min (Fig. 3a). According to Sim et al. [10], the compound having retention time of 3.60 min was dirhamnosyl component and that which had 4.86 min as retention time was a monorhamnosyl component.

The HPLC chromatogram of the blank sample was shown for the comparison of this result (Fig. 3b).

The mass spectrometric analysis gave confirmation of these observations. FAB-MS of the dirhamnolipid exhibited a peak at *m/z* 674.04 (Fig. 4), and monorhamnolipid exhibited a peak at *m/z* 541.60 (Fig. 5).

The component with R_f value 0.19 corresponding a dirhamnolipid gave a molecular ion at *m/z* 674 ($M+Na^+$) together with a characteristic fragment ion at *m/z* 527 ($M - C_{10}H_{18}O_2 + Na_2^+$, loss of terminal lipid) confirming the presence of the $C_{10}.C_{10}$ dirhamnolipid ($Rha_2C_{10}C_{10}$) [20]. The monorhamnolipid with R_f value 0.36 gave a molecular ion at *m/z* 541 ($M + Na^+$) and a fragment ion at *m/z* 381 confirming the presence of a $C_{10}.C_{10}$

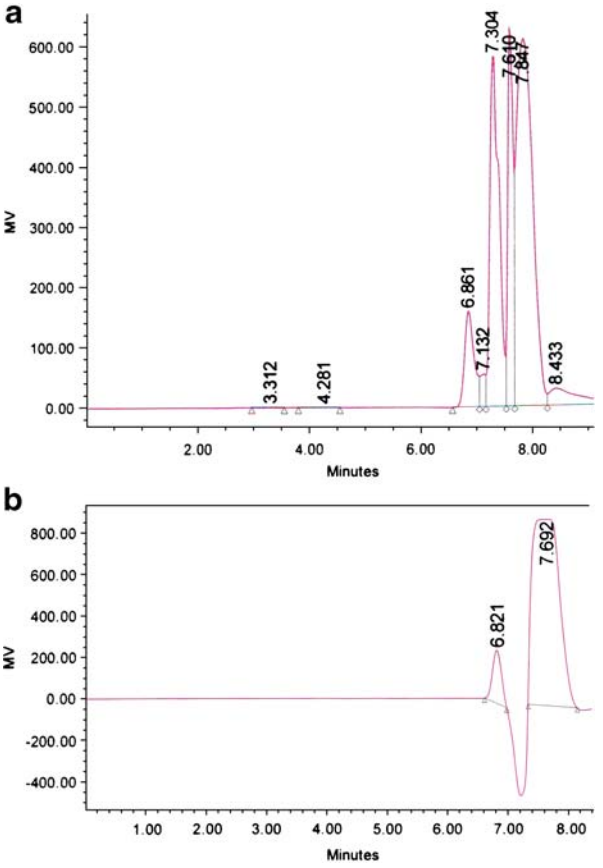

Fig. 3 **a** HPLC chromatogram of the mixture of rhamnolipid spots (R_f 0.19 and 0.36) using a Waters C 18 column (4.6×250 mm) with Waters 717 plus auto sampler and 2487 refractive index detector. **b** HPLC chromatogram of the blank sample using a Waters C 18 column (4.6×250 mm) with Waters 717 plus auto sampler and 2487 refractive index detector

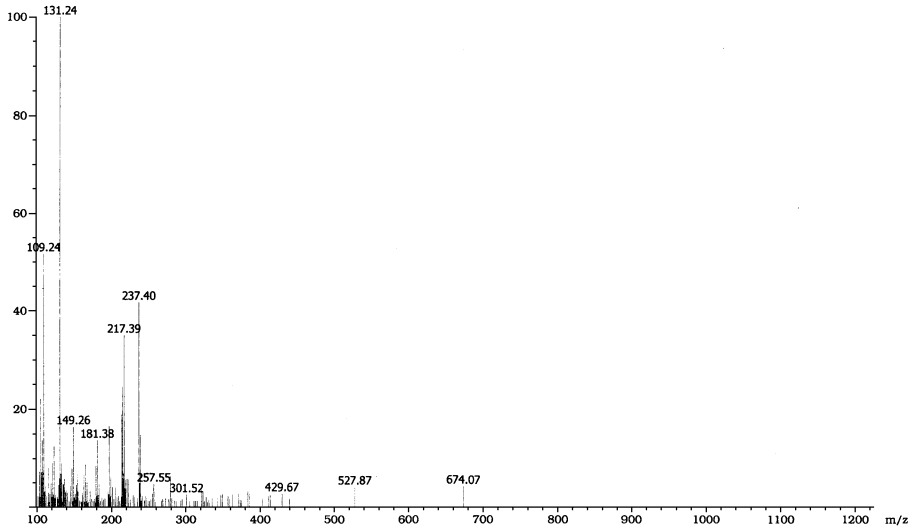

Fig. 4 FAB mass spectrum in the positive ion mode for Rha2-C_{10}-C_{10} (*m*/*z* 674)

monorhamnolipid. These data are consistent with the structure previously reported for the C_{10}.C_{10} monorhamnolipid [20]. Fragmentation of the major C_{10}.C_{10} rhamnolipid under FAB-MS conditions generated dissociated ions at *m*/*z* 381(loss of terminal C_{10} lipid) and *m*/*z* 237 (further loss of rhamnose). The fragmentation patterns obtained in our study were similar with previous reports [20], and both these main molecular ions were consistent with the structures expected for rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid (Rha-C_{10}-C_{10}) and β-α-rhamnosyl (1→2) rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid (Rha_2-C_{10}-C_{10}). The other component with R_f 0.59 gave molecular ion *m*/*z* 479 (spectrum not shown)

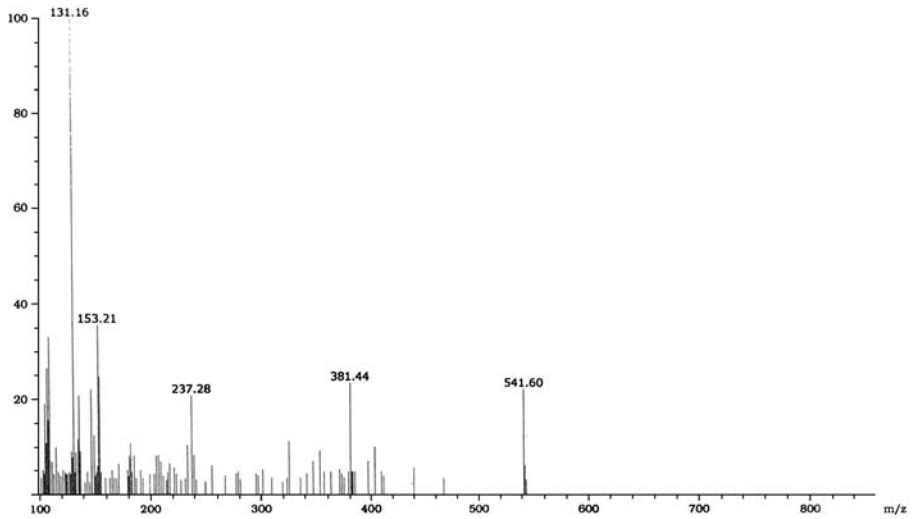

Fig. 5 FAB mass spectrum in the positive ion mode for Rha-C_{10}-C_{10} (*m*/*z* 541)

consistent with Rha_2-C_{10}, which indicated the rupture of the ester bond between the two alkylic chains of dirhamnolipid [4, 25].

The structural characterization of biosurfactants produced by *P. aeruginosa* MTCC 2297 was revealed by FAB-MS. It shows the presence of dirhamnolipid and monorhamnolipid as major components. Rha_2C_{10} was also present as another component. Although the properties of rhamnolipids depended on the distribution of their homologues, little was known about the contribution of each individual homologue in the surface properties of rhamnolipid mixtures.

The orange peel, the natural waste material used for the production of the biosurfactant in this study, was supplied at 3% concentration (weight/volume). However, only 40% (weight/volume) of the supplied orange peel was used by the organism for the production of biosurfactant. After 7 days of optimum incubation, the residual processed orange peel turned into a single, slimy, and brownish mass retaining 60% of the initial weight supplied. The waste materials after the extraction of the biosurfactant were sterilized and were discarded. However, this processed waste material is nutritively rich and may be used as a great biofertilizer, once large-scale operation is put into practice.

Conclusions

To the best of our knowledge, this report is the first one that describes the production of biosurfactant using orange peel as carbon substrate. The orange peel was utilized by *P. aeruginosa* MTCC 2297 as a very effective carbon source and produced rhamnolipid biosurfactant, which was similar to those characterized from other *Pseudomonas* strains. Any slight variation in the compositions of orange peel from different seasons could not produce significant effects on the production and surface-active properties of the biosurfactant.

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