

# Antimicrobial Applications of Rhamnolipid Biosurfactant Produced from *Achromobacter* sp. (PS1) Isolate Using Lignocellulosic Hydrolysate

Sam Joy <sup>1</sup> , Shashi Sharma <sup>1,\*</sup> 

<sup>1</sup> Amity Institute of Biotechnology, Amity University, Sector- 125, Noida, UP- 201313, India; drjoysam@gmail.com(S.J.);

\* Correspondence: ssharma@amity.edu (S.S.);

Scopus Author ID 57193424723

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**Abstract:** Heterogeneous mixture of partially purified rhamnolipid (RL) produced from *Achromobacter* sp. (PS1) using lignocellulosic rice straw (RS) sugar hydrolysate medium revealed six different congeners- Rha- C<sub>10</sub>-C<sub>10</sub>, Rha-C<sub>8</sub>-C<sub>10</sub>/Rha-C<sub>10</sub>-C<sub>8</sub>, Rha- C<sub>12</sub>-C<sub>10</sub> / Rha- C<sub>10</sub>-C<sub>12</sub>, referring mono-rhamnolipids amounting to total 68.23 % and Rha-Rha-C<sub>10</sub>-C<sub>10</sub>, Rha-Rha-C<sub>8</sub>-C<sub>10</sub>/Rha-Rha-C<sub>10</sub>-C<sub>8</sub>, Rha-Rha-C<sub>10</sub>-C<sub>12</sub>/Rha-Rha-C<sub>12</sub>-C<sub>10</sub>, referring di-rhamnolipids amounting to 31.73 %, with Mono to Di- RL in the ratio of 2.1:1. This mixture's antimicrobial action containing more mono-rhamnolipids analyzed using broth macro-dilution method exhibited a broad-spectrum antibacterial activity showing  $\geq 90$  % growth inhibition of both Gram-positive and Gram-negative pathogenic bacteria at MIC ranging from 1.25 mg/mL to 10 mg/mL of total rhamnolipids. This might be due to the more hydrophobic character of mono-rhamnolipids containing a single rhamnosyl group and showing high surface activities. On the other hand, the non-antifungal activity may be attributed to the lower percentage of di-rhamnolipids in the partially purified mixture.

**Keywords:** partially purified rhamnolipid; lignocellulose; antimicrobial application; broth macro-dilution method; *Achromobacter*.

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## 1. Introduction

Biosurfactants are surface-active secondary metabolites produced by microorganisms and plants. These are categorized into low-molecular-weight and high-molecular-weight polymers [1]. Biosurfactants find broad applications in various industries because of low toxicity, biodegradability, stability, and sustainability of production from cheap renewable feedstock [2, 3]. With respect to properties, biosurfactants show superior properties such as foaming, emulsification, wetting, dispersion, stabilization, and detergency, including antimicrobial and anti-tumor activities compared to chemical counterparts [4-8].

In the last decade, the global biosurfactant market showed a steady increase in compound annual growth rate (CAGR) of 5.5 % from 2018 – 2024, which projects its expected market utilization to cross over 540 kilotons 2024, surpassing 2700 million USD industry. Among various categories of biosurfactants, "rhamnolipids", a glycolipid biosurfactant, alone witnesses the highest gain of over 8.0 % in the current global biosurfactant market because of their excellent and superior properties [9].

Rhamnolipid biosurfactants have been considered relevant molecules for several biomedical applications. Their surface-active properties can destabilize membrane integrity

and induce pore formation in lipid bilayer membranes of microorganisms, thereby resulting in membrane disruption and cell disruption death [8]. Apart from these applications, rhamnolipids have also been explored as anti-adhesive agents preventing the growth and biofilm formation of pathogenic agents on medical equipment, catheters, and surgical implants and as safe vehicles for efficient drug delivery [5, 10, 11].

Despite the numerous industrial applications of rhamnolipids, they are not widely used primarily because the model organism for rhamnolipid production in *Pseudomonas aeruginosa*, which is the third most common nosocomial pathogen with a rating at biosafety level 2 (BSL-2) [12] and associated with chronic and fatal lung disease in cystic fibrosis patients [13, 14]. The pathogenicity factor and the high production costs of 5 to 20 USD/kg/L compared to the production costs for chemical surfactants, which lie between 1 and 3 USD/kg/L, become the limiting factors in imposing restrictions associated with the commercialization of rhamnolipids [15]. This is also reflected by the current price of 10 mg of rhamnolipid as \$227 in Sigma Aldrich and \$200 in AGAE Technologies [16]. Other limiting factors hindering the market success of rhamnolipids include high substrate costs (e.g., pure sugars & hydrocarbons), low product concentrations, and severe stable foam formation under aerated and agitated conditions [17].

Our findings have been able to isolate a high-yielding rhamnolipid-producing microorganism from genus *Achromobacter* (NCBI accession no. KT735240), which has been reported to possess rare biosurfactant synthetic abilities [18, 19]. This organism shows 99 % similarity to *Achromobacter insolitus* [18], which has a rating as biosafety level 1 (BSL-1) [20], contrary to *P.aeruginosa* (model organism) with a rating as biosafety level 2 (BSL-2) [12]. In the current study, the rhamnolipid produced utilizing lignocellulosic substrate from *Achromobacter* sp. (PS1) isolate has been evaluated for its antimicrobial applications.

## 2. Materials and Methods

### 2.1. Microorganism and chemicals.

The bacterial culture *Achromobacter* sp. (PS1) was routinely sub-cultured and maintained on nutrient agar plates. All chemicals, solvents, and reagents used were of analytical grade. The rhamnolipid standard JBR 215 (15% solution in water) was purchased from Jeneil biosurfactant Company (Saukville, WI, USA), while R95Dd Rhamnolipid (95% purity) was from Sigma-Aldrich (India). For saccharification of lignocellulosic rice straw residues, advanced-saccharin SEB-enzyme containing a cocktail of lignocellulolytic enzymes -cellulases (365 FPU/g),  $\beta$ -glucosidases (571 CBU/g), and xylanases (7000 ABXU/g) was purchased from Advanced enzymes (India).

### 2.2. Rhamnolipid biosurfactant production and extraction.

Rhamnolipid biosurfactant was produced in lignocellulosic sugar hydrolysate obtained from enzymatically hydrolyzed ammonia-soaked pre-treated rice straw. The sugar hydrolysate was supplemented with essential optimized nutrients viz.  $\text{NaNO}_3$  (8.7g/L); KCl (1.1g/L); NaCl (1.1g/L);  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.00028g/L);  $\text{K}_2\text{HPO}_4$  (4.4g/L);  $\text{KH}_2\text{PO}_4$  (3.4g/L);  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.5g/L) and beef extract (0.16g/L). The partially purified viscous honey-colored rhamnolipid biosurfactant was synergistically extracted from culture filtrate using sweep-floc coagulation and acidification method with Rf values of 0.74 and 0.36 corresponding to the standard Jeneil rhamnolipid [21].

This partially purified rhamnolipid used for antimicrobial studies was structurally confirmed using tandem-MS. The critical micelle concentration was determined by plotting the surface tension of dilutions made in deionized water against a biosurfactant concentration curve.

### 2.3. Antimicrobial application of the rhamnolipid biosurfactant.

The macro-dilution broth method was adopted as per Clinical and Laboratory Standards Institute (CLSI) protocols [22].

#### 2.3.1. Inoculum preparation.

The inoculum for each test pathogenic organism was prepared by making a broth suspension of isolated colonies selected from a 24-hour plate in either Mueller-Hinton broth (MHB) or Roswell Park Memorial Institute (RPMI1640) medium for bacteria and fungus, respectively. The turbidity was adjusted using a sterile medium to achieve turbidity equivalent to a 0.5 McFarland standard. This was carried out by visually comparing the inoculum tube with the BaSO<sub>4</sub> 0.5 McFarland standard tube against a card with a white background and contrasting black lines. This adjusted inoculum suspension was then used for inoculating the test tubes containing rhamnolipid dissolved in a sterile medium (MHB/RPMI), resulting in a final suspension containing approximately  $5 \times 10^5$  CFU/mL [23].

#### 2.3.2. Macro-dilution (Tube) Broth Method.

The partially purified rhamnolipid biosurfactant stock solution was prepared by dissolving 0.4 g each in 20 mL of Mueller-Hinton broth (MHB) and Roswell Park Memorial Institute (RPMI1640) medium for bacteria and fungus, respectively. This was then serially diluted in tubes containing sterile MHB/ RPMI1640 medium to obtain a final rhamnolipid concentration ranging from 10 – 0.01 mg/mL for examining the antimicrobial evaluation action against the test strains - Gram-positive: *Micrococcus flavus* (NCIM 2376), *Staphylococcus aureus* (NCIM 5021), *Bacillus subtilis* (NCIM 2063), *Streptococcus faecalis* (NCIM 5024); Gram-negative: *Pseudomonas aeruginosa* (NCIM 2200), *Serratia marcescens* (NCIM 5246), *Escherichia coli* (NCIM 2931), *Alcaligenes faecalis* (NCIM 2105), *Salmonella typhimurium* (NCIM 2501), *Proteus mirabilis* (NCIM 2241) and fungal strains: *Candida albicans* and *Cryptococcus neoformans*. The sterility controls the growth control were also maintained for each tested strain. The growth inhibition percentage was calculated by measuring the optical density (O.D) at 600nm, using the following equation [23].

$$\text{Growth inhibition (\%)} = [1 - (\text{ODT}/\text{ODC})] \times 100 \quad \text{Eq. (1)}$$

where, ODT represents the optical density of the test sample with a biosurfactant, and ODC is the optical density of control (without biosurfactant). The lowest concentration of biosurfactant, which inhibited 90% of the growth compared to control, was used to calculate MIC 90. Triplicate assays were performed for each microorganism and biosurfactant concentrations.

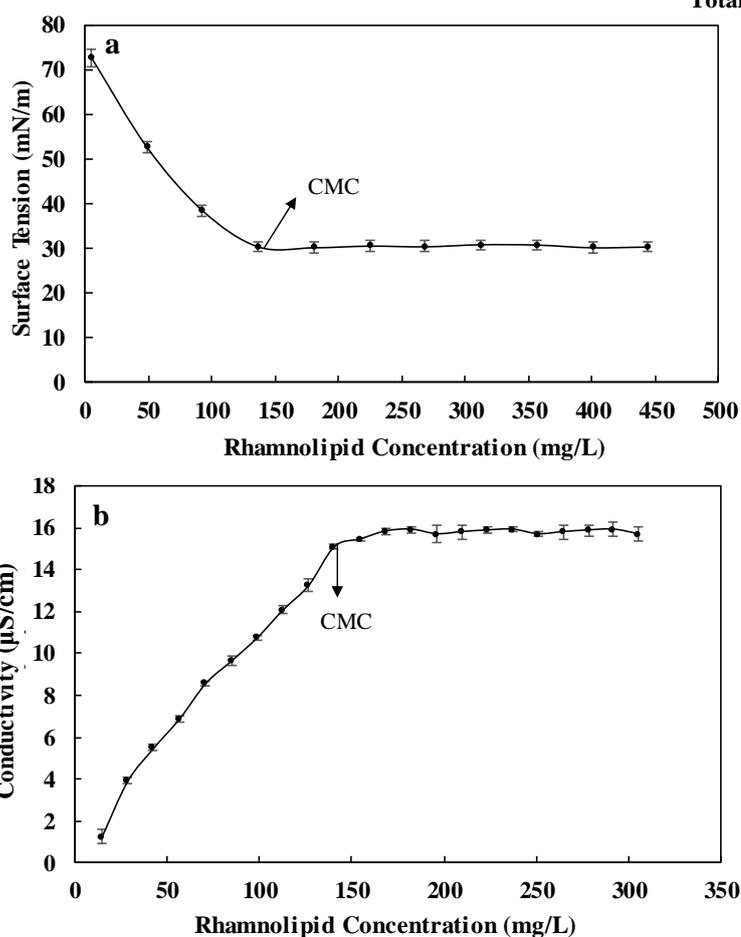
### 3. Results and Discussion

#### 3.1. Rhamnolipid characterization.

It is known that the production medium composition significantly affects the structure and composition of rhamnolipids and hence the application part [24]. The tandem mass spectrometric (MS) analysis of the rhamnolipid biosurfactant ( $3.55 \pm 0.06$  g/L) produced from rice straw hydrolysate in the proposed study showed the presence of six different types of congeners (Table 1) with 68.23 % mono-rhamnolipids and 31.73 % Di-Rhamnolipids in the ratio of 2.1:1.

**Table 1.** Tandem-MS results of  $[M + Na]^+$  and  $[M + K]^+$  ions corresponding to mono and di-rhamnolipids.

	Pseudo molecular ion mass ( $m/z$ )		Congeners	Relative intensity (%)
	$[M + Na]^+$	$[M + K]^+$		
Mono-rhamnolipid	527.5	543.4	Rha-C <sub>10</sub> -C <sub>10</sub>	46.34
	499.1	514.3	Rha-C <sub>8</sub> -C <sub>10</sub> /Rha-C <sub>10</sub> -C <sub>8</sub>	7.93
	555.3	571.4	Rha-C <sub>12</sub> -C <sub>10</sub> /Rha-C <sub>10</sub> -C <sub>12</sub>	13.96
			<b>Total</b>	<b>68.23</b>
Di-rhamnolipid	673.4	689.3	Rha-Rha-C <sub>10</sub> -C <sub>10</sub>	22.00
	644.9	661.2	Rha-Rha-C <sub>8</sub> -C <sub>10</sub> /Rha-Rha-C <sub>10</sub> -C <sub>8</sub>	4.09
	701.3	717.4	Rha-Rha-C <sub>10</sub> -C <sub>12</sub> /Rha-Rha-C <sub>12</sub> -C <sub>10</sub>	5.64
			<b>Total</b>	<b>31.73</b>



**Figure 1.** CMC determination of the rhamnolipid biosurfactant (a) surface tension measurements; (b) conductivity measurement.

Li *et al.* [24] reported efficient production of di-rhamnolipid (Rha-Rha-C<sub>10</sub>-C<sub>10</sub>) as the predominant component with soybean oil and glycerol as carbon source, accounting for 64.80 % and 85.70 % of total products, respectively, from *P. aeruginosa* YM4. The critical micelle

concentration (CMC) of rhamnolipid products was also found to vary with the content of di-rhamnolipid, whereby lower CMC values corresponded to higher di-rhamnolipid contents. This observation was supported by a low CMC value of 50 mg/L of rhamnolipids containing 85.70 % of di-rhamnolipid. Further, it was reported that higher di-rhamnolipid content leads to better viscosity-reducing activity.

Samadi *et al.* [25] also reported similar results of rhamnolipid composition with mono-rhamnolipids forming 68.35 % of the total congeners in the rhamnolipid produced from *Pseudomonas aeruginosa* MN1. The ratio of mono to di-rhamnolipid congeners in the rhamnolipid biosurfactant is reported to affect the surface and emulsifying activities. Mono-rhamnolipid congeners are reported to be more surface-active than di-rhamnolipids due to their less hydrophilic nature resulting from the absence of the second rhamnosyl group [25, 26]. Abbasi *et al.* [27] reported that bacterial mono-RL interacts with dielaidoylphosphatidylethanolamine (DEPE) model membranes, destabilizes the lamellar configuration, and changes the lipid/ water interface characteristics by modification of the hydrogen-bonding pattern. Zhong *et al.* [28] showed that the adsorption capacity of all the cells to monorhamnolipid was much stronger than to dirhamnolipid. Among the six different rhamnolipid congeners, the mono-rhamnolipid Rha-C10-C10 was followed by di-rhamnolipid Rha-Rha-C<sub>10</sub>-C<sub>10</sub> was found to be the most abundant congeners with a relative percentage of 46.34 % and 22 %, respectively (Table 1).

The critical micelle concentration (CMC) of rhamnolipid is also affected by the length of the fatty acid chain and the number of rhamnose units [29]. In our study, the CMC of the partially purified rhamnolipid was determined to be 136 mg/L and 141 mg/L using surface tension and conductivity measurements, respectively (Figure 1).

### 3.2. Antimicrobial evaluations.

The antibacterial potential was analyzed using the partially purified rhamnolipid at a concentration of 10 mg/mL – 0.01 mg/mL. Amongst the Gram-negative pathogenic bacteria examined, the rhamnolipid exhibited 91.01 % growth inhibition for *Serratia marcescens* at MIC of 1.25 mg/mL, while 90.14 % and 96.20 % for *Escherichia coli* and *Pseudomonas aeruginosa* respectively at MIC of 2.5 mg/mL. As reported, different factors such as the congeners' composition, the length of the acyl chains, and the presence of unsaturation affect the antimicrobial activity of both RLs and RLs mixture [30]. For the other Gram-negative bacteria- *Alcaligenes faecalis*, *Salmonella typhimurium*, and *Proteus mirabilis*, MIC of 5.0 mg/mL was found to be effective. The effective high concentration may be justified by the report of Buonocore *et al.* [30], stating that generally, these bacteria are resistant to anionic surfactants because their outer membrane is hardly permeable to hydrophobic and amphipathic molecules. In the case of the Gram-positive pathogenic bacteria, growth inhibition of  $\geq 90$  % was observed towards MIC of 5.0 mg/mL for *Micrococcus flavus* and 10 mg/mL for *Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus faecalis*, respectively. This evidence is in accordance with the literature and can be explained by the ability of biosurfactants to disrupt the membrane structure of Gram-positive bacteria by disturbing interactions with phospholipids and membrane proteins [30]. The rhamnolipid biosurfactant observed no antifungal activity in the case of the two fungal strains, *Candida albicans* and *Cryptococcus neoformans* (Table 2).

**Table 2.** Percentages of growth inhibition obtained with the partially purified rhamnolipid at different concentrations.

Microorganism	Percentage growth inhibition at different Rhamnolipid (RL) concentration									
	RL (mg/mL)									
	10.0	5.0	2.5	1.25	0.62	0.31	0.15	0.070	0.03	0.01
<b>Bacterial strains</b>										
<i>Micrococcus flavus</i> (NCIM 2376)	98.52 ± 1.26	90.14 ± 1.39	84.24 ± 3.53	76.49 ± 2.44	68.31 ± 0.71	59.15 ± 1.13	50.32 ± 1.04	43.33 ± 1.32	37.71 ± 0.84	30.11 ± 1.19
<i>Staphylococcus aureus</i> (NCIM 5021)	90.50 ± 2.70	78.62 ± 3.12	62.44 ± 3.30	49.19 ± 3.63	40.17 ± 1.17	32.15 ± 1.54	27.15 ± 1.08	22.37 ± 1.19	20.15 ± 1.43	17.35 ± 1.03
<i>Bacillus subtilis</i> (NCIM 2063)	97.55 ± 1.58	88.57 ± 3.46	77.96 ± 2.31	71.84 ± 3.43	68.43 ± 0.32	61.92 ± 1.48	59.37 ± 1.51	41.11 ± 0.39	40.21 ± 0.84	39.14 ± 2.31
<i>Streptococcus faecalis</i> (NCIM 5024)	96.59 ± 2.32	85.91 ± 2.01	58.64 ± 2.57	45.45 ± 1.29	36.32 ± 2.45	30.27 ± 2.69	25.31 ± 31	20.11 ± 1.32	13.05 ± 2.01	10.13 ± 1.13
<i>Pseudomonas aeruginosa</i> (NCIM 2200)	99.16 ± 0.50	99.45 ± 1.14	96.20 ± 1.13	86.00 ± 1.70	80.43 ± 1.51	78.27 ± 2.57	75.31 ± 3.02	72.19 ± 1.15	68.32 ± 1.51	65.17 ± 1.48
<i>Serratia marcescens</i> (NCIM 5246)	100.00 ± 0.0	99.43 ± 1.27	95.63 ± 1.06	91.01 ± 2.53	86.51 ± 1.30	80.24 ± 1.51	75.33 ± 1.32	71.21 ± 1.51	70.81 ± 1.84	68.49 ± 1.55
<i>Escherichia coli</i> (NCIM 2931)	98.59 ± 0.49	98.23 ± 0.25	90.14 ± 1.49	80.63 ± 2.73	76.28 ± 2.44	70.14 ± 3.31	75.32 ± 1.91	71.16 ± 1.59	65.23 ± 3.21	60.31 ± 3.31
<i>Alcaligenes faecalis</i> (NCIM 2105)	99.13 ± 0.61	91.77 ± 1.22	84.42 ± 2.45	70.56 ± 2.75	62.15 ± 1.44	58.16 ± 1.32	45.13 ± 0.89	40.27 ± 1.51	36.61 ± 1.37	30.21 ± 1.21
<i>Salmonella typhimurium</i> (NCIM 2501)	98.34 ± 2.75	90.23 ± 1.17	65.89 ± 1.87	50.33 ± 0.94	45.17 ± 2.90	39.32 ± 1.97	31.75 ± 2.65	26.32 ± 2.62	21.11 ± 1.05	17.31 ± 3.21
<i>Proteus mirabilis</i> (NCIM 2241)	96.68 ± 3.31	90.74 ± 2.63	60.11 ± 1.75	50.29 ± 3.06	45.32 ± 1.44	40.11 ± 2.08	36.29 ± 2.31	31.15 ± 2.19	29.51 ± 1.82	26.41 ± 3.05
Streptomycin (Standard)	99.17 ± 0.13	99.32 ± 1.39	98.17 ± 1.61	97.57 ± 2.44	94.31 ± 1.18	91.14 ± 1.93	90.31 ± 0.87	87.43 ± 0.19	85.31 ± 1.94	83.31 ± 1.45
<b>Fungal Strains</b>										
<i>Candida albicans</i>	2.14 ± 0.56	1.17 ± 0.61	0.51 ± 0.3	0.41 ± .21	-	-	-	-	-	-
<i>Cryptococcus neoformans</i>	1.52 ± 0.48	0.97 ± 1.12	0.93 ± 0.51	0.59 ± 0.41	-	-	-	-	-	-

This can be explained on the basis of observation made by Rodrigues *et al.* [31], wherein they demonstrated that the di-RL congeners are responsible for the antifungal activity, whereas the mono-RLs exhibited only a weak inhibitory activity, and in our heterogeneous mixture of rhamnolipids, the ratio of mono-RL is more. It was also observed that the antifungal activity is lost during the recovery of the RLs from the culture medium and could be restored by adding NaCl, which also altered their aggregation behavior [31].

This variation in the growth inhibition among the pathogens examined may be related to the differences in cell membrane composition [32, 33]. Das *et al.* [34] also reported that the degree of hydrophobicity of the rhamnolipid biosurfactant plays an important role in exhibiting antibacterial action. This they explained by the change in the proportion of mono to di rhamnolipids in the order of 1:1, 1:2, and so on, which resulted in the enhancement of the polar nature of the compound with the corresponding decrease in its antibacterial action. Similarly, the change in proportion from di to mono rhamnolipid, on the other hand, increased the antibacterial zone diameter, especially of Gram-negative bacteria, and the increment accounted for this enhancement in the hydrophobic nature of the mixture, which supposedly favored its penetration through the lipids of the bacterial cell wall. This justification supports our observations of more than 90% growth inhibition for almost all Gram-negative bacteria with increasing concentrations of partially purified rhamnolipid from 1.25mg/mL to 5.0 mg/mL as the ratio of mono-rhamnolipid congener to dirhamnolipid congener is higher, being 2.1:1 as observed from the results of structural characterization using tandem-MS.

#### **4. Conclusions**

This present study demonstrates the broad-spectrum antibacterial activity of the partially purified heterogeneous mixture of rhamnolipids produced economically from sugar hydrolysates of cheap lignocellulosic rice straw residues. This will prove the efficacy of bioproduct in antibiotic formulations for utilization in pharmaceutical, cosmetic, and food industries. Further, the fractionization of the heterogeneous mixture into Mono-RL and Di-RL and their respective cellular actions will help understand the mechanisms involved and in engineering the cell as per the application required.

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## Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results".

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