

Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide

Neissa M. Pinzon · Lu-Kwang Ju

Received: 3 April 2009 / Revised: 26 May 2009 / Accepted: 27 May 2009 / Published online: 23 June 2009
© Springer Science+Business Media B.V. 2009

Abstract Rhamnolipids, produced predominantly by *Pseudomonas aeruginosa*, are biosurfactants with important applications. For efficient culture screening according to rhamnolipid productivity, the method using agar plates containing methylene blue (MB) and cetyl trimethylammonium bromide (CTAB) was re-examined. An alternative set-up using a fixed underneath light source and image analysis software improved the detection of the circles formed due to complexation between anionic rhamnolipids and cationic MB/CTAB. The roles and effects of MB and CTAB concentrations and pH on the complexation phenomena are reported.

Keywords Biosurfactants · Cetyl trimethylammonium bromide · Methylene blue · *Pseudomonas aeruginosa* · Rhamnolipids

Introduction

Rhamnolipids are biosurfactants produced mainly by *Pseudomonas aeruginosa*. They reduce surface and

interfacial tensions and offer excellent detergent, emulsifying/solubilizing, foaming, and dispersing properties. Being biodegradable, they are particularly suitable for bioremediation (Benincasa 2007; Zhu and Zhang 2008) and enhanced oil recovery (Harvey et al. 1990; Wang et al. 2007). The increased interest in rhamnolipid production and applications warrant the development of fast and effective strain-screening methods for rhamnolipid productivity. For this purpose, Siegmund and Wagner (1991) proposed the use of agar plates containing methylene blue (MB) and cetyl trimethylammonium bromide (CTAB) (SW plates). On these plates blue halos would form around the rhamnolipid-producing colonies. Ideally the halo size would correlate with the amount of rhamnolipids produced (Siegmund and Wagner 1991). Since then others have used the SW plates to study the effect of medium composition on rhamnolipid production and to screen for productive strains (Wild et al. 1997; Gunther et al. 2005). When the method was evaluated for use in this laboratory, the edges of the blue halos were not always clearly distinguishable and could shift depending on the intensity and location of the light source as well as the background color. In this study the SW-plate method was revisited for improved understanding. A set-up using fixed, underneath illumination, such as that of a dissecting microscope or a UV transilluminator, and common image analysis software, is recommended for easier detection. Some potential limitations of this semi-quantitative method were

N. M. Pinzon · L.-K. Ju (✉)
Department of Chemical and Biomolecular Engineering,
The University of Akron, Akron, OH 44325-3906, USA
e-mail: LukeJu@Uakron.edu

identified when the modified set-up was used with different *P. aeruginosa* strains. The complexation between anionic rhamnolipids and MB/CTAB as well as the effect of pH on the SW agar plates were also investigated.

Materials and methods

Ten *P. aeruginosa* strains were used: PAO1, ATCC 9027, and 8 strains isolated from soil samples taken at a biodiesel plant in Ralston, Iowa. The latter strains were designated as E03-36, E03-40, F04-63, F04-67, H05-03, H05-25, H05-45, and ST55 [ARS (NRRL) Culture Collection]. Prior to plating, the cultures were grown in shaker flasks at 34°C for 24 h using the following medium (per l): 20 g glucose or glycerol, 0.7 g KH_2PO_4 , 0.9 g Na_2HPO_4 , 2 g NaNO_3 , 0.4 g $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 2 ml of a trace element solution, which contained, per l, 2 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and 0.6 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. The SW agar plates were prepared by adding 0.2 g CTAB, 0.005 g MB, and 12 g agar to 1 l of the above medium (Siegmund and Wagner 1991). Following the improved procedure of Gunther et al. (2005), shallow wells were cut on the agar plate surface using the heated tip of a 5 ml Eppendorf pipette. Ten microliters of the inoculum were added into each well. The plates were incubated for 48 h at 34°C and then stored in the refrigerator for at least 24 h (typically 48 h). The cold storage tended to darken the blue color, making visible recognition less difficult (Gunther et al. 2005). A fixed light source such as that of a dissecting microscope (Olympus SZX12 ILLB100) or a UV transilluminator (Fisher Scientific) was used to light the plates. Image analysis software was used to determine the halo diameters on the pictures taken with a digital camera connected to the UV transilluminator or the dissecting microscope. The same procedure was also used to observe the halo formation when standard solutions of different rhamnolipid concentrations (e.g., 11, 23, 57 and 114 g/l) were added to the wells on the agar plates. In some experiments the CTAB concentration was varied (at 0, 0.05, 0.1, 0.2, 0.3, and 0.5 g/l) to investigate the role and effect of CTAB.

MB and CTAB were purchased from Fisher Scientific. The rhamnolipid standard was provided by Ecochem Canada Ltd. (Delia, AB, Canada). Using

HPLC-MS (HP 1100 series + Bruker Daltonics Esquire LC-MS analyzer) with a mobile phase of 55:45 (v/v) acetonitrile:water, the standard was found to contain two dominant rhamnolipids: L-rhamnolipid- β -hydroxydecanoyl- β -hydroxydecanoate and L-rhamnolipid-L-rhamnolipid- β -hydroxydecanoyl- β -hydroxydecanoate. Their relative peak areas of total-ion intensity were 48.4% and 51.6%, respectively.

Results and discussion

Use of dissecting microscope or UV transilluminator for clear observation of SW plates

The blue halos formed on SW plates changed in color and size with the intensity and incidence angle of the light source. The halo areas could be more reproducibly determined from the pictures taken with either a dissecting microscope or a UV transilluminator, as shown in Fig. 1, where each plate had four wells, added with 10 μl standard solutions containing different rhamnolipid concentrations. Observed under the dissecting microscope, the circles formed around the wells had at least four distinguishable layers (Fig. 1a): an innermost light area, a bluest halo, a lighter blue zone outside the darker blue halo, and an outermost brown ring. Similar four-layered structures were observed with the UV transilluminator but the colors were different (Fig. 1b). (The outermost layer was light and not always discernible on pictures.) Without the fixed underneath illumination, these layers were not always evident and would vary depending on the background and lighting condition.

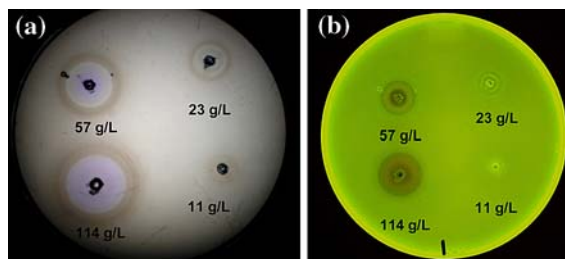


Fig. 1 SW agar plates with four wells, each added with 10 μl of a standard solution: picture taken with the plate lighted on (a) a dissecting microscope and (b) a UV-transilluminator

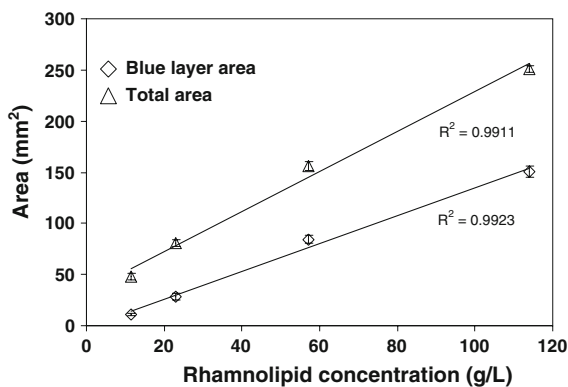


Fig. 2 Linear correlation of the complexation-affected area with the rhamnolipid concentration. The total area is for all four layers; the blue layer area is for the combined innermost light layer and the bluest halo. The results are the averages and standard deviations obtained from three agar plates

In Fig. 2 both the total areas surrounding the wells (including all four layers) and the blue areas (i.e., the innermost light area and the bluest halo area, which together correspond to the blue halo area used originally by Siegmund and Wagner (1991)), determined with the dissecting microscope, were plotted against the rhamnolipid concentrations added to the wells. Good linear correlations, with $R^2 > 0.99$, were obtained with either of the two types of areas. The standard deviations of the areas determined with the help of dissecting microscope or UV transilluminator were much smaller than those determined without the fixed underneath illumination (data not shown).

Effects of CTAB concentration

The phenomena observed on SW agar plates were known to be associated with the ion-pairing complexation between anionic rhamnolipids and cationic MB and CTAB (Siegmund and Wagner 1991) but the individual roles of MB and CTAB were not clearly established. With aqueous solutions, instead of the complex agar plates, the following observations were obtained (Pinzon and Ju 2009): (1) Aqueous MB solutions showed absorbance bands near 292, 615, and 664 nm. (2) CTAB had no detectable absorbance and the mineral salts medium used in SW agar plates had only one very weak absorbance band near 300 nm. (3) Rhamnolipid solutions had no absorbance at concentrations below 100 mg/l. At higher concentrations, the “absorbance” appeared and expanded to longer

wavelengths with increasing concentration, presumably due to light scattering of the micelles formed. (4) With solutions of 5 mg MB/l (same as in SW plates) and varying rhamnolipid or CTAB concentrations, the absorbance at 664 nm increased only within a narrow range (1:1 to 2:1) of the molar ratio between rhamnolipids and the combined MB and CTAB, supporting the occurrence of ion-pairing complexation.

Clearly, MB was included not only for being a cationic compound but also for its characteristic blue color. However, only very low MB concentrations could be used; at higher concentrations, the intense blue would mask the color change from ion-pairing complexation. Using MB alone would therefore limit the applicability of the method to detection of very low rhamnolipid levels. Addition of the absorbance-free CTAB enabled detection at the levels produced by *P. aeruginosa* colonies.

The effect of CTAB concentration was investigated with plates containing 5 mg MB/l and different CTAB concentrations (0, 0.05, 0.1, 0.2, 0.3, and 0.5 g/l). Rhamnolipid solutions of 10, 25, 50, and 125 g/l were added to the wells of each plate. The total areas of the circles surrounding these wells, revealed on the UV transilluminator, are summarized in Table 1. For the same rhamnolipid concentration, the circles were smaller on the plates of higher CTAB concentrations. On the plates of 0.3 and 0.5 g CTAB/l, the only circles detectable were those from at least 50 g rhamnolipids/l. The circles from lower rhamnolipid concentrations became more recognizable on plates of lower CTAB concentrations but the

Table 1 Total affected areas surrounding the wells added with 10 μ l of rhamnolipid (RhL) solutions of different concentrations, on agar plates with 5 mg methylene blue/l and various concentrations of cetyl trimethylammonium bromide (CTAB)

CTAB (g/l)	RhL (g/l)			
	10	25	50	125
0	ND	ND	ND	ND
0.05	10	35	145	489
0.1	6	30	120	307
0.2	0	11	32	180
0.3	0	0	25	154
0.5	0	0	12	117

Note: Areas are reported in mm^2

ND Not detectable

circles from high rhamnolipid concentrations became very diffuse and the edges harder to distinguish. On the plate of no CTAB, no circles could be detected while the whole plate looked darker. Totally 3.6 μmol rhamnolipids were added to the four wells of each plate. Without CTAB, the plate contained only 0.3 μmol of MB. The added rhamnolipids must have spread over the entire plate.

According to the above understanding, the CTAB concentration can be adjusted (without changing the MB concentration) for different applications. For example, if the purpose is to ensure complete deactivation of genes responsible for rhamnolipid synthesis, the CTAB concentration may be reduced significantly to reveal the potential residual production with larger complexation circles.

Effect of pH

Circle formation was optimal at pH 7 and 9. The circles were not apparent at pH 3 and 5, because the ion-pairing requires rhamnolipids to be present as anionic ions while their pKa were in the range of 4.3–5.5 (depending on the rhamnolipid structure and concentration) (Lebron-Paler et al. 2006). At pH 11, the circles were formed with comparable sizes to those at pH 7 and 9 but the agar plates at this pH were a much paler blue making the circle identification more difficult. These findings indicated that the strength of buffer to be used in the SW plates should depend on whether significant acid production might be expected and, if so, the buffering capacity of the medium would need to be increased.

Molar complexation ratios

An attempt was made to determine the molar ratios of rhamnolipids-to-combined MB and CTAB, i.e. (MB + CTAB), involved in the complexation. The total circle area (including all four layers) around each well was used to determine the number of moles of (MB + CTAB) that participated in the complexation. The resultant molar complexation ratios are given in Table 2. The molar ratios of rhamnolipids-to-(MB + CTAB) turned out to be not 1:1 but to increase with increasing rhamnolipid concentration. The ratio was as high as 3.2:1 when the concentration of rhamnolipid solution added was 114 g/l (Table 2).

Table 2 Molar complexation ratios of rhamnolipids-to-(MB + CTAB) and SDS-to-(MB + CTAB)

Surfactant concentration (g/l) ^a	Molar complexation ratio	
	Rhamnolipids: (MB + CTAB) ^b	SDS: (MB + CTAB) ^c
11	1.7 ± 0.1	1.3
23	2.0 ± 0.1	1.3
57	2.6 ± 0.1	1.2
114	3.2 ± 0.1	1.4

^a Concentration of either rhamnolipids or sodium dodecyl sulfate (SDS) in the 10 μl solution added to the well in an agar plate

^b Molar ratios determined from three agar plates

^c Molar ratios determined from one agar plate

To check if the deviation from 1:1 complexation was caused by experimental or computational errors, the same procedures were applied to SW plates with the wells added with solutions of different sodium dodecyl sulfate (SDS) concentrations. Clear four-layered complexation circles were also observed. The molar complexation ratios of SDS:(MB + CTAB) determined with these plates were much closer to 1:1, ranging from 1.2:1 to 1.4:1, and had no apparent dependency on the SDS concentration (Table 2). The anionic SDS has a simple structure—a straight C12 hydrocarbon with a sulfate end group. The SDS-MB/CTAB complexes are expected to be rather water insoluble and the ion-pairing stable. On the other hand, the rhamnolipid molecule has the sugar moiety of one or two rhamnose linked to a chain of one or two β -hydroxyalkanoic acid (mainly C10). Even after being complexed with MB/CTAB, the sugar moiety continues to provide hydrophilicity. The large, concentration-dependent molar ratios observed with rhamnolipids probably reflected the more complicated nature of the complexation between rhamnolipids and MB and CTAB.

Besides the complicated complexation nature, the mechanisms responsible for formation of the unique four-layered circles are unknown. Furthermore, the complexation behaviors used for rhamnolipid detection with SW plates were in a transitional, time-dependent state. These factors, together with the varying molar complexation ratios, make the SW-plate method unsuitable for quantitative analysis. The method is recommended only for strain screening and other qualitative purposes.

Table 3 Affected areas and estimated amounts of rhamnolipids produced by different *P. aeruginosa* strains on SW agar plates

Strain	Area (mm ²)	Rhamnolipids (μmol) ^a	Rhamnolipids (mg) ^b	Extent of pigment formation
E03-36	86	0.39	0.23	+
E03-40	129	0.59	0.34	+
H05-25	108	0.49	0.28	+
H05-45	100	0.45	0.26	++
H05-03	102	0.46	0.27	++
F04-63	98	0.45	0.26	++
F04-67	50	0.23	0.13	+
ST55	59	0.27	0.15	++
PAO1	101	0.46	0.27	+
ATCC 9027	ND	ND	ND	+++

^a Calculated using a molar complexation ratio of 2.3:1 rhamnolipids:(MB + CTAB), which is the average ratio obtained from those reported in Table 2

^b Estimated with an average rhamnolipid MW of 576, assuming similar composition as the rhamnolipid standard

+, Pigments visible only inside the well of initial inoculation; ++, pigments visible in the area immediately surrounding the well; +++, pigments visible in an extensive area outside the well, preventing detection of complexation circles

Pigment formation

For demonstration, the SW plates were used to evaluate the rhamnolipid productivity of 10 *P. aeruginosa* strains. The total complexation areas due to rhamnolipid production in 48-h incubation of the cultures are summarized in Table 3. Using the average molar complexation ratio of 2.3:1 (from Table 2), the amounts of rhamnolipids produced are also estimated. Further included in Table 3 (last column) are qualitative descriptions for the extents of pigment formation by different strains. Many *P. aeruginosa* strains produced fluorescent and non-fluorescent pigments that deposited around the colonies. These pigments could affect the recognition of the complexation circle edge (Wild et al. 1997). Two extreme examples are shown in Fig. 3: one had high rhamnolipid productivity and minimal pigment formation; the other produced an excessive amount of pigments that prevented the detection of complexation circles. As noted in Table 3, four of the tested strains, PAO1, E03-40, E03-36, and H05-25, were good rhamnolipid producers without excretion of large amounts of pigments.

In general, all of the tested strains showed better growth on plates containing glycerol as the carbon source than on the glucose-based plates. The only exception was the ATCC 9027 strain, which grew similarly well on both substrates. The complexation

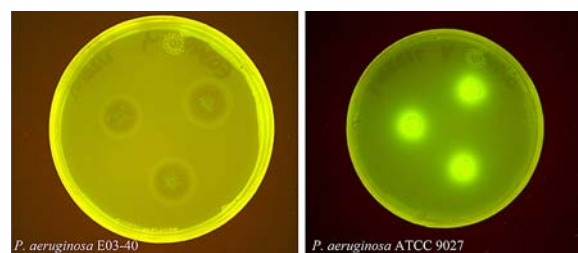


Fig. 3 UV-transilluminated pictures of agar plates inoculated with *P. aeruginosa* E03-40 (left) and ATCC 9027 (right), respectively. E03-40 strain gave clear rhamnolipid complexation circles with minimal pigment formation while ATCC 9027 produced excessive amounts of pigments, which prevented the detection of rhamnolipid complexation

circle formation was also easier to detect on the glycerol-based plates.

Acknowledgements The study was supported by the US Department of Transportation, Office of the Secretary, Grant No. DTOS59-07-G-00050. The authors thank Dr. Tsung Min Kuo (USDA-ARS-NCAUR, Peoria, IL) for providing the strains isolated from the biodiesel plant soil samples, Dr. Yang Yun (Biomedical Engineering) for the use of UV transilluminator, and Ms. Jennifer Lilly and Mr. Aaron Cook for their assistance in carrying out experimental procedures.

References

- Benincasa M (2007) Rhamnolipid produced from agroindustrial wastes enhances hydrocarbon biodegradation in contaminated soil. *Curr Microbiol* 54(6):445–449

- Gunther NWIV, Nunez A, Fett W, Solaiman DKY (2005) Production of rhamnolipids by *Pseudomonas chlororaphis*, a nonpathogenic bacterium. *Appl Environ Microbiol* 71(5):2288–2293
- Harvey S, Elashvili I, Valdes JJ, Kamely D, Chakrabarty AM (1990) Enhanced removal of Exxon Valdez spilled oil from Alaskan gravel by a microbial surfactant. *Biotechnology* 8(3):228–230
- Lebron-Paler A, Pemberton JE, Becker BA, Otto WH, Larive CK, Maier RM (2006) Determination of the acid dissociation constant of the biosurfactant monorhamnolipid in aqueous solution by potentiometric and spectroscopic methods. *Anal Chem* 78(22):7649–7658
- Pinzon NM, Ju L-K (2009) Analysis of rhamnolipid biosurfactants by methylene blue complexation. *Appl Microbiol Biotechnol* 82(5):975–981
- Siegmund I, Wagner F (1991) New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnol Tech* 5(4):265–268
- Wang Q, Fang X, Bai B, Liang X, Shuler PJ, Goddard WA, Tang Y (2007) Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. *Biotechnol Bioeng* 98(4):842–853
- Wild M, Caro AD, Hernandez AL, Miller RM, Soberon-Chavez G (1997) Selection and partial characterization of a *Pseudomonas aeruginosa* mono-rhamnolipid deficient mutant. *FEMS Microbiol Lett* 153(2):279–285
- Zhu L, Zhang M (2008) Effect of rhamnolipids on the uptake of PAHs by ryegrass. *Environ Pollut* 156(1):46–52