Comparative analysis of rhamnolipids produced by *Pseudomonas aeruginosa* NCAIM (P) B 001380 on different carbon sources by HPLC-ESI-MS

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This study represents analysis of carbon sources effect on composition of rhamnolipid mixture produces by strain *P. aeruginosa* NCAIM (P) B 001380 isolated from high alkaline mineral cutting oil. Strain was grown on PPAS medium supplemented with different carbon sources (2%), two simple (glucose and glucose with addition of kerosene) and two complexs, waste, sources (frying sunflower oil and sunflower oil mill effluent, SME). Isolated rhamnolipid mixtures were analyzed by HPLC ESI-MS. Results showed that retention times depend on lipidic component of rhamnolipid, not only on molecular weight. In all, or almost all, rhamnolipid mixtures were present mono-rhamno-di-lipidic congeners: Rha-C8-C8, Rha-C8-C10/Rha-C10-C8, Rha-C10-C10:1/Rha-C10:1-C10, Rha-C8-C12/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-C14:1/Rha-C14:1-C10/Rha-C12-C12:1/Rha-C12:1-C12 and Rha-C10-C10-CH3 and di-rhamno-di-lipidic congeners: Rha-Rha-C8-C10 (all sources except frying sunflower oil), Rha-Rha-C10-C10, Rha-Rha-C10-C12:1/Rha-Rha-C12:1-C10,Rha-C10-C10:1/Rha-C8-C2:1//Rha-C12:1-C8 (all sources except sunflower frying oil), Rha-Rha-C10-C12/Rha-Rha-C12-C10, Rha-Rha-C10-C14:1/Rha-Rha-C14:1-C10/Rha-Rha-C12-C12:1/Rha-Rha-C12:1-C12, Rha-Rha-C12-C12 and Rha-Rha-C10-C10-CH3. Some rhamnolipidic congeners were detected only sporadically. Mono-rhamno-mono-lipidic Rha-C10 was detected on glucose and kerosene and Rha-C14:2 on SME, whereas rare observed di-rhamno-di-lipidic congeners were: Rha-Rha-C10 (SME and glucose), Rha-Rha-C8-C8 (SME and glucose with addition of kerosene), Rha-Rha-C14-C14 (frying sunflower oil). Rha-Rha-C14-C16/Rha-Rha-C16-C14 (frying sunflower oil and SME). This comparative analysis indicated that cultivation conditions, such as carbon source, had effect on composition of rhamnolipid mixtures and that differences were reflected in of mono- and di-rhamno-mono-lipidic and di-rhamno-di-lipidic congeners.

Introduction

Rhamnolipids (RLs), microbial secondary metabolites, are amphiphatic compounds with tensioactive properties. These microbial products appear to play a role whenever a microbe encounters an interface. Biosurfactants are important for motility, cell–cell interactions and cellular differentiation, substrate accession, as well as avoidance of toxic elements and compounds. They may also 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 (Van Hamme *et al*, 2006).

Rhamnolipid (RL) production is possible from most carbon sources supporting bacterial growth. Nevertheless, oil of vegetable origin, such as soybean, corn, canola, and olive, provides the highest productivity. Among water-soluble substrates, mannitol is especially effective. Elevated C/N and C/P ratios promote rhamnolipids production, while high concentrations of divalent cations, especially iron, are inhibitory. Actually, nitrogen-limiting conditions do not favor rhamnolipids production per se, like phosphate-limited conditions, but production starts with the exhaustion of nitrogen. Production of rhamnolipids is inhibited by the presence of NH4+, glutamine, asparagine, and arginine as nitrogen source and promoted by NO3−, glutamate and aspartate (Tahzibi *et al*, 2004, Soberón-Chávez *et al*, 2005, Chayabutra *et al*, 2001).

Materials and methods

Microorganism

Strain P. aeruginosa NCAIM (P) B 001380, early named as san ai was isolated from industrial mineral metal-cutting oil (Karadzic *et al*, 2004).

Culture conditions

The strains were activated in nutrient agar at 30 ºC for 24 h and transferred to a 500 mL Erlenmeyer flask, containing 100 mL Kay's mineral medium (Gunther *et al*,2005). The flask was incubated at 30°C for 20 hours and shaken at 250 cycles min-1.

Actively grown culture was used to inoculate proteose peptone-ammonium salt (PPAS) medium (Gunter *et al*, 2005) with at 30 °C for 96 h. As a 2% source of carbon were used: sunflower oil, sunflower frying oil, sunflower mil effluent (SME), glucose, glucose + kerosene.

Determination RL concentration

Concentration of RL was determined spectrophotometrically with orcinol reaction using rhamnose as a standard as previously described. (Wang *et al*, 2007,,Wilhelm *et al*, 2007).

Isolation of RL

Mixture of RL was isolated from fermentation broth by acidic precipitation, followed with extraction with mixture of chloroform and methanol (2:1) (Heyd *et al*, 2008) and used for HPLC-MS-ESI analysis.

HPLC-MS-ESI

Mass spectra of RL from chloroform methanol extract of culture filtrate were recorded on MS system consisting of a HPLC (Agilent 1200 Series, Agilent Technologies) and 6210 Time-of-Flight LC/MS (Agilent Technologies), using column Zorbax Eclipse Plus C18 and DAD detector. 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. Data were processed by means of MassHunter Workstation.

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Results and discussion

Results showed that retention times in condition of gradient elution with formic acid and acetonitrile, depend on lipidic component of rhamnolipid, not only on molecular weight. In all, or almost all, rhamnolipid mixtures were present mono-rhamno-di-lipidic congeners: Rha-C8-C8, Rha-C8-C10/Rha-C10-C8, Rha-C10-C10:1/Rha-C10:1-C10, Rha-C8-C12/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-C14:1/Rha-C14:1-C10/Rha-C12-C12:1/Rha-C12:1-C12 and Rha-C10-C10-CH3 and di-rhamno-di-lipidic congeners: Rha-Rha-C8-C10 (all sources except frying sunflower oil), Rha-Rha-C10-C10, Rha-Rha-C10-C12:1/Rha-Rha-C12:1-C10,Rha-C10-C10:1/Rha-C8-C2:1//Rha-C12:1-C8 (all sources except sunflower frying oil), Rha-Rha-C10-C12/Rha-Rha-C12-C10, Rha-Rha-C10-C14:1/Rha-Rha-C14:1-C10/Rha-Rha-C12-C12:1/Rha-Rha-C12:1-C12, Rha-Rha-C12-C12 and Rha-Rha-C10-C10-CH3. Some rhamnolipidic congeners were detected only sporadically. Mono-rhamno-mono-lipidic Rha-C10 was detected on glucose and kerosene and Rha-C14:2 on SME, whereas, rare observed di-rhamno-di-lipidic congeners were: Rha-Rha-C10 (SME and glucose), Rha-Rha-C8-C8 (SME and glucose with addition of kerosene), Rha-Rha-C14-C14 (frying sunflower oil) and Rha-Rha-C14-C16/Rha-Rha-C16-C14 (frying sunflower oil and SME) (Table 1.). Fig 1. shows MS spectra of same detected congeners.

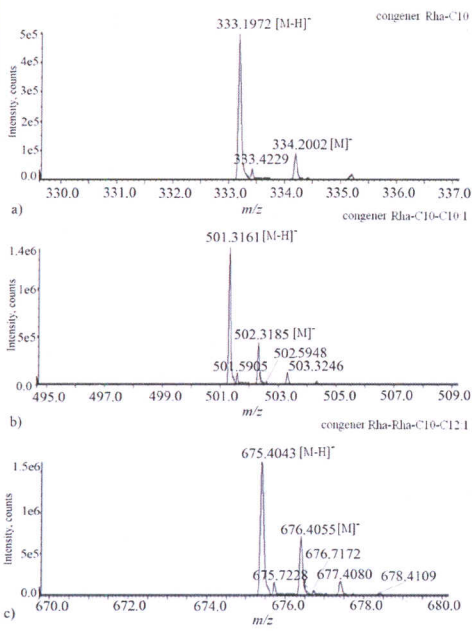

Figure 1. MS spectra of rhamnolipid congeners detected in rhamnolipid mixture of *P. aeruginosa* NCAIM (P) B 001380: a) mono-rhamno-mono-lipidic Rha-C10, b) mono-rhamno-di-lipidic Rha-C10-C10:1/Rha-C10:1-C10 and c) di-rhamno-di-lipidic Rha-Rha-C10-C12:1/Rha-Rha-C12:1-C10.

Table 1. List of detected RL congeners of *P. aeruginosa* NCAIM (P) B 001380 produced on PPAS medium with different carbon sources with retention time

RL congener, molecular weight	Sample (retention time)			
	Frying sunflower oil	SME	Glucose	Glucose + kerosene
Rha-C10, 334.41	-	-	-	7,53
Rha-C14:2, 386.48	-	9,60	-	-
Rha-C8-C8, 448.55	9,38	9,39	9,38	9,41
Rha-C8-C10/ Rha-C10-C8, 476.60	10,43	10,45	10,45	10,46
Rha-C10-C10:1/Rha-C10:1-C10, 502.64	11,11	11,12	11,13	11,14
Rha-C10-C10//Rha-C8-C12/Rha-C12-C8, 504.65	11,54	11,57	11,55	11,57
Rha-C10-C12:1/Rha-C12:1-C10, 530.69	12,20	12,19	12,19	12,21
Rha-C10-C12/Rha-C12-C10, 532.71	12,63	12,64 12,83	12,64	12,62
Rha-C10-C14:1/Rha-C14:1-C10//Rha-C12-C12:1/Rha-C12:1-C12	13,21	13,16	13,15	12,99
Rha-C10-C14/Rha-C14-C10//Rha-C12-C12, 560.76	13,64	13,60	13,59	13,61
Rha-C10-C10-CH3, 518.68	12,10	12,11	12,10	12,10
Rha-Rha-C10, 480.55	-	7,20	7,20	-
Rha-Rha-C8-C8, 594.69	-	8,85	-	8,85
Rha-Rha-C8-C10/Rha-Rha-C10-C8, 622.74	-	9,83	9,83	9,84
Rha-Rha-C10-C10:1/Rha-Rha-C10:1-C10//Rha-C8-C10:1/Rha-C12:1-C8, 648.78	-	10,44	10,45	8,33 10,46
Rha-Rha-C10-C10, 650.79	10,82	10,90	10,83	10,86
Rha-Rha-C10-C12:1/Rha-Rha-C12:1-C10, 676.83	11,45	11,27 11,48	11,26 11,47	11,24 11,49
Rha-Rha-C10-C12/Rha-C12-C10, 678.84	11,92	11,95	11,92	11,94
Rha-Rha-10-C14:1/Rha-Rha-C14:1-C10//Rha-Rha-c12-C12:1/Rha-Rha-C12:1-C12, 704.89	12,53	12,52	12,51	12,53
Rha-Rha-C12-C12, 706.90	12,99	12,97	12,97	12,98
Rha-Rha-C14-C14, 763.00	12,95	-	-	-
Rha-Rha-C14-C16/Rha-C16-C14, 791.06	12,77	13,35	-	-
Rha-Rha-C10-C10-CH3, 664.82	11,36	11,27 11,36	11,26 11,37	11,28 11,39

Conclusion

This comparative analysis indicated that cultivation conditions, such as carbon source (purity and complexity) had effect on composition of rhamnolipid mixtures and that differences were reflected in mono-rhamno-mono-lipidic and mono- and di-rhamno-di-lipidic congeners. Rarely present congeners were Rha-C10 (glucose), Rha-C14;1 (SME), Rha-Rha-C10 (SME and glucose) and Rha-Rha-C8-C8 (SME and glucose + kerosene). Generally, the lowest diversity of detected RL structures showed medium with frying sunflower oil (17), the highest medium substituted with SME (21), and simple carbon sources (glucose and glucose with kerosene) were in the middle (18, 19, respectively). Investigation of effect of medium composition (organic, mineral or combined) on diversity of rhamnolipid structures is underway.