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Procedure for Rhamnolipids Quantification Using Methylene-blue

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[Abstract] Rhamnolipids produced by *Pseudomonas aeruginosa* (*P. aeruginosa*) represent a group of biosurfactants with various applications (*e.g.,* bioremediation of oil spills, cosmetics, detergents and cleaners). The commonly used colorimetric methods for rhamnolipid quantification, including anthrone, phenol−sulfuric acid and orcinol based quantification (Helbert and Brown, 1957; Chandrasekaran and BeMiller, 1980), are laborious and operationally hazardous because of the strong acid/chemical emanation which can cause deterioration of instruments measurements (*e.g.,* spectrophotometer). Therefore, the methylene-blue-based analysis appears as a promising alternative to safely quantify whole rhamnolipid molecules based on chemical complexation reaction (Pinzon and Ju, 2009). Indeed, methylene blue and rhamnolipids form a complex in a water-chloroform phase system. The rhamnolipids-methylene blue complex is partitioned into the chloroform phase which will develop a blue color that can be quantified at 638 nm to deduce rhamnolipids concentration. Here, we describe a variant of methylene-blue-based rhamnolipids quantification procedure that allows spectrophotometric quantification on standard 96-well plastic microplate contrarily to original methylene blue procedure that requires specific and expensive microplate due to chloroform chemical properties.

Materials and Reagents

1. Borate Buffer Packs BupH™ (Thermo Fisher scientific, catalog number: 28384)
2. Centrifuge round bottom reaction tubes (30 ml) (Krackeler Scientific, COREX®, catalog number: 1-8445-30)
3. Greiner CELLSTAR® 96 well plates (clear polystyrene wells flat bottom) (Sigma-Aldrich, catalog number: M3687-60 EA)
4. Microcentrifuge tube [2 ml, transparent polypropylene (PP) with Lid] (Sigma-Aldrich, Brand®, catalog number: Z628034-500 EA)
5. Sterile culture tube (PP) (14 ml) (The Lab Depot Inc., catalog number: TLDT8235)
6. Centrifugal Filter (0.1 µm pore size, Non-Sterile) with Durapore® PVDF Membrane UFC40VV25 Ultrafree® (2 ml) (Merck Millipore Corporation, catalog number: UFC40VV25)
7. Aluminium foil
8. Micropipettor (10 µl,100 µl and 1,000 µl) tips
9. *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 Wild-type (strain PAO0001)

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(http://www.pseudomonas.med.ecu.edu/)

10. Chloroform (≥ 99%) (Carl Roth GmbH & Co. KG, catalog number: 3313.1)
11. Ethyl acetate (> 99.5%) (Merck Millipore Corporation, EMSURE®, catalog number: 1096231000)
12. Hydrochloric acid (HCL) standard (1 N solution) (Sigma-Aldrich, Fluka analytical, catalog number: 318949-500 ml)
13. HCl standard (0.2 N solution in water) (Sigma-Aldrich, Fluka analytical, catalog number: 343102-500 ml)
14. Luria Bertani (LB) Broth (Lennox) Powder microbial growth medium (Sigma-Aldrich, catalog number: L3022-250 G)
15. Methylene blue solution [1.4% (w/v) in 95% ethanol] (Sigma-Aldrich, catalog number: 1808-50 ml)
16. 3-(N-morpholino) propanesulfonic acid (MOPS) (99.5%) (Sigma-Aldrich, catalog number: M1254-100 G)
17. Rhamnolipids (90%, Standard) (Sigma-Aldrich, catalog number: R90)
18. Sodium hydroxide (NaOH) solution
19. LB-MOPS broth (see Recipes)
20. Borax buffer (50 mM) (see Recipes)
21. Methylene blue aqueous solution (see Recipes)

Equipment

1. Micropipettor (100 µl-1,000 µl)
2. Micropipettor (10 µl-100 µl)
3. Autoclave
4. Stopwatch
5. Measuring Pipette (5 ml) (Jaytec Glass Limited, catalog number: WJ.485)
6. 96-well microplate spectrophotometer (*e.g.* Molecular Devices, model: SpectraMax M2 device)
7. Vacuum concentrator (*e.g.* Savant SPEEDVAC SVC 200H: 200 *x g*)
8. Tabletop centrifuge
9. Vortex mixer

Procedure

A. *P. aeruginosa* PAO1 culture and rhamnolipids extraction

1. *P. aeruginosa* PAO1 was grown with agitation (175 rpm) at 37 °C for 18 h in a sterile culture tube (14 ml) containing 5 ml LB-MOPS medium (initial A_{600} of the culture was between 0.020 and 0.025).
2. After incubation, culture of *P. aeruginosa* in mid-stationary phase was centrifuged

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(3,200 *x g*, 24 °C, 5 min) to obtain supernatant and pellet. 4 ml of supernatant was filtered (0.1 µm filters, 20 *x g*) to remove cells. Then, pH of cell-free supernatant was adjusted to 2.3 ± 0.2 using 1 N HCl (≈ 80 µl).

3. Rhamnolipids were extracted by mixing 4 ml of supernatant with 4 ml of ethyl acetate. The non-miscible mixture was vigorously vortexed (600 rpm) for 20 sec and phase separation was realized by centrifugation in a tabletop centrifuge at speed 100 *x g* for 1 min.
4. The upper, rhamnolipid-containing organic/ethyl acetate phase was transferred to a new reaction tube (30 ml). Ethyl acetate phase should be carefully removed without taking any liquid from the top aqueous layer.
5. Extraction procedure was repeated three times and ethyl acetate extracts were combined in the reaction tube and evaporated to dryness in speedvac centrifugal evaporator (generally evaporated in 30 min at 200 *x g* speed) and immediately quantified.

B. Rhamnolipids quantification

1. Formation of chloroform-methylene blue complex

   Dry rhamnolipid-extract was dissolved in 4 ml chloroform and mixed with 400 µl methylene blue solution (freshly prepared). Tubes were vigorously mixed by vortexing for 5 min, then incubated at ambient temperature for 15 min to allow for color formation.

   Note: The chloroform phase will develop a blue color, proportional to the concentration of rhamnolipids. Cover tubes with aluminum foil to minimize chloroform evaporation during incubation [Figure 1(d)].

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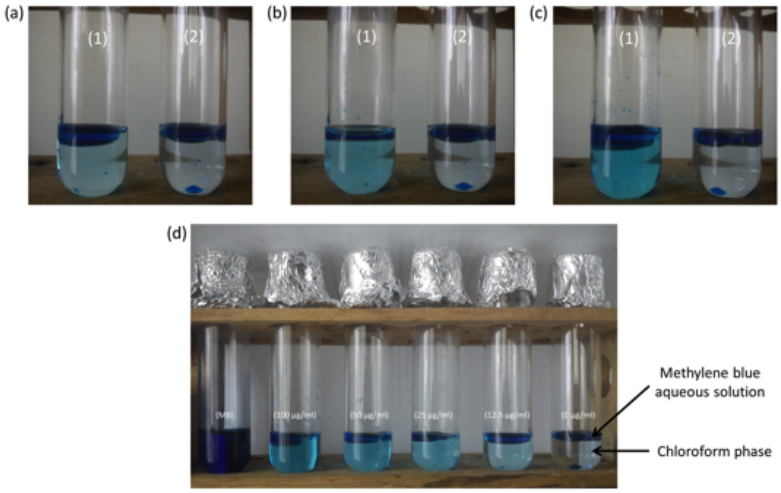

Figure 1. Expected results during chemical complexation reaction step. (a). Methylene blue complexation in chloroform containing 100 µg/ml of rhamnolipids (1) and chloroform without rhamnolipids (2) at $T_{5\ min}$. (b). Methylene blue complexation in chloroform containing 100 µg/ml of rhamnolipids (1) and chloroform without rhamnolipids (2) at $T_{10\ min}$. (c). Methylene blue complexation in chloroform containing 100 µg/ml of rhamnolipids (1) and chloroform without rhamnolipids (2) at $T_{15\ min}$. (d). Methylene blue aqueous solution (MB) and chloroform containing 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 0 µg/ml of rhamnolipids complexed with methylene blue. T represents incubation time for complexation.

2. Measurements step

   One ml of the chloroform (lower) phase was transferred in a clear microcentrifuge tube (2 ml) and mixed by vortexing for 20 sec with 500 µl of HCl 0.2 N. Phase separation was then realized by centrifugation in a tabletop centrifuge at speed 100 *x g* for 1 min and the solution was left at room temperature for 10 min to optimize methylene blue extraction in HCl. Finally, 200 µl of the upper acidic phase, containing a portion of the complexed methylene blue, was transferred in a 96-well microplate and the absorbance was measured at 638 nm with SpectraMax M2 device against an HCl 0.2 N blank.

3. Rhamnolipids calibration curve generation

   Rhamnolipids standard solution was prepared by dissolving 8 mg of rhamnolipids in 4 ml of chloroform. This solution is used to generate different concentration of rhamnolipids by dilution in chloroform (*i.e.* 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml). For each concentration, formation of chloroform-methylene blue complex and measurements steps were carried out to generate calibration curve equation (Figure 2; Table 1).

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Representative data

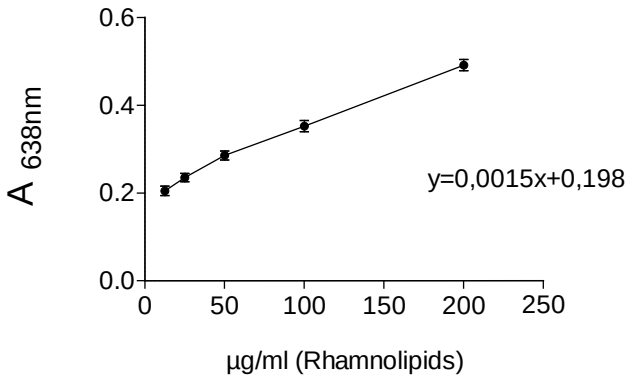

Figure 2. Expected calibration curve of different concentration of rhamnolipid (12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml). Absorbance of complexed methylene blue was measured at 638 nm with SpectraMax M2 device against an HCl (0.2 N) blank. Complexation and measurements were repeated three times and error bars represent the standard error of the mean.

Table 1. Absorbance of complexed methylene blue measured at 638 nm with SpectraMax M2 device against an HCl (0.2 N) blank

	Rhamnolipids standard					Rhamnolipids extracted from *P. aeruginosa* culture
Concentration (µg/ml)	200	100	50	25	12.5	
Absorbance at 638 nm	0.494	0.335	0.293	0.234	0.199	0.398
	0.487	0.356	0.284	0.235	0.202	0.396
	0.492	0.365	0.279	0.235	0.213	0.408

The applicability of methylene blue rhamnolipid method was previously verified by comparison of the analysis results with those obtained from the commonly used anthrone reaction technique (Pinzon and Ju, 2009). We show in Table 2 the main difference between both methods that highlighted the advantages of methylene blue rhamnolipid method.

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Table 2. Comparative table between methylene blue rhamnolipid and anthrone methods

	Methylene blue rhamnolipid method (Pinzon and Ju, 2009)	Anthrone method (Helbert and Brown, 1957; Chayabutra *et al.*, 2001)
Temperature reaction	Room temperature	95-100 °C
Use of concentrated sulfuric acid	No	Yes
Type of measure and quantified compound	Direct/measure of molar rhamnolipids concentration	Indirect/ measure of rhamnose concentration in the rhamnolipids, need a correction factor.
Crucial key step	Careful chloroform phase transfer and upper acidic phase transfer	Careful preparation of the anthrone reagent in concentrated sulfuric acid, Careful drop of anthrone reagent to the $NaHCO_3$ solution
Risk for operator	Lower	Higher
Specificity and Interference	Not communicated (Presence of Nitrate (NO_3^-) decreases absorbance level)	Around 89% (presence of NO_3^-, Fe, Cl^- and other carbohydrate over-estimate rhamnose level)

Notes

1. Incubation time for blue color development in chloroform phase should be equal for each tested sample.
2. During chloroform phase transfer avoid touching the methylene blue solution with tips (Video 1).

Video 1. Lower chloroform phase transfer and upper acidic phase transfer

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3. During upper acidic phase transfer, avoid pipetting chloroform which will attack/dissolve 96-well microplate. For that, prefer a clear microcentrifuge tube or transfer the chloroform/HCL solution into a clear tube for better visualization of the separated phase before pipetting the upper acidic phase. Check that all wells are free of chloroform before absorbance measurement.
4. During all transfer phases use new tips for each sample.

Recipes

1. LB-MOPS broth
   Add 25 g of dry LB powder and 10 g of MOPS to 1,000 ml of deionized water
   Adjust pH to 7.2 ± 0.2 by adding NaOH solution
   Autoclave for sterilization
2. Borax buffer solution
   Dissolve one borate buffer pack in 500 ml of deionized water (each pack makes 50 mM borate, pH 8.5)
   Store at 2-8 °C
3. Methylene blue aqueous solution
   Add 200 µl of methylene blue solution to 4.8 ml of deionized water
   Adjust pH to 8.6 ± 0.2 by adding 15 µl of a 50 mM borax buffer
   Store at room temperature

Acknowledgments

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