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Disruption of *Yarrowia lipolytica* biofilms by rhamnolipid biosurfactant

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Abstract

Background: *Yarrowia lipolytica* is an ascomycetous dimorphic fungus that exhibits biofilm mode of growth. Earlier work has shown that biosurfactants such as rhamnolipids are efficient dispersants of bacterial biofilms. However, their effectiveness against fungal biofilms (particularly *Y. lipolytica*) has not been investigated. The aim of this study was to determine the effect of rhamnolipid on a biofilm forming strain of *Y. lipolytica*. Two chemical surfactants, cetyl-trimethyl ammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) were used as controls for comparison.

Results: The methylene blue dye exclusion assay indicated an increase in fungal cell permeability after rhamnolipid treatment. Microtiter plate assay showed that the surfactant coating decreased *Y. lipolytica* biofilm formation by 50%. Rhamnolipid treatment disrupted pre-formed biofilms in a more effective manner than the other two surfactants. Confocal laser scanning microscopic studies showed that biofilm formation onto glass surfaces was decreased by 67% after sub-minimum inhibitory concentration (sub-MIC) treatment with rhamnolipids. The disruption of biofilms after rhamnolipid treatment was significant ($P < 0.05$) when compared to SDS and CTAB.

Conclusion: The results indicate a potential application of the biological surfactant to disrupt *Y. lipolytica* biofilms.

Keywords: Biofilm, Biosurfactant, CTAB, Rhamnolipid, SDS, *Yarrowia lipolytica*

Background

Yarrowia lipolytica earlier referred to as *Endomycopsis lipolytica*, *Saccharomycopsis lipolytica* or *Candida lipolytica* is a hemiascomycetous fungus belonging to the Saccharomycetales order. It is often isolated from environments that are rich in hydrophobic substrates [1]. The organism inhabits soil [2], seawater [3] and refrigerated meat products [4]. The fungus is found in the oral cavity, pulmonary tract and intestines of healthy individuals. It is also an opportunistic pathogen that causes oral candidiasis, candidemia and catheter related infections [5]. From biomedical point of view, the eradication of this organism thus becomes important. The fungus forms biofilms on different surfaces in the presence of a variety of substrates [6]. It is well known that microorganisms in the biofilm mode of growth often resist a variety of antimicrobial agents. There is thus a need to explore alternative means of disrupting biofilms. A variety of chemicals including biocides and surfactants have

been used to control biofilms [7]. Chemical surfactants find applications in areas of medical care. For example, cetyl trimethyl ammonium bromide (CTAB) is used as a disinfectant in medical settings [8]. Sodium dodecyl sulfate (SDS) is effective by mediating leakage of cellular material from microorganisms [9].

Widespread use of chemical surfactants is discouraged due to their inherent toxicity. In this context, biosurfactants are being favored [10]. The latter group of surfactants offer several advantages in being relatively non-toxic, effective under different environmental conditions and in being biocompatible [11,12]. Biosurfactants have been used to disrupt bacterial biofilms [13,14]. However the reports on the efficacy of biosurfactants on fungal biofilms are limited [15]. We hypothesized that rhamnolipids may be effective against biofilms of *Y. lipolytica*. The yeast strain used in the current investigation forms biofilms on a variety of water-soluble and -insoluble substrates. The objective of this work was therefore to test the effectiveness of rhamnolipids in (i) preventing biofilm formation and (ii) in disrupting pre-established biofilms of *Y. lipolytica*. The results have been compared with two chemical surfactants.

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Results and discussion

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of surfactants

The chemical and biological surfactants displayed antifungal activity against the cells of *Y. lipolytica* NCIM 3589. Rhamnolipid and CTAB displayed a minimum inhibitory concentration (MIC) of $5\% \pm 0.1$ w/v and minimum fungicidal concentration (MFC) value $>10\% \pm 0.1$ w/v, while SDS showed MIC and MFC values of $0.62\% \pm 0.05$ w/v. SDS was more effective as an antifungal agent compared to rhamnolipids and CTAB. This anionic surfactant is known to possess detergent and antimicrobial properties [16]. The surfactant permeabilizes cells by targeting the cytoplasmic membranes and by affecting membrane-bound enzymes [16]. Rhamnolipids are anionic biosurfactants that disrupt cells by interacting with the phospholipid components of the biological membranes [17,18]. Rhamnolipids derived from *Pseudomonas aeruginosa* are known to possess antifungal activity against some plant pathogenic fungi such as *Cercospora kikuchii*, *Cladosporium cucumerinum*, *Colletotrichum orbiculare*, *Cylindrocarpon destructans*, *Magnaporthe grisea* and *Phytophthora capsici* [19]. The rhamnolipids inhibited spore germination and prevented hyphal growth in *P. capsici* at concentrations of $50 \mu\text{g ml}^{-1}$. A cationic surfactant, CTAB displayed lower antifungal activity towards *Y. lipolytica* as compared to SDS or rhamnolipids. In the present investigation, lower antifungal activity of CTAB could be a result of reversal of fungal cell surface charge and not due to cell lysis, as observed with SDS [9].

Increase in cell permeability after treatment with surfactants

Rhamnolipids display antimicrobial and surfactant properties [14]. They are known to increase cell permeability of *P. aeruginosa*, *Escherichia coli* and *Bacillus subtilis* [20]. In the present case, rhamnolipids were found to be less effective in increasing the cell permeability of *Y. lipolytica*, as

compared to SDS. SDS displayed higher permeability even at concentrations lower than MIC values (Figure 1, Table 1). In Figure 1, white arrows point towards non-permeabilized cells and the black arrows depict permeabilized cells. The increase in permeability observed after treatment with SDS may be due to the possible formation of molecular aggregates in the membrane and creation of trans-membrane pores [21]. CTAB was least effective in permeabilizing cells. This reduced permeability of the dye could be due to the robust nature of the fungal cell walls [22].

Effect of surfactant pre-coating on biofilm growth

Anti-adhesive activity of microbial surfactants has been reported earlier [11,14,23]. Pre-coating of microtiter plate wells with the surfactants effectively reduced the development of *Y. lipolytica* biofilms. Adhesion of *Y. lipolytica* cells to the microtiter plate wells was inhibited to 50% with rhamnolipids at MIC concentration (5%) (Figure 2, asterisk). Rhamnolipids showed significant anti-adhesive ability ($P < 0.05$) as compared to CTAB that inhibited 29% at MIC value of 5% (Figure 2, black arrow). With SDS at MIC (0.625%) the inhibition was less than 10% (Figure 2, black triangle). Rhamnolipids are known to decrease *Listeria monocytogenes* attachment when preconditioned onto Polytetrafluoroethylene (PTFE) surfaces [24]. We have also recently demonstrated the ability of rhamnolipids in disrupting *Bacillus pumilus* biofilms by removing exopolymeric substances [14]. SDS is known to display anti-adhesive ability by affecting the hydrophobic bonds that help in attachment of cells to the surfaces [25]. In the present study however, SDS showed lower anti-adhesive ability than rhamnolipids. Although CTAB is reported to bind to the negatively charged microbial surfaces, alter their surface charge and prevent the binding of the cells to the surfaces [26], this surfactant was not as effective in preventing *Y. lipolytica* adhesion. In the present study, the anti-adhesive effect of rhamnolipids was found to be

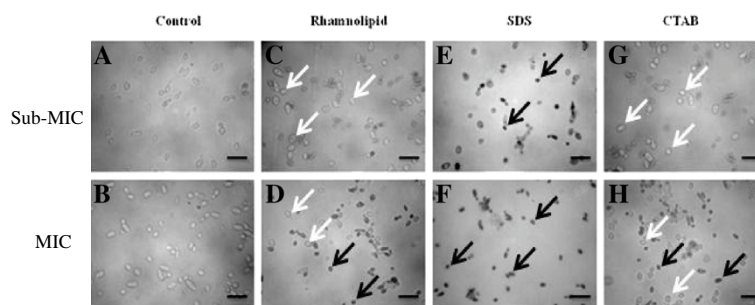


Figure 1 Morphological features of *Y. lipolytica* NCIM 3589 cells. Control cells (A and B); after treatment with rhamnolipid (C, D); SDS (E, F); and CTAB (G, H) at sub-MIC and MIC concentrations, respectively, for 1 h. The sub-MIC and MIC concentrations of 2.5% and 5% w/v respectively for rhamnolipid and CTAB and 0.3% and 0.62% w/v concentration of SDS were used. Bar indicates 10 μm . The upper panel indicates sub-MIC concentrations and lower panel shows MIC concentrations of the surfactants tested. White arrow shows non-permeability of methylene blue within the yeast cells whereas the black arrow depicts increase in cell permeability and uptake of the dye after treatment with surfactants.

Table 1 Methylene blue dye exclusion by *Y. lipolytica* cells post treatment with ½ MIC and MIC of surfactants for 1 h

| Surfactants | Cells stained at ½ MIC (%) | Cells stained at MIC (%) |
|-------------|----------------------------|--------------------------|
| Rhamnolipid | 20 ± 2 | 25 ± 2 |
| SDS | 70 ± 4 | 95 ± 2 |
| CTAB | 7 ± 2 | 14 ± 4 |

significantly higher as compared to SDS and CTAB suggesting the potential of rhamnolipids as anti-adhesive agents in the treatment of fungal biofilms.

Disruption of preformed biofilms of *Y. lipolytica*

The preformed biofilms of *Y. lipolytica* were treated with the surfactants for 1, 2 or 3 h. Biofilms of *Y. lipolytica* formed for 3 days in microtiter plate wells were disrupted effectively (55% with rhamnolipid, 35% with CTAB and 40% with SDS at respective MIC values) within 1 h of treatment with the surfactants (Figure 3a asterisk, arrow and triangle, respectively). At concentrations of surfactants lower than MIC, rhamnolipid displayed effective dispersion of biofilms (46%), followed by SDS (38%) and CTAB (25%). At higher concentrations (>2.5%), the effect of SDS was slightly better than that of the rhamnolipid. However, over a period of time, the efficacy of rhamnolipids was found to be similar to that of SDS (Figure 3b and 3c). CTAB was less effective in controlling biofilms, possibly due to its chemical interaction with fungal proteins present in the exopolymeric matrix [27]. There are also reports that show the efficacy of CTAB over SDS in controlling bacterial biofilms [27]. The present study showed that *Y. lipolytica* biofilms could be removed effectively by SDS compared to CTAB.

Biofilms of *Y. lipolytica* formed on glass slides for 3 days were disrupted by using sub-MIC concentrations of the surfactants. The concentrations of surfactants were selected on the basis of the microtiter plate results. Rhamnolipids effectively disrupted *Y. lipolytica* biofilms on glass surfaces upto 76% with SDS, 53% and with CTAB 38% disruption was observed. The disruption with rhamnolipids was found to be statistically significant ($P < 0.05$) as compared to SDS and CTAB. Rhamnolipids have earlier shown to be effective against bacterial biofilms [14], however there are limited reports on their effect on fungal biofilms. In the present study rhamnolipids were found to be effective in disrupting biofilms of *Y. lipolytica* compared to SDS and CTAB, suggesting their potential application as biofilm disrupting agents (Figure 4). The effectiveness of rhamnolipids against biofilms even at low concentrations makes them a good candidate for therapeutic applications.

Conclusions

Rhamnolipids have potential to disrupt *Y. lipolytica* biofilms as compared to chemical surfactants, SDS and CTAB. The results suggest potential of rhamnolipid bio-surfactants as anti-adhesive and preformed biofilm disrupting agents and their possible role in the treatment of fungal infections.

Methods

Culture and growth conditions

A biofilm forming strain of *Y. lipolytica* NCIM 3589 was used in the experiments [6]. Cells were grown in 50 ml YEPD broth (yeast extract 0.3%, peptone 0.5% and dextrose 1%) in 250 ml Erlenmeyer flasks at 30°C on shaker at 150 rpm for 24 h.

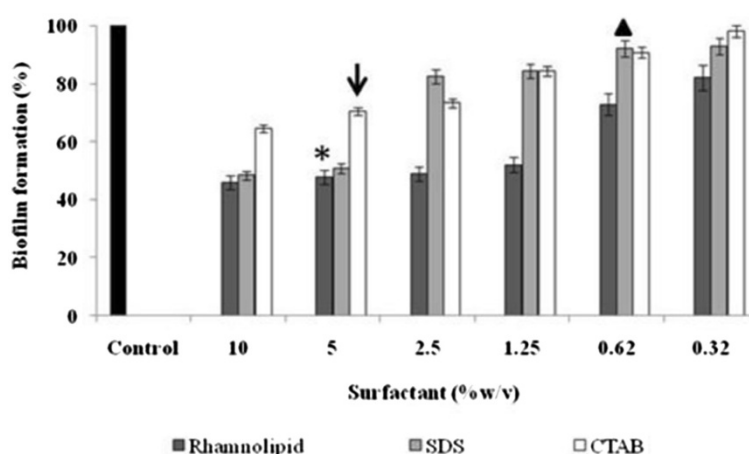


Figure 2 Inhibition of *Y. lipolytica* NCIM 3589 biofilms by surfactants. Observations in microtiter plate wells after pre-coating with different concentrations of rhamnolipid, SDS or CTAB. Control indicates biofilm formation by *Y. lipolytica* in microtiter plates untreated with the surfactants. The OD values are normalized with reference to control (considered as 100%). Where asterisk (*), triangle (▲) and arrow (↓) indicates MIC concentrations of rhamnolipid, SDS and CTAB respectively.

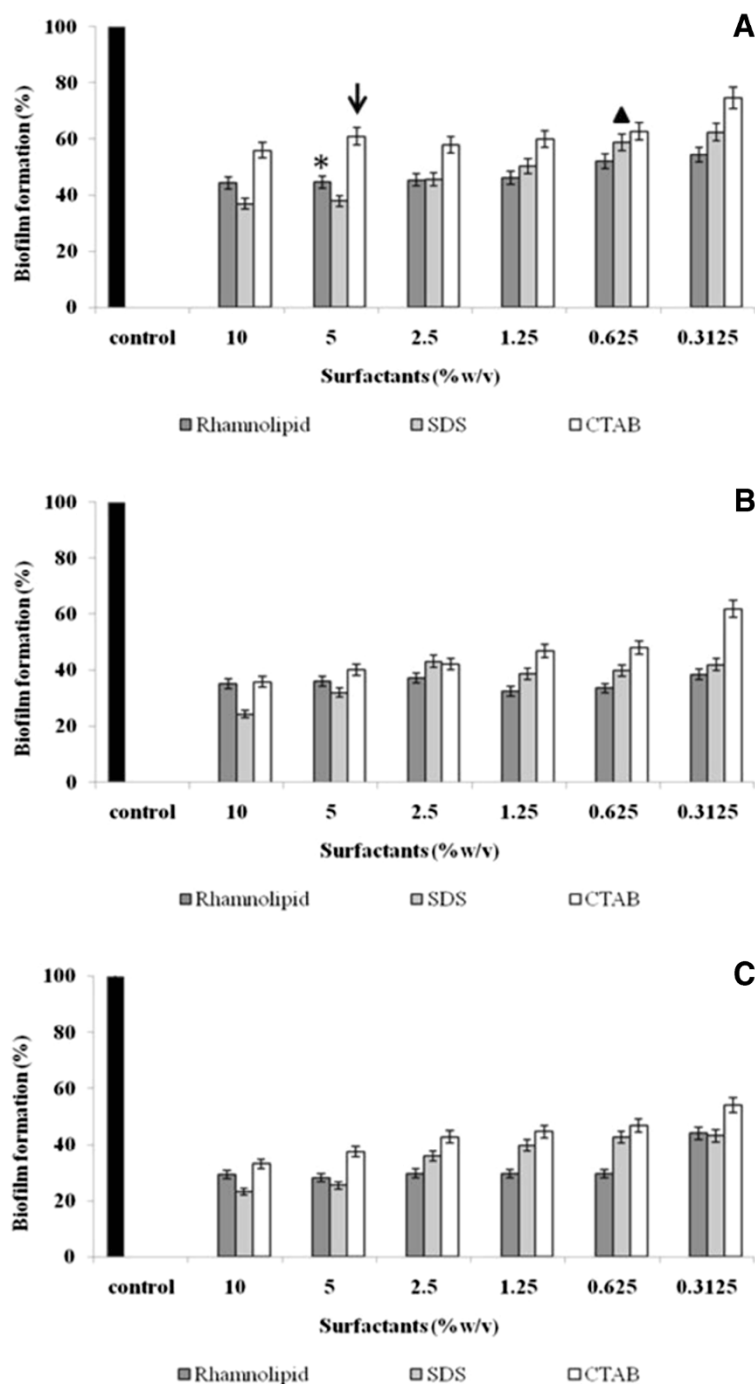


Figure 3 Effect of surfactants on preformed biofilms of *Y. lipolytica* NCIM 3589. Observations with rhamnolipid, SDS and CTAB after (A) 1 h (B) 2 h and (C) 3 h of incubation. Where, star (*), triangle (▲) and arrow (↓) indicates MIC concentrations of rhamnolipid, SDS and CTAB respectively.

Chemical agents

Rhamnolipid biosurfactant, a mixture of mono and di-rhamnolipids was used [14]. CTAB and SDS were obtained from HiMedia, India. Stock solutions of the surfactants were prepared in sterile distilled water; filter sterilized through 0.22 μ filters and used further for experimentation.

Determination of MIC and MFC values

MIC of surfactants against *Y. lipolytica* was determined by broth microdilution assay in sterile 96 well microtiter plates (Tarsons, India) [14]. Briefly, pre-grown (36 h) cells of *Y. lipolytica* were added to the microtiter plate wells containing YEPD medium to achieve the final cell

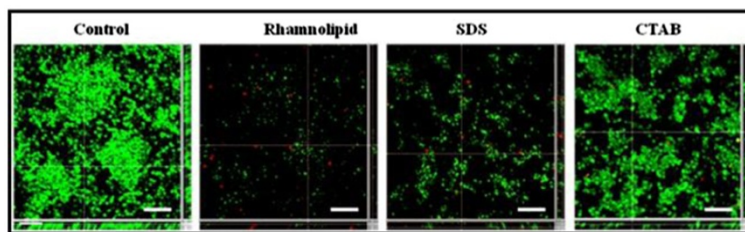


Figure 4 Representative CLSM images of *Y. lipolytica* NCIM 3589 biofilms. Observations of control cells and cells treated with sub-MIC concentration of rhamnolipid (2.5% w/v), SDS (0.3% w/v) and CTAB (2.5% w/v) for 1 h, showed live cells stained with SYTO9 and dead cells stained with PI. Bar indicates 30 μm .

numbers of 1×10^7 cells ml^{-1} . Surfactants (rhamnolipid, SDS and CTAB) were added to these wells at varying concentrations (0.005 to 10% w/v). The final volume in the microtiter plate wells was maintained to 200 μl . The plates were incubated at 30°C for 48 h and after the incubation period, growth in presence of the surfactants was estimated as OD₆₀₀ using a microtiter plate reader (Multiskan, Thermo Lab systems). Wells without surfactants and those lacking the cells were used as controls. The MFC was determined by streaking the culture grown in presence of different concentrations of the bio-surfactant onto YEPD agar plates. The plates were incubated for 48 h and growth was recorded. Minimum inhibitory concentration (MIC) was determined as the lowest concentration without visible growth and the minimum fungicidal concentration (MFC) as the lowest concentration showing no growth on the agar surface. The experiments were performed in triplicate and the mean values were obtained.

Fungal cell permeability analysis by methylene blue dye exclusion

In order to determine the effect of surfactants on yeast cell permeability, the method of Hammer *et al.* (2004) was followed with few modifications [28]. *Y. lipolytica* cells were grown in YEPD broth for 24 h. The cells were harvested, washed twice with sterile distilled water and resuspended in PBS to achieve 10^7 cells ml^{-1} . Aliquots were distributed equally in sterile flasks containing rhamnolipids, SDS or CTAB at MIC and sub-MIC concentrations. The flasks were incubated at 30°C under shaking conditions. After 1 h, 100 μl of these samples were withdrawn and to this 20 μl of 0.05% w/v methylene blue (prepared in sterile distilled water) was mixed and incubated for 1 min at room temperature. Methylene blue stained samples were placed onto the glass slides and the cells were examined microscopically by using an Axio Scope-A1 microscope with a photographic attachment (ProgRes[®] Capture Pro 2.7) at a magnification of 400 \times . A minimum of 500 cells in consecutive visual fields were examined and the percentage stained cells was calculated manually [28]. Cells untreated

with the surfactants were used as controls for the experiment.

Effect of surfactant pre-coating on biofilm formation

Surfactants (100 μl containing 0.3-10% w/v concentration) were added to wells of the polystyrene microtiter plates and incubated for 12 h at 4°C to facilitate effective coating [23]. After the incubation period, the wells were emptied of residual surfactants, rinsed with sterile distilled water and air dried in a laminar air flow for 5 min. Cells (100 μl containing $\sim 10^7$ cells ml^{-1}) of *Y. lipolytica* were added to the microtiter plate wells and incubated for 24 h at 30°C. After the incubation period, the microtiter plate wells were emptied of the non-adherent cells and the plates were rinsed with sterile distilled water. The adherent cells were quantified by using the crystal violet assay [14]. All experiments were carried out in triplicates with two biological replicates and average values indicating standard deviation are presented here.

Disruption of preformed biofilms

Y. lipolytica biofilms were allowed to form in sterile polystyrene 96 well microtiter plate wells for 3 days [6]. After the incubation period, planktonic cells were removed and varying concentrations (0.3-10% w/v) of rhamnolipid, SDS and CTAB were individually added to the wells. The plates were further incubated at 30°C for 1, 2 or 3 h. After each time interval, the microtiter plate wells were emptied of the non-adherent cells and rinsed with sterile distilled water. The residual biofilms were quantified by using the standardized crystal violet assay [14]. All experiments were performed in triplicates with two biological replicates and the data is presented as average values indicating standard deviation.

Confocal laser scanning microscopy (CLSM)

Y. lipolytica biofilms were formed on sterile microscopic glass slides as described earlier [29]. Cells were inoculated in sterile petriplates containing the growth medium (YEPD) to reach a cell density of 1×10^7 cells ml^{-1} . Sterile microscopic glass slides were placed in the petriplates

and incubated on a rocker at 30°C for 3 days. After the growth period, glass slides were removed and placed in another petri dish containing growth medium supplemented with 0.3% w/v concentration of rhamnolipids, SDS or CTAB. Biofilms were treated for 1 h in presence of the surfactants. Untreated biofilms were used as controls and the biofilm coverage thus obtained was considered to be 100%. The slides were removed, rinsed twice with sterile distilled water and stained with LIVE/DEAD BacLight staining kit containing SYTO9 and Propidium Iodide (PI) (Molecular Probes, Eugene, Oregon, US) as per the manufacturer's instructions. The slides were observed under a CLSM (SP2 AOBS, Leica Microsystems, Germany). A 63× 1.2 NA water immersion objective was used with 488 nm Ar laser excitation and 500–640 nm band pass emission setting. Multiple (20) images were scanned and analyzed using the image processing software; *ImageJ* (<http://rsb.info.nih.gov/ij>). The observations were made in triplicates and representative images are presented here.

Statistical analysis

The effect of rhamnolipid on biofilms was analyzed statistically by the Students *t*-test and treatments were considered significantly different if $P \leq 0.05$.

Abbreviations

CTAB: Cetyl-trimethyl ammonium bromide; SDS: Sodium-dodecyl-sulphate; MIC: Minimum inhibitory concentration; MFC: Minimum fungicidal concentration; PTFE: Polytetrafluoroethylene; NCIM: National collection of industrial microorganisms; YEPD: Yeast extract peptone dextrose; OD: Optical density; CLSM: Confocal laser scanning microscopy.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

DHD and SD performed the experimental work and acquired the data. YVN performed the CSLM analysis. VPV and SSZ made contributions to conception and design. ARK analyzed and interpreted the data. DHD, SD, VPV and SSZ have been involved in drafting the manuscript and revising it critically for important intellectual content. All authors have given final approval for the version to be published.

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