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Production and characterization of rhamnolipids from *Pseudomonas aeruginosa* san-ai

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Abstract: The production and characteristics of rhamnolipid biosurfactant obtained by the strain *Pseudomonas aeruginosa* san-ai were investigated. With regard to the carbon and nitrogen sources, several media were tested to enhance the production of rhamnolipids. Phosphate-limited proteose peptone–ammonium salt (PPAS) medium supplemented with sunflower oil as a source of carbon and mineral ammonium chloride and peptone as nitrogen sources greatly improved the production of rhamnolipid, from 0.15 on basic PPAS (C/N ratio 4.0) to 3 g L^{-1} on optimized PPAS medium (C/N ratio 7.7). Response surface methodology analysis was used for testing the effect of three factors, *i.e.*, temperature, concentration of carbon and nitrogen source (mass %), in the optimized PPAS medium on the production of rhamnolipid. The isolated rhamnolipids were characterized by infrared (IR) spectroscopy and electrospray ionization mass spectrometry (ESI–MS). The IR spectra confirmed that the isolated compound corresponded to the rhamnolipid structure, whereas MS indicated that the isolated preparation was a mixture of mono-rhamno-mono-lipidic, mono-rhamno-di-lipidic and di-rhamno-di-lipidic congeners.

Keywords: rhamnolipids; *Pseudomonas aeruginosa*; renewable sources.

INTRODUCTION

Biosurfactants are microbial secondary metabolites that appear to play a role whenever a microbe encounters an interface.[1] Biosurfactants are important for motility, cell–cell interactions (biofilm formation, maintenance and maturation, quorum sensing, amensalism and pathogenicity) and cellular differentiation, substrate accession (*via* direct interfacial contact and pseudosolubilization of substrates), as well as avoidance of toxic elements and compounds. They may also

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be used as carbon and energy storage molecules, as a protective mechanism against high ionic strength, and may simply be byproducts released in response to environmental changes (*e.g.*, extracellular coverings).[2]

Almost all surfactants currently in use are chemically derived from petroleum. However, biosurfactants have several advantages over the chemical surfactants, such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH and salinity, and the ability to be synthesized from renewable feedstock.[1] Due to these properties, biosurfactants are becoming important biotechnology products for industrial and medical applications.[3] They can be used as emulsifiers, de-emulsifiers, wetting and foaming agents, functional food ingredients and as detergents in petroleum, petrochemicals, environmental management, agrochemicals, foods and beverages, cosmetics and pharmaceuticals, and in the mining and metallurgical industries. Surfactants also play an important role in enhanced oil recovery by increasing the apparent solubility of petroleum components and effectively reducing the interfacial tensions of oil and water *in situ*.[4]

The main factor limiting commercialization of biosurfactants is associated with their non-economic large-scale production. To overcome this obstacle and to compete with synthetic surfactants, an inexpensive substrate and effective microorganism have to be intensively developed for biosurfactant production. Agro-industrial wastes are considered as promising substrates for biosurfactant production, which could alleviate many processing industrial waste management problems.[5] The fact should be noted that although the literature mentions a number of microbe producers with potential to be advantageous for increasing production and efficiency, in practice, this has only been confirmed for a few genera such as *Bacillus*, *Candida* and *Pseudomonas*.[1] Regardless of these problems, the production of microbial surfactants follows the trend of green chemistry and forms the basis of modern industrial processes. The creation of an ecological society, which is in harmony with its surroundings, is now, with green chemistry, the greatest challenge for science and mankind.

Biosurfactants can be divided into two classes: low-molecular-mass molecules, which efficiently lower surface and interfacial tension, and high-molecular-mass polymers, which are more effective as emulsion stabilizing agents. The classes of low-mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high-mass ones include polymeric and particulate surfactants. Most biosurfactants are either anionic or nonionic and the hydrophobic moiety is based on long-chain fatty acids or their derivatives whereas the hydrophilic portion can be a carbohydrate, amino acid, phosphate or cyclic peptide. Bacteria are the predominant group of surfactant-producing organisms.[3] *Pseudomonas* species synthesize both classes of surfactants, low- and high-molecular-mass molecules, but are commonly mentioned as rhamnolipid (RL) producers.[2,6]

Rhamnolipids (RLs) belong to class of low-molecular-mass molecules. The principal rhamnolipids: mono-rhamno-di-lipidic congener and di-rhamno-di-lipidic congener, consist of one or two L-rhamnose units and two units of β-hydroxydecanoic acid (RL1 and RL2 in Fig. 1), while mono-rhamno-mono-lipidic congener and di-rhamno-mono-lipidic congener, consisting of one or two L-rhamnose and one unit of β-hydroxydecanoic acid, are biosynthesized only under certain cultivation conditions (RL3 and RL 4 in Fig. 1).[7] Rhamnolipids are secondary metabolites, and as such, their production coincides with the onset of the stationary phase of microbial growth.[8] Rhamnolipid production seems possible from most carbon sources supporting bacterial growth. Nevertheless, oil of vegetable origin, such as soybean, corn, canola, and olive, provides the highest productivity. Elevated C/N and C/P ratios promote the production of rhamnolipids, while high concentrations of divalent cations, especially iron, are inhibitory. Production of rhamnolipids is inhibited by the presence of NH_4^+, glutamine, asparagine, and arginine as nitrogen source and promoted by NO_3^-, glutamate and aspartate.[9]

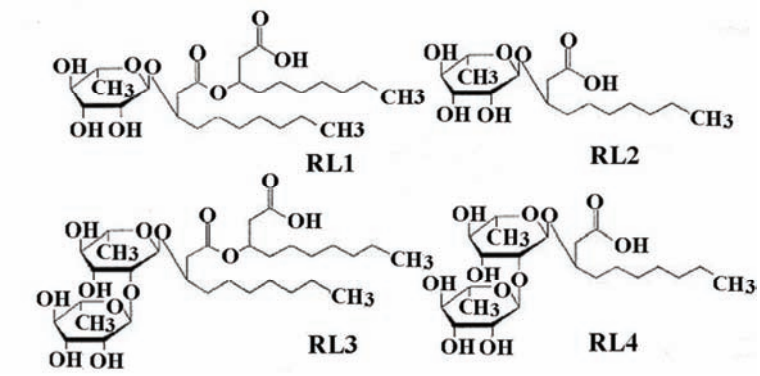

Fig. 1. Structure of rhamnolipid: RL1 (mono-rhamno-di-lipidic congener), RL2 (mono-rhamno-mono-lipidic congener), RL3 (di-rhamno-di-lipidic congener) and RL4 (di-rhamno-mono-lipidic congener).

Pseudomonas sp. are well known for their ability to produce rhamnolipid biosurfactants with potential surface active properties when grown on different carbon substrates and, therefore, are promising candidates for large scale production of biosurfactants.[6,7] In addition to tensioactive properties, rhamnolipids are compounds which play a vital role in regulating the cell population density-dependent control of genes expression, termed quorum sensing (QS) or cell-to-cell communication.[10] Except these, biosurfactants in the mentioned physiological process are involved as transcription factors, signal molecules and as a range of other secondary metabolites, among others extracellular lipase, the expression of which on a genetic level is regulated together with the rhamnolipids themselves.[11]

The aim of this research was to optimize the medium with regard to sources of carbon and nitrogen for improved production of rhamnolipids by the strain *P. aeruginosa* san-ai and to characterize the obtained rhamnolipids by FTIR and MS analysis. This is the first investigation of the production of rhamnolipids by a strain isolated from an unusual environment, *i.e.*, an extremely alkaline environment with a high amount of hydrocarbons. The dynamics of the production of RL by *P. aeruginosa* san-ai during submerged growth, as well comparison of the productivity of a referent strain and strains isolated from similar environments, was also investigated.

EXPERIMENTAL

Microorganisms

P. aeruginosa san-ai strain was isolated from industrial mineral metal-cutting oil.[12] *P. aeruginosa* 67 was isolated from a biopile constructed in Oil Refinery Pančevo,[13] Serbia, whereas *P. aeruginosa* ATCC 27853 was used as the referent strain.

Culture conditions

The strains were cultivated on nutrient agar (Torlak, Serbia) at 30 °C for 24 h and transferred to a 500 mL Erlenmeyer flask, containing 100 mL of Kay's mineral medium (3 g L^{-1} $NH_4H_2PO_4$, 2 g L^{-1} K_2HPO_4, 2 g L^{-1} glucose, 0.5 mg L^{-1} $FeSO_4$ and 1 g L^{-1} $MgSO_4$).[14] The flask was incubated at 30 °C for 20 h and shaken at 250 cycles min^{-1} on a horizontal shaker Kuhner (Switzerland). An actively growing culture was used to inoculate the basic medium.

Selection of the basic medium

Investigation of production media was realized in three steps: *i*) selection of the basic medium, *ii*) selection of the sources of N and C and *iii*) final optimization of the C and N ratio by the response surface methodology (RSM).

To select the basic medium, an actively growing culture from Kay's medium was dispensed (1 %) into 500 mL Erlenmeyer flasks containing 100 mL of one of three media: LB (Lurie–Bertani), MSM (mineral salt medium) and PPAS (phosphate-limited proteose peptone––ammonium salt) as a modification of PPGAS (phosphate-limited peptone–glucose–ammonium salt).[14] The composition of the LB medium was 5 g L^{-1} NaCl, 5 g L^{-1} yeast extract and 10 g L^{-1} peptone I.[12] The MSM medium contained 4 g L^{-1} NH_4NO_3, 4.08 g L^{-1} KH_2PO_4, 5.68 g L^{-1} Na_2HPO_4, 7.77×10^{-4} g L^{-1} $CaCl_2$, 0.2 g L^{-1} $MgSO_4\cdot7H_2O$, 1.49×10^{-3} g L^{-1} ethylenediaminetetraacetate disodium salt (sodium EDTA) and 5.56×10^{-4} g L^{-1} $FeSO_4\cdot7H_2O$.[15] The PPAS medium consisted of 1.07 g L^{-1} NH_4Cl, 1.49 g L^{-1} KCl, 14.54 g L^{-1} Tris HCl, 0.20 g L^{-1} $MgSO_4$ and 10 g L^{-1} peptone.[14] As a source of carbon, 0.7 % of olive oil (Carapelli, Italy) was added to all the listed basic media[7] and fermentation was realized at 30 °C for 96 h.

Selection of optimal type of nitrogen and carbon source

The effect of different sources of carbon and nitrogen was investigated using PPAS, as the basic medium selected in the previous step. To elucidate the effects of C-sources (2 % w/w), glucose, sunflower oil (Vital, Serbia), olive oil, metal cutting oil, kerosene, frying sunflower oil, ethanol, glycerol, or sunflower mill effluent (Plima M, Serbia) were investigated. Two fractions from sunflower mill effluent were tested: the oil emulsion (residue after oil degumming, composed of water, oil and phosphatides), and the fatty acids after neutralization

and saponification (composed of neutral oil, fatty acids and waxes.) For selection of optimal nitrogen source, peptone I (Torlak, Serbia) from the PPAS medium (with 2 % of olive oil) was replaced with (1 % w/w): whey, meat extract, yeast extract, soy flour (Soja Vita, Serbia) and tryptone (Torlak, Serbia).

An actively growing culture from Kay's medium was dispensed (1 %) into 500 mL Erlenmeyer flasks containing 100 mL of medium and the fermentation was performed at 30 °C for 96 h. All experiments were realized in tetraplicate.

Determination of the carbon and nitrogen contents of the media

The total contents of carbon and nitrogen were determined using a Vario EL III CHNS/O elemental analyzer (Germany).

Growth of bacterial strains

Bacterial growth was monitored as the change of optical density (*OD*) at 580 nm, measured on a Gilford 250 spectrophotometer (Oberlin, Ohio, USA), using sterile medium as the reference.[16]

Determination of the RL concentration

After removal of the biomass, 0.25 mL of 500 mM glycine buffer, pH 2, was added to 0.25 mL of supernatant. The mixture was well stirred and centrifuged for 10 min at 10000 rpm (Denver Instrument, USA). The supernatant was discarded and precipitate resuspended in 0.5 mL of a mixture of chloroform/methanol (2:1), with intense agitation for 5 min. The suspension was centrifuged for 5 min at 10000 rpm and 0.25 mL of supernatant was transferred to a new Eppendorf tube. After evaporation of the solvent mixture, the precipitate remaining was dissolved in water. The concentration of RL (c_{rl}) was determined spectrophotometrically by the orcinol reaction using rhamnose as a standard. The orcinol reagent (0.19 % orcinol in 53 % (v/v) sulfuric acid) was prepared immediately before use. The reaction mixture, composed of 150 μL of sample and 1350 μL of reagents, was well stirred, warmed for 30 min at 80 °C, and kept for 15 min at room temperature. The absorbance was measured at 421 nm using a Gilford 250 instrument. c_{rl} was calculated based on the assumption that 1 μg of rhamnose corresponds to 2.5 μg of RL.[17,18]

Isolation of RL

RL was isolated from the fermentation broth after separation of the bacterial cells by centrifugation. The crude preparation of RL was obtained by acidic precipitation using 1 M HCl (final pH: 2). The precipitate was collected by centrifugation at 5000 rpm for 10 min (Sorvall, rotor SS-1, UK) and the RLs were dissolved in a mixture of chloroform and methanol (2:1). Clear supernatant obtained after centrifugation at 5000 rpm for 10 min (Janetzki T32c, Germany)[16] was vacuumed to dryness and used for FTIR and MS analysis.

Response surface methodology (RSM)

Response surface methodology (RSM) was applied for data analysis using Design Expert software (version 8.0.5). The PPAS medium with sunflower oil as the carbon source and peptone I as the nitrogen source was used. The plan applied in this study involved 17 experiments conducted according a Box–Behnken design.[19] The effects of the concentrations of the C and N sources and temperature were tested in following ranges: concentration of sunflower oil, as the carbon source (1–6 %), concentration of peptone I, as the nitrogen source (0.5–4 %) and temperature (20–40 °C). The response value was the concentration of rhamnolipid (c_{rl}) expressed as g L^{-1}. The concentration of rhamnolipid was measured on the fourth day of fermentation.

Lipase enzyme assay

The lipase activity (c_e) was measured spectrophotometrically using an assay based on the hydrolysis of *p*-nitrophenyl palmitate (pNPP, Sigma Aldrich, USA) at a concentration of 0.79 mmol pNPP mL^{-1}. The reaction mixture was composed of 900 μL of pNPP solution and 100 μL of lipase solution. The pNPP solution was prepared as follows: 30 mg of pNPP in 10 mL of 2-propanol was added to 90 mL of 0.05 M phosphate buffer pH 8.0 supplemented with 207 mg of Na-deoxycholate and 100 mg of gumarabic. The enzyme reaction mixture was incubated at 30 °C and the absorbance measured at 410 nm during the first 3 min of reaction. One unit (1 U) is defined as that quantity of enzyme that (under the test conditions) liberates 1 μmol pNPP min^{-1}.[12]

FTIR analysis

The IR spectra were recorded on a Perkin-Elmer 31725 X FTIR spectrophotometer using KBr discs.

HPLC–MS-ESI analysis

Mass spectra of RL were recorded on MS system consisting of an HPLC (Agilent 1200 Series, Agilent Technologies) and a 6210 Time-of-Flight LC/MS (Agilent Technologies), using Zorbax Eclipse Plus C18 column and a DAD detector. The mobile phase was a mixture of solvent A (0.2 % formic acid in water) and B (acetonitrile) in a gradient mode: 0–1.5 min 95 % A, 1.5–12 min 95–5 % A, 12–15 min 5 % A, 15–16 min 5–95 % A. The data were processed by means of a Mass Hunter Workstation.

RESULTS AND DISCUSSION

Strain *P. aeruginosa* san-ai, isolated from an unusual extremely alkaline environment with high content of hydrocarbons (mineral cutting oil), was investigated to determine its capability to produce rhamnolipids (RLs) on different sources of carbon and nitrogen.[12] Potential of *P. aeruginosa* san ai to produce RLs was compared to that of a referent strain ATCC 27853 and the strain *P. aeruginosa* 67, isolated from a biopile with a high level of petroleum hydrocarbons.

Selection of the basic medium

Pseudomonas sp. produce rhamnolipids as secondary metabolites and the production, among other things, depends on the general medium composition, particularly on the sources of carbon and nitrogen, as well as the total C/N ratio.[8,20–24] LB, MSM and PPAS medium were investigated to find the optimal base for testing the influence of carbon and nitrogen sources on the production of RLs by *P. aeruginosa* san-ai. All media were supplemented with olive oil as a source of carbon, which according to the literature provides the greatest production of rhamnolipids.[8] The production of rhamnolipids on LB, MSM, and PPAS medium was found to be 15.5, 10.7, and 1010.4 mg L^{-1}, respectively, indicating that PPAS is the optimal base to improve rhamnolipid production, which is in good agreement with previously reported data.[14]

Influence of carbon source on the production of RL

Results obtained by testing sunflower oil, olive oil, metal cutting oil, kerosene, frying sunflower oil, ethanol, glucose, glycerol, a combination of glucose and sunflower, and sunflower mill effluent on the production of RL by *P. aeruginosa* san-ai in PPAS medium are shown in Fig. 2a. The histogram shows that the yield of RL was the highest on sunflower (1.35 g L^{-1}) and olive oil (1.01 g L^{-1}). A high production of RL was achieved on the frying sunflower oil (0.96 g L^{-1}), suggesting the possibility of using this substrate as a renewable source. Low production

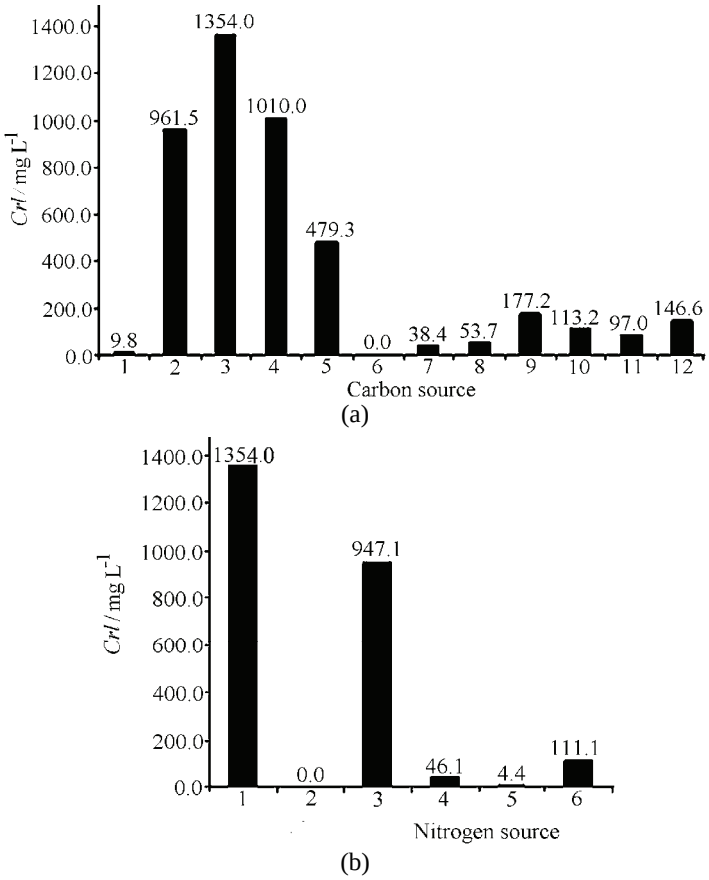

Fig. 2. a) Effect of different sources of carbon on the production of RL by *P. aeruginosa* san-ai. PPAS medium with (2 %): 1 – kerosene, 2 – frying sunflower oil, 3 – sunflower oil, 4 – olive oil, 5 – sunflower oil and glucose, 6 – metal cutting oil and glucose, 7 – ethanol, 8 – glycerol, 9 – glucose, 10 – sunflower mill effluent suspension, 11 – sunflower mill effluent emulsion; 12 – 0.5 % glucose (basic PPAS[14]). b) Effect of different sources of nitrogen on the production of RL by *P. aeruginosa* san-ai. PPAS medium with 2 % of sunflower oil, supplemented with (1 %): 1 – peptone I (control), 2 – whey, 3 – meat extract, 4 – yeast extract, 5 – soy flour, 6 – tryptone.

was detected on sunflower oil mill effluent and the other sources of carbon (kerosene, ethanol, glycerol, metal cutting oil, a combination of sugar and sunflower oil, glucose). Thus, sunflower oil was selected as the optimal carbon source.

Using PPAS medium supplemented with sunflower oil as a source of carbon and six different nitrogen sources: whey, meat extract, yeast extract, soybean flour, tryptone, peptone I, their effect on the production of RL by *P. aeruginosa* san-ai was tested. Preliminary experiments (data not shown) with urea, sodium nitrate and ammonium chloride as unique N-sources, showed that these sources were not suitable for a minimal growth of the culture, giving extremely low productions of RL. Fig. 2b shows that the highest production of rhamnolipid was achieved using peptone I (1.35 g L^{-1}) and meat extract (0.95 g L^{-1}). Production of RL on yeast extract, soy flour, tryptone and the whey was low or undetectable. Therefore, peptone I was selected as the optimal nitrogen source.

After selection of the optimal carbon (sunflower oil) and the optimal nitrogen sources (peptone I), *P. aeruginosa* san-ai on improved PPAS medium gave an RL production of 3.1 g L^{-1}. Under the same conditions, the productions of RL by *P. aeruginosa* ATCC 27853 and *P. aeruginosa* 67 were found to be 1.2 and 0.8 g L^{-1}, respectively. Compared to *P. aeruginosa* san-ai, both strains exhibited lower productions of RL. Interestingly, despite being isolated from an environment with a high content of hydrocarbon derivates (similar to the natural environment of *P. aeruginosa* san-ai), strain 67 produced less RL than strain san-ai.

This study showed that good substrates for the production of RL were vegetable oils, including oil wastes, as a carbon source and peptone I as an organic nitrogen source, which differs significantly from producing strains reported in the literature. Namely, glucose, glycerol and olive oil in combination with inorganic nitrogen were found to be preferential sources for RL production giving a high yield of 1.2–7.65 g L^{-1} of RL, respectively.[22–27] In addition, industrial waste and byproducts, such as whey waste, with yield of 0.92 g L^{-1} of RL, and molasses and corn steep liquor, both with yield of 0.25 g L^{-1} of RL, showed themselves to be relatively good substrates.

Comparison of the production of rhamnolipids by *P. aeruginosa* grown in LB, as a commonly used medium and the optimized PPAS medium, which were found to be 15 and 3000 mg L^{-1}, respectively, clearly shows the potential of *P. aeruginosa* san-ai for enhanced RL production.

Dynamics of fermentation on optimized medium

Production of RL by *P. aeruginosa* san-ai on optimized medium (PPAS supplemented with sunflower oil, as carbon source and ammonium chloride and peptone I as nitrogen sources) for a period of 8 days was monitored and the results are shown in Fig. 3a. Obviously, a significant production of RL begins after the third day of fermentation, when the bacterial population is in a stationary phase of growth, which is consistent with the fact that RL, as a secondary metabolite,

was produced after the phase of intensive growth. c_{rl} varied considerably during the fermentation with a maximum production of RL on the fourth day (3.0 g L^{-1}) with an *OD* of 2.2, while on the seventh day, the yield of 4.6 g L^{-1} RL, with a culture *OD* of 0.25, was the result of cell lysis.

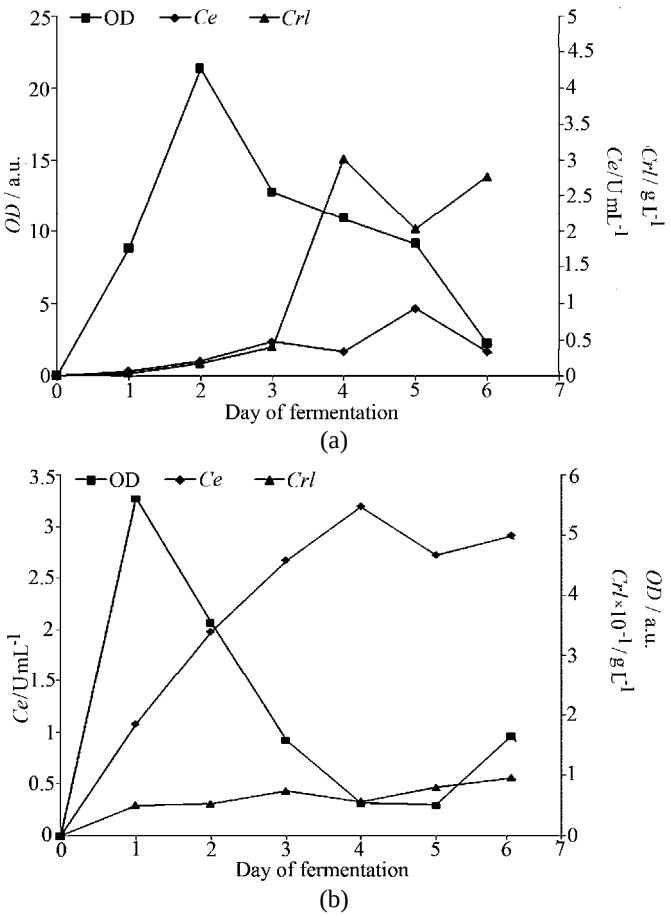

Fig. 3. Dynamics of the growth (*OD*), production of RL (c_{rl}) and lipase activity (c_e) of *P. aeruginosa* san-ai on: a) optimized PPAS medium (sunflower oil and peptone I) and b) LB medium.

Comparison of the dynamics of the fermentation (*OD*, c_{rl} and c_e) on the optimized PPAS medium (Fig. 3a) and the commonly used LB medium (Fig. 3b) showed differences. The bacterial culture was more active on the optimized medium, giving five times higher growth and favoring rhamnolipid production (30 times higher) than the LB medium. Interesting, on the other hand, the composition of the LB medium was more suitable for enzyme synthesis, giving a higher lipase production. The observed differences between the growth of the culture

and the production of rhamnolipids and lipase are related to cell-to-cell communication. Namely, rhamnolipids play a vital role in regulating the cell population density-dependent control of genes expression, termed quorum sensing (QS).[10] Observed cell density changes and exchanged activity of extracellular lipase, as indicators of QS response on environmental change clearly suggest that the effect of nutritional compounds should be considered in the context of environmental regulation of the *P. aeruginosa* QS system.[28]

RSM Analysis

The highest production of RL achieved by the one-factor-at-a-time approach was found to be 3 g L^{-1}. Analysis of the effect of temperature and the concentrations of the carbon and nitrogen sources on RL production on the optimized PPAS medium was made by the RSM experimental design methodology. A similar approach based on the combination of the one-factor-at-a-time and the statistical experimental design methodology for enhanced RL production was recently reported.[19] For the RSM analysis, 17 experiments were conducted under a three-factorial Box–Behnken (BC) design. The value of the *F*-test of 3.51 and *P* value of 0.0389 indicate that the model was adequate for describing the obtained experimental results, the *P* value BC model term (combination of temperature and concentration of nitrogen source) of 0.0167 was significant. The equation of the statistical model is:

$$Y = 1.42 - 0.50A - 0.78B - 0.36C + 1.06\,AB + 0.37AC + 1.44BC$$

where *A*, *B*, *C* and *Y* correspond to the sunflower oil and peptone I content, %, temperature, °C, and c_{rl}, g L^{-1}, respectively. The results of the experimental runs and points are given in Table I. The 3D response surface shows the effect of pep-

TABLE I. Experimental RSM design according to the Box–Behnken method, for rhamnolipid production on optimized medium with a total C/N ratio of medium per run

Run	Carbon source %	Nitrogen source %	Temperature °C	Total C/N ratio	c_{rl} / g L^{-1}
1	6.00	4.00	30	9.78	0.36
2, 4, 9,12, 14	3.50	2.25	30	8.73	1.59
3	3.50	0.50	20	12.83	4.07
5	6.00	0.50	30	19.51	0
6	3.50	4.00	20	7.10	0.83
7	6.00	2.25	20	12.55	0.26
8	1.00	2.25	20	4.83	0.68
10	3.50	4.00	40	7.10	1.56
11	6.00	2.25	40	12.55	1.72
13	1.00	0.50	30	5.68	3.00
15	3.50	0.50	40	12.83	0.05
16	1.00	2.25	40	4.83	0.67
17	1.00	4.00	30	4.39	0.56

tone I and temperature (sunflower oil, 3.5 %) (Fig. 4). The optimal response predicted by the RSM was found to be 4.07 g L^{-1} of RL, obtained with 3.5 % sunflower oil and 0.5 % peptone I (C/N ratio 12.83), at 20 °C. With 1 % sunflower oil and 0.5 % peptone I (C/N ratio 5.68) at 30 °C, a rhamnolipid concentration of 3.00 g L^{-1} was achieved.

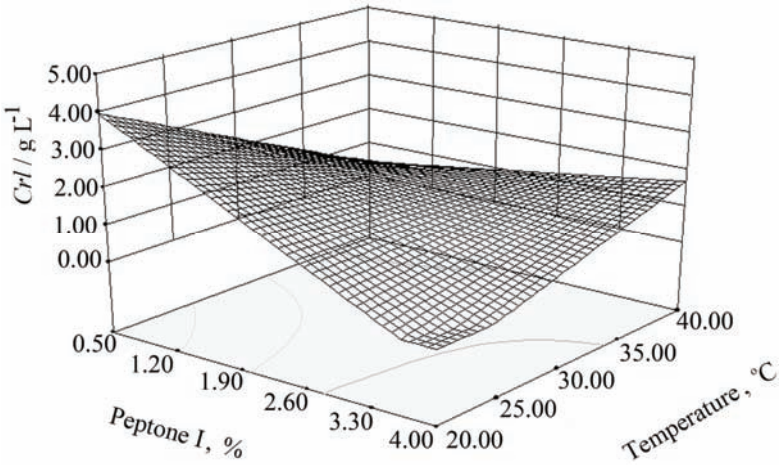

Fig. 4. 3D surface graph: effect of temperature and concentration of nitrogen source, with a 3.5 % concentration of the carbon source, on rhamnolipid production by *P. aeruginosa* san-ai.

Comparison of C/N ratio in the basic, one-factor-at-a-time optimized and RSM optimized PPAS medium for the highest yield showed a C/N ratio of 4.0, 7.7 to 12.83, respectively. This correlates with the fact that the evaluated C/N conditions gave higher yield of rhamnolipids.[7–9] However, this C/N ratio is strongly affected by temperature, as Table I indicates.

A previously reported optimization of rhamnolipid production by *P. aeruginosa* san-ai on LB medium using RSM analysis gave a yield of only 138 mg L^{-1}.[29] Thus, the present combined one-factor-at-a-time and statistical approach to enhance the production of RL gave an over 30 times better yield than using only RSM on a single basic medium.

Characterization of the RL

IR analysis. The IR spectrum of rhamnolipid from *P. aeruginosa* san-ai is shown in Fig. 5. The fingerprint areas between 400–1500 cm^{-1} showed the deformation C–OH band at 1384 cm^{-1}, the O–H in plane deformation at 1315 cm^{-1}, the O–C–O symmetric band at 1047 cm^{-1}, the C–O stretching at 1168, 1127 and 1047 cm^{-1}, C–H deformations at 1451, 1238 and 808 cm^{-1} and CH_3 rocking at 983 cm^{-1} for RL. There are also the typical stretching vibrations of the COO^- group. The strong symmetric stretching C=O band of the carboxylate group of

RL was at 1739 cm^{-1}. The IR spectra of RL gave absorption bands at 3360 cm^{-1} for symmetric O–H stretching. The spectrum also showed vibrations at 2928 cm^{-1} and 2856 cm^{-1} typical for the C–H stretching vibrations of CH_2 and CH_3 groups. The results are in a good agreement with a typical IR spectrum of rhamnolipids.[30]

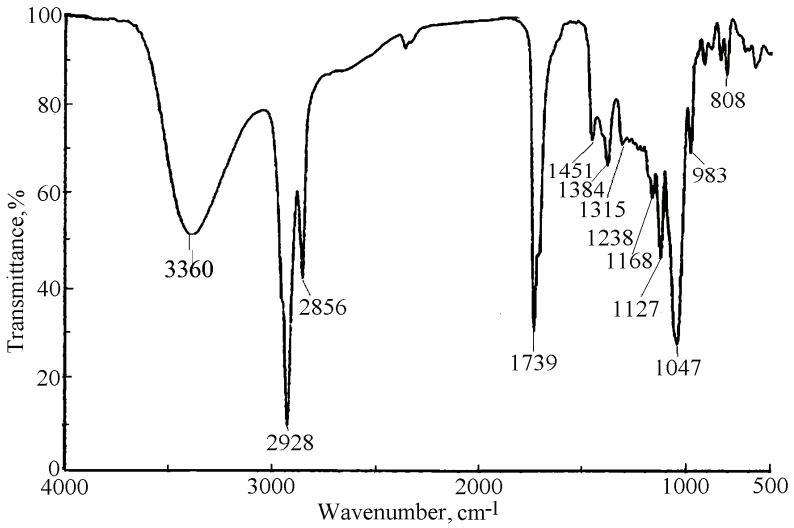

Fig. 5. IR Spectrum of rhamnolipid showing the following vibrations: C–H stretching asym. (2928 and 2856 cm^{-1}), C=O stretching (1739 cm^{-1}), C–H deformations (1451, 1238 and 808 cm^{-1}), C–H/O–H deformation (1384 cm^{-1}), O–H in plane deformation (1315 cm^{-1}), C–O stretching (1168, 1127 and 1047 cm^{-1}), CH_3 rocking (983 cm^{-1}).

HPLC–ESI-MS analysis. A list of the rhamnolipid congeners detected by HPLC–MS-ESI analysis with their molecular formulas, molecular weights, retention time and abundance of M^- and $[M–H]^-$ is given in Table II. The mass spectra of Rha-C10, Rha-C10-C10:1/Rha-C10:1-C10 and Rha-Rha-C10-C12:1/Rha--Rha-C12:1-C10 observed by ESI–MS in the negative mode are given in Figs. 6–8. Table II shows a list of the detected rhamnolipid congeners with their molecular formulas, weights and relative abundance of M^- and $[M–H]^-$. The strain *P. aeruginosa* san ai produces a unique mixture of rhamnolipids composed of: Rha-C8, Rha-C10, Rha-C12, Rha-C8-C10/Rha-C8-C10, Rha-C10-C10:1/Rha-C10:1-C10, Rha-C8-C12/Rha-C12-C8//Rha-C10-C10, Rha-C10-C12:1/Rha-C12:1-C10, Rha-C10-C12/Rha-C12-C10, Rha-C10-C14/Rha-C14-C10//Rha-C12-C12, Rha-C10-C10-CH3, Rha-Rha-C10-C10, Rha-Rha-C10-C12/Rha-Rha-C12-C10, Rha-Rha-C10-C12:1/Rha-Rha-C12:1-C10, Rha-Rha-C10-C14:1/Rha-Rha-C14:1-C10//Rha-Rha-C12-C12:1 and Rha-Rha-C10-C10-CH3. Thus, the most frequent were mono-rhamno-di-lipidic (7 detected), followed by di-rhamno-di-lipidic (5

detected) and mono-rhamno-mono-lipidic (3 detected) congeners. The relative abundance of M^- and $[M–H]^-$ of mono-rhamno-mono-lipidic, mono-rhamno-di-lipidic and di-rhamno-di-lipidic structures were 1.83, 53.15 and 44.77 %, respectively.[31] The most abundant (12 %) individual congeners were ions of Rha-C8-C10/Rha-C8-C10, Rha-C8-C12/Rha-C12-C8//Rha-C10-C10, Rha-C10-C12/ /Rha-C12-C10, Rha-Rha-C10-C10, Rha-Rha-C10-C12:1/Rha-Rha-C12:1-C10.

TABLE II. List of rhamnolipid congeners detected by HPLC–MS-ESI analysis with their molecular formulas, molecular weights, retention time and abundance of M^- and $[M–H]^-$

Rhamnolipid congener	Molecular formula	Molecular weight g mol^{-1}	Retention time, min	Relative abundance of M^- + $[M–H]^-$, %
Rha-C8	$C_{14}H_{26}O_7$	306.17	10.29	0.06
Rha-C10	$C_{16}H_{30}O_7$	334.20	8.05	1.61
Rha-C12	$C_{18}H_{34}O_7$	362.23	9.22	0.16
Rha-C8-C10/Rha-C10-C8	$C_{24}H_{44}O_9$	476.30	11.16	12.64
Rha-C10-C10:1/Rha-C10:1-C10	$C_{26}H_{46}O_9$	502.31	11.88	6.95
Rha-C8-C12/Rha-C12-C8//Rha-C10-C10	$C_{26}H_{48}O_9$	504.33	12.34	12.71
Rha-C10-C12:1/Rha-C12:1-C10	$C_{28}H_{50}O_9$	530.34	12.98	4.40
Rha-C10-C12/Rha-C12-C10	$C_{28}H_{52}O_9$	532.36	13.43	12.48
Rha-C10-C14/Rha-C14-C10//Rha-C12-C12	$C_{30}H_{56}O_9$	560.39	14.40	1.50
Rha-C10-C10-CH3	$C_{27}H_{50}O_9$	518.34	12.89	2.47
Rha-Rha-C10-C10	$C_{32}H_{58}O_{13}$	650.39	11.55	12.47
Rha-Rha-C10-C12:1/Rha-Rha-C12:1-C10	$C_{34}H_{60}O_{13}$	676.40	11.98	12.32
Rha-Rha-C10-C12/Rha-Rha-C12-C10	$C_{34}H_{62}O_{13}$	678.42	13.30	0.13
Rha-Rha-C10-C10-CH3	$C_{33}H_{60}O_{13}$	664.40	12.11	8.64
Rha-Rha-C10-C14:1/Rha-Rha-C14:1-C10//Rha-Rha-C12-C12:1	$C_{36}H_{64}O_{13}$	704.43	13.76	11.21

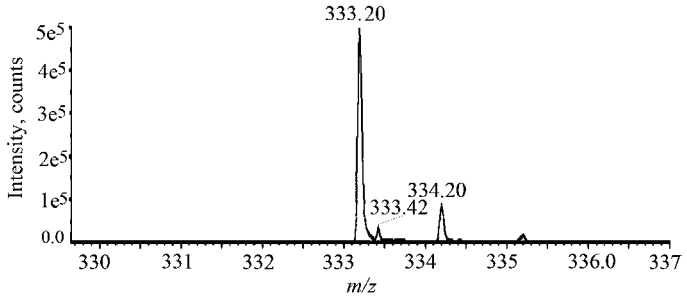

Fig. 6. MS spectrum of mono-rhamno-mono-lipidic Rha-C10 rhamnolipid congener detected in the rhamnolipid mixture produced by *P. aeruginosa* san-ai.

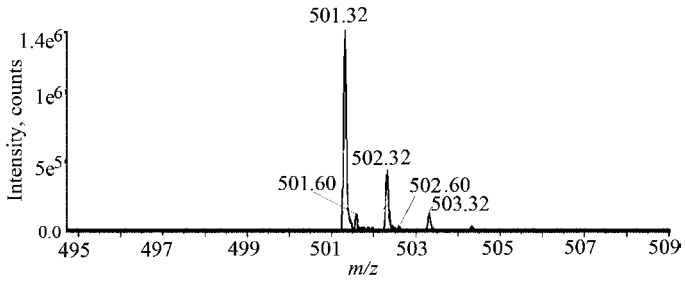

Fig. 7. MS spectrum of mono-rhamno-di-lipidic Rha-C10-C10:1/Rha-C10:1-C10 rhamnolipid congener detected in the rhamnolipid mixture produced by *P. aeruginosa* san-ai.

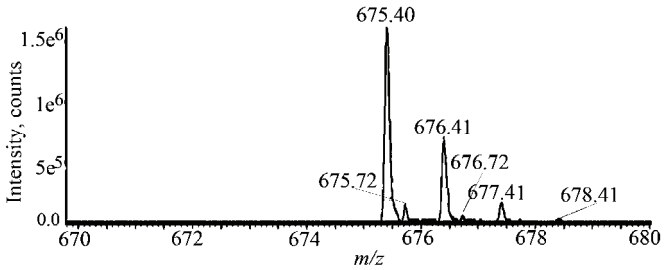

Fig. 8. MS spectrum of di-rhamno-di-lipidic Rha-Rha-C10-C12:1/Rha-Rha-C12:1-C10 rhamnolipid congener detected in the rhamnolipid mixture produced by *P. aeruginosa* san-ai.

CONCLUSIONS

Rhamnolipid production by the *P. aeruginosa* san-ai strain was significantly enhanced on optimized PPAS medium. The tested carbon and nitrogen sources indicated that the strain *P. aeruginosa* san-ai also grew and produced RL on waste raw materials (oil from the fryers and sunflower mill effluent). PPAS medium supplemented with sunflower oil as a source of carbon and ammonium chloride and peptone as nitrogen sources greatly improved rhamnolipid production, from 0.15 g L^{-1} on basic PPAS (C/N ratio 4.0) to 3 g L^{-1} on the optimized PPAS medium (C/N ratio 7.7). A comparison of the production of rhamnolipids by *P. aeruginosa* grown in LB, a commonly used medium, and optimized PPAS showed the production to 15 and 3000 mg L^{-1}, respectively, clearly showing the potential of *P. aeruginosa* san-ai for enhanced RL production. Further elevation of RL production on optimized PPAS was achieved by RSM analysis of the concentrations of the carbon and nitrogen sources, and temperature. The best yield of 4.07 g L^{-1} was achieved at 20 °C with a carbon concentration of 3.5 % and a 0.5 % concentration of nitrogen sources with a C/N ratio of 12.83. Compared to the referent *P. aeruginosa* ATCC 27853 strain and strain 67 isolated from biopile, the strain *P. aeruginosa* san-ai has a much better potential for RL production.

As confirmed by MS analysis, the rhamnolipid produced by *P. aeruginosa* san-ai is a mixture of different rhamnolipidic congeners (mono-rhamno-mono-

-lipidic, mono-rhamno-di-lipidic and di-rhamno-di-lipidic). The mono-rhamno--di-lipidic congener had the highest relative abundance of M^- and $[M–H]^-$.

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И З В О Д

ПРОДУКЦИЈА И КАРАКТЕРИЗАЦИЈА РАМНОЛИПИДА СОЈА *Pseudomonas aeruginosa* SAN-AI

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У овом раду је описана продукција и карактеризација рамнолипидног биосурфактанта соја *Pseudomonas aeruginosa* san-ai. Испитан је утицај различитих извора угљеника и азота на продукцију рамнолипида. Подлога протеозе пептон-амонијум соли са ограниченом количином фосфата (PPAS) са сунцокретовим уљем као извором угљеника и амонијум-хлоридом и пептоном као извором азота, знатно повећава продукцију рамнолипида, од 0,15, на PPAS са стандардним саставом (однос C/N 4,0), до 3 g L^{-1} на оптимизованој PPAS подлози (однос C/N 7,7). Методом планираног експеримента (*response surface methodology*) испитан је ефекат три параметра: температуре, концентрације извора угљеника и азота на оптимизованој подлози. Карактеризација изолованог препарата рамнолипида урађена је помоћу IR и ESI-MS анализа. IR анализа је потврдила присуство структурних елемената карактеристичних за рамнолипиде. MS анализа показала је да реч о смеши у којој су присутне моно-рамно-моно-липидне, ди-рамно-моно-липидне и ди-рамно-ди-липидне компоненте.

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