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Comparative Assessment of Bio-surfactants for Potential Application in Antifungal Edible Coating

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ABSTRACT

Background: The use of edible coatings has achieved increasing attention as an eco-friendly and health-conscious method for enhancing the shelf life and quality of food products. Biosurfactants are amphiphilic compounds produced by microorganisms. These compounds possess both hydrophilic and hydrophobic moieties, allowing them to interact with both polar and nonpolar substances.

Methods: This study investigated the antifungal efficacy of rhamnolipid, sophorolipid and surfactin coatings with synergistic potential Aloe vera gel against *Botrydiplodia theobromae*. In present study, petri plates were supplemented with different biosurfactants at varying concentrations with Aloe vera gel used to control the *B. theobromae*.

Result: The solutions of rhamnolipid, sophorolipid and surfactin (50 mg/L), Aloe vera gel (25%) and synergistic effect of rhamnolipid, sophorolipid and surfactin (10 mg/L) with aloe vera gel (25%) were evaluated for their biocontrol activity on *B. theobromae* and found that rhamnolipid alone showed comparatively low antifungal activity while rhamnolipid with aloe vera showed high antifungal activity with lower rhamnolipid concentration. The results indicated that a combination of rhamnolipid and aloe vera gel exhibited the highest antifungal activity against *B. theobromae* without significant phytotoxicity or cytotoxicity. It was observed that the biosurfactants cause rupture of mycelia as its evident from the microscopy. These findings suggest that rhamnolipid-based edible coatings could serve as a promising alternative to chemical fungicides for the management of post-harvest losses.

Key words: Aloe vera, Biosurfactants, Rhamnolipid, Sodium surfactin, Sophorolipid.

INTRODUCTION

Biosurfactants are surface-active compounds produced by microorganisms such as bacteria, fungi and yeast and are amphiphilic by nature (Jimoh *et al.,* 2019). These biosurfactants can take on various structures, including glycolipids, mycolic acids, polysaccharide-lipid complexes, lipoproteins/lipopeptides and phospholipids (Xi *et al.,* 2021; Fardami *et al.*, 2022). The type of biosurfactant produced depends on the molecular composition of the microorganism. For example, *Pseudomonas* spp. produce rhamnolipids, *Torulopsis* spp. produce sophorolipids and *Bacillus* spp. also produce surfactin (Roy *et al.*, 2017). While chemically synthesized surfactants are widely available, they have several drawbacks compared to biosurfactants, such as higher environmental toxicity, lower biodegradability, carcinogenicity and adverse effects on soil quality (Markande *et al.,* 2021; Demirbilek *et al.,* 2022; Alkooranee *et al.,* 2020). *Botryodiplodia theobromae* is a widespread fungal pathogen responsible for considerable post-harvest losses in banana fruit. This cosmopolitan species can infect bananas at different stages of growth. The disease can spread quickly, particularly under favorable conditions like high humidity and temperature (Renganathan *et al.,* 2018 and Kumah *et al.,* 2020). Many researchers (Sarwar *et al.,* 2018, Goswami *et al.,* 2019 and Kang *et al.,* 2017) proved that biosurfactants was found to retain surface-active properties under the extreme conditions and highly potent antifungal agents (Kahraman *et al.*, 2012; Roy *et al.*, 2017. De Santana-Filho *et al.,* 2015 and de Freitas *et al.*, 2019). Microbial surfactants have been observed to control plant pathogens, demonstrating their broad-spectrum activity and potential applications in sustainable agriculture (Jiang *et al.,* 2016, Zhang *et al.,* 2020 and Krishnan *et al.,* 2019). The potential of the biosurfactants depends on their molecular composition, chain length and type of producing microorganisms (Cameotra and Makkar, 2004). This present research explores their promising antifungal activity against *Botryodiplodia theobromae*. This study

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focuses on the different types of biosurfactants and their application.

MATERIALS AND METHODS

The present study was conducted at Amity University Rajasthan and J.C. Bose university of Science and Technology, YMCA in year 2022-2024. The fungal pathogen *Botryodiplodia theobromae* strain ITCC 7162, was originally isolated from papaya fruit in 2013 at Navsari Agricultural University, Gujarat, India and the same was procured from the Indian Type Culture Collection (ITCC), Delhi, for evaluating the antifungal efficacy of biosurfactants based coating formulation. The fungal strain was cultivated and maintained on potato dextrose agar (PDA) media in accordance with ITCC guidelines. Rhamnolipid (RL), Sophorolipids (SL) and Surfactin (SF) were previously produced, while fresh aloe vera gel was extracted for use in the present study.

Antifungal potential of different biosurfactants

The method used to assess the effect of biosurfactants was adopted from Xi *et al.* (2021) and Baccile *et al.* (2020). The stock solutions of RL, SL and solutions were prepared in physiological saline (pH 7.0) at a concentration of 50 mg/L (w/v). The stock solution was sterilized using membrane filtration (pore size 0.22 µm, Pall Corporation, USA). For the antifungal assay, the prepared solutions were added to PDA media at the desired concentrations and the plates were allowed to solidify. Control plates without biosurfactants were also prepared. A fungal mycelial plug was obtained using a sterilized stainless steel cork borer (8 mm) and transferred to the media surface. After 48 hrs of incubation at 25°C, the radius of mycelial growth was measured using a digital micrometre (Aerospace, India) in both treated and control plates. The antifungal potential was calculated as the percentage reduction in radial mycelial growth.

$$\% \text{ of Inhibition} = \frac{C - T}{C} \times 100$$

Where,
C = Control.
T = Treatment.

Antifungal potential of the biosurfactants based food coating solution

To observe synergistic effect aloe vera (AL) gel (25% w/v) was mixed with RL, SL and SF solutions. For the antifungal assay, the prepared solutions were added to PDA media at the desired concentrations and the plates were allowed to solidify. The same process was repeated as mentioned for the antifungal potential assessment in previous section and the antifungal potential was calculated based on the percentage reduction in mycelial growth compared to control plates. The assay was also conducted to evaluate the synergistic potential of different biosurfactants in combination with AL gel. PDA plates were prepared containing AL gel combined with different biosurfactants, in addition to the control plates.

Mechanism of action of biosurfactants

Microscopy

To investigate the effects of biosurfactants on fungal mycelia, fungal samples were incubated in tubes containing biosurfactants suspension. The treatment suspensions were incubated for 15 minutes at ambient temperature. The fungal specimens were stained with lacto-phenol cotton blue for better visualization. The damage to the mycelia was then observed under a microscope at 40 X and 100 X magnifications with immersion oil (Shamly *et al.,* 2014).

Scanning electron microscopy (SEM) analysis

For higher resolution imaging with SEM, samples were fixed by immersion in 5% (v/v) glutaraldehyde for 24 hrs at 4°C and post-fixed with 1% (w/v) osmium tetroxide for 2 hrs at 4°C. The fixed samples were dehydrated using a graded acetone series and then coated with gold before being examined with a Scanning Electron Microscope (TM-1000, Hitachi, Tokyo) (Mohammed *et al.,* 2018).

Edible coating trial on banana Surface

The optimized biosurfactants concentration was applied using atomized spraying on banana fruit and the fungal pathogen (*Botryodiplodia theobromae*) was spiked on the surface. Further, banana samples were incubated at 50± 10% relative humidity in plant growth chambers at 37°C (Sharma *et al*., 2018). After incubation on day 4, the fruits were cut on the place of spiked fungal pathogens and were observed for the symptoms of the spoilage caused by the fungal pathogen.

Phytotoxicity assay

This study aimed to evaluate the phytotoxicity of biosurfactants on two different plant seeds namely, *Brassica nigra* (black mustard) and *Triticum aestivum* (wheat). The biosurfactants stock was used for the treatment of the seeds to assess the phytotoxicity. Solutions were prepared in PBS (pH 7.0) and were sterilized using membrane filtration. Seeds of *B. nigra* and *T. aestivum* were surface-sterilized with sodium hypochlorite (2% solution) and rinsed thoroughly with sterile distilled water. The sterile petri plate was cushioned with the cellulose filter papers and 25 seeds of each plant were placed in Petri dishes containing 10 mL of biosurfactants stock and a control (sterile distilled water). The petri plates were incubated in the dark at 27°C for 10 days (Sharma *et al*., 2014).

Cytotoxicity assessment

The CaCo-2 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with, *e.g.,* fetal bovine serum, antibiotics at 37°C in a humidified atmosphere containing 5% CO_2. The biosurfactants stock was prepared using in DMSO and stored for further analysis. Cells were seeded into 96-well culture plates at a density of 1×10^4 cells/well and allowed to adhere

overnight. The biosurfactants solutions were added to the wells and DMSO served as a negative control and sodium dodecyl sulfate (SDS) were used as positive controls. Absorbance was measured at 570 nm using a microplate spectrophotometer.

RESULTS AND DISCUSSION

Antifungal assay of biosurfactants containing edible coating

In my experiment, a notable difference in growth was observed between media plates with biosurfactants and the control plates without biosurfactants. It was clearly evident that there was difference in growth measurement in all the treated cases, while there was no effect on fungal growth in non-biosurfactants augmented plates. After 72 hrs of incubation, plates containing RL showed only 21.99%±0.02, SF containing plates displayed 25.3%±0.44, while SL augmented plates showed an inhibition of 28%±0.26 in comparison to the growth equated on control plates.

But there was a synergistic increase in biosurfactants antifungal potential while mixing of aloe vera gel with previously tested concentration of biosurfactants After 72 hrs, control plates showed normal growth of *Botryodiplodia theobromae*. RL+AV exhibited the inhibition of 58.8%±0.14, SS+AV demonstrated the inhibition of 29.4%±0.2 while SL displayed an inhibition of 29.5%±0.15. It indicates that the amalgamation of biosurfactants and aloe vera gel were observed for synergistic behaviour in terms of their antifungal potential.

Aloe vera, as edible coating constituents, was earlier reported as effective agent in reducing the transpiration and softening in fruits during storage possibly by lowering weight loss and maintaining fruit firmness (Valverde *et al*., 2005; Martínez-Romero *et al*., 2006). Escamilla-García *et al*. (2018) previously demonstrated that starch-based coatings containing nisin and lactic acid ethyl ester (LAE) effectively inhibit both bacteria and fungi. Coated papayas exhibited a significant decrease in yeast population (3 Log_{10} CFU g/l) compared to uncoated papayas. The population was increased in uncoated papayas to 4.7 Log_{10} CFU g/l indicated complete spoilage.

Similarly, Adetunji *et al*. (2019) evaluated the bio control potential of an edible coating containing 2% rhamnolipid and 2% Aloe vera gel against *Penicillium digitatum* NSP01. The rhamnolipid and Aloe vera based edible coating could serve as an alternative substitute for chemical antifungal agents used in the prevention of post-harvest losses agricultural productivity.

Various strains of the *Bacillus* are known for producing lipopeptides based biosurfactants such as surfactin which have significant antifungal potential. *B. subtilis* inhibited the mycelial growth of *Colletotrichum capsici* by 61.5% after 96 hours of incubation compared to the control (Kumar *et al*., 2021). In another attempt, Solanki *et al*. (2022), evaluated the potential of biosurfactants as a coating substance for the post-harvest storage of Jamun fruit. Convincingly, bio-surfactant can be utilised as a capable edible coating agent, which result in delay metabolic activities, preserves firmness and quality of fruit and so increases the shelf life of fruit during storage.

In present study, it was observed that there was a considerable difference and degree of antifungal potential of the RL, SL and SF. This is possibly due to the compositional difference and interactions of various food matrixes with RL, SL and SF. It was clear from the Table 1 and established that the rhamnolipid has shown the maximal synergistic antifungal potential with aloe vera as compared to the SL and SF. So, it was decided to continue rest of the other analysis with rhamnolipids only which was based on the significant antifungal activity against *Botryodiplodia theobromae*.

Table 1: Antifungal potential of rhamnolipid, surfactin and sophorolipid.

Fungal pathogen	Rhamnolipid (50 mg/L)	Surfactin (50 mg/L)	Sophorolipids (50 mg/L)	Rhamnolipids + AL	Surfactin + AL	Sophorolipids + AL
Botrydiplodia *theobromea*	21.19±0.20	25.33±0.445	28±0.26	58.8±0.14	29.4±0.20	29.5±0.15

AL - Aloe vera.

Table 2: Phytotoxicity of RL, SF and SL against *Brassica nigra* and *Triticum aestivium* plant seeds.

Samples	Seed germination	Germination index	Vigor index
Rhamnolipids (*B. nigra*)	100±0.25	106±0.4	1260±0.32
Surfactin (*B. nigra*)	100±0.37	104±0.32	1279±0.53
Sophorolipids (*B. nigra*)	100±0.23	103±0.27	1301±0.4
SDS (*B. nigra*)	23±0.4	23±0.47	200±0.46
Rhamnolipids (*T. aestivium)*	100±0.36	108±0.67	1305±0.19
Surfactin (*T. aestivium)*	100±0.43	104±0.32	1201±0.43
Sophorolipids (*T. aestivium)*	100±0.13	106±0.21	1312±0.65
SDS (*T. aestivium)*	20±0.56	20±0.54	210±0.51

Phytotoxicity assay

The seed germination in RL, SL and SF was 100% all the seeds were found germinating during the test while in SDS the seed germination was only limited to the 20% ±0.41 (Table 2).

RL showed the vigor index was 1260±0.32, in SF vigor index was 1279±0.53, while SF the vigor index was 1300±0.4 and in SDS controls the vigor index was 200 ±0.46. Based on the provided seed germination, germination index and vigor index, it's evident that all the biosurfactants tested significantly outperform SDS in promoting seed vigor. The findings are made acquainted with a mean value and standard deviation.

In RL, SL and SF, the seed germination of *Brassica nigra* was 100%, 100% and 100% was respectively, while in case of SDS the seed germination was 20%±0.56 (Table 2). So, it's clear that RL, SF and SL significantly outperform SDS in promoting seed germination. The germination index in RL, SF and SL 108±0.67, 104±0.32 and 106±0.21 while in SDS the germination index was 20±0.54. Based on the provided germination indices, it's clear that RL, SF and SL significantly outperform SDS in promoting seed germination. Rhamnolipids shows the vigor index was 1305±0.19. In Surfactin vigor index was 1201±0.43, while Sophorolipids the vigor index was 1312 ±0.65 and in SDS the Vigor index was 210±0.5. Based on the provided vigor indexes, it's clear that RL, SF and SL significantly outperform SDS in promoting seed Vigor.

The results show that RL, SF and SL sustained high seed germination rates, germination indexes and Vigor indexes, whereas SDS resulted in significantly lower values for all parameters.

De Almeida *et al.* (2019), observed the germination (GI), a combined measure of seed germination and root elongation, was used to assess the toxicity of the vegetable biosurfactant on cabbage. A GI value of 80% has been established as a threshold for indicating the absence of phytotoxicity. The results demonstrated that the tested biosurfactant solutions did not adversely affect cabbage seed germination or root elongation. The GI values for biosurfactant solutions containing 1.0, 0.05 and 0.025 g/L were all 100%, indicating no inhibitory effects.

Various authors exploring the effects of biosurfactants on phytotoxicity, particularly concerning seed germination and plant growth (Sharma *et al.,* 2014)

Cytotoxicity assessment on human gut epithelial cell

In assessment of the cytotoxicity of the biosurfactants, it was observed that the RL, SF and SL showed 93.9%± 0.18, 94%±0.21 and 89%±0.23 viability, respectively. At the same time SDS solution has only 39%±0.23, viability on incubation, while in distilled water viability was considered the 100% viability to compare the viability in all the treatments (Fig 1). These results indicated that treatment with RL, SF and SL resulted in slight reductions in cell viability as compared to the distilled water, but its way better in comparison with the chemical surfactant (SDS) which showed drastic decline in the viability among the tested compounds.

Panchariya (2021), analysed biosurfactants concentrations of 5 µg/mL, 25 µg/mL and 50 µg/mL had no significant impact on cell viability, even showing a slight increase. Concentrations of 100 and 200 µg/mL led to a dramatic decrease in cell survival, suggesting a toxic effect on higher concentrations.

So, viability of more than 90% and above can be considered non-cytotoxic as reported earlier by various reports. It was observed earlier that the cytotoxicity scale appears to be a 5-point scale, directly correlated with cell viability. Where 0= Represents 90% cell viability (lowest cytotoxicity) and 4= Represents <10% cell viability (highest cytotoxicity) (Rodríguez *et al.,* 2020).

Microscopic observation

The mycelia of treated *Botrydiplodia theobromae* with the rhamnolipid and aloe vera gel coating formulation exhibited significant damage. In contrast, the untreated mycelia showed no visible changes in hyphal morphology. However, the treated mycelia displayed extensive cytoplasmic leakage and their morphology was severely disrupted (Fig 2).

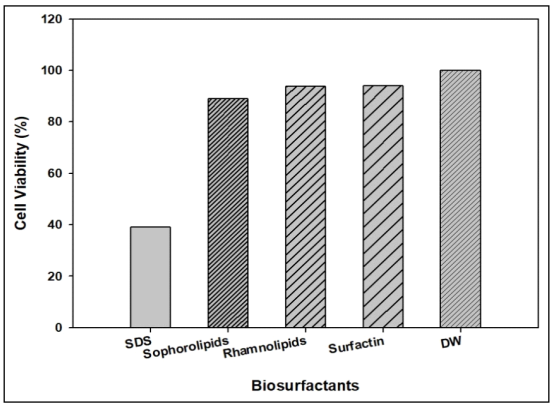
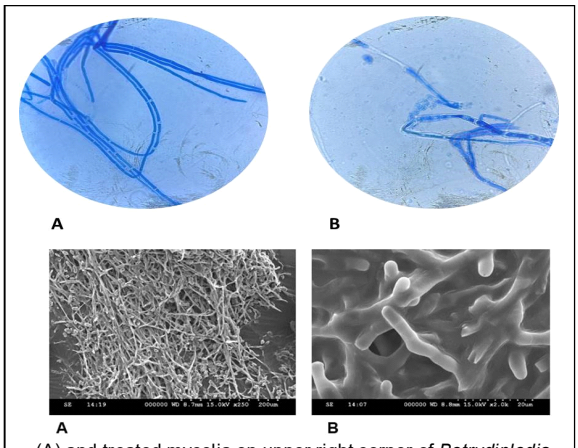


Fig 1: Cytotoxicity of the biosurfactants to CaCo-2 cell lines.



(A) and treated mycelia on upper right corner of *Botrydiplodia theobromae*. SEM analysis of control on lower left (A) and treated mycelia on lower right corner (B) of *Botrydiplodia theobromae.*

Fig 2: Microscopic images of control upper left corner.

The SEM analysis provided valuable insights into the morphological characteristics of *Botrydiplodia theobromae*. The detailed visualization of conidial structures, including surface striations and septa, enhances our understanding of this pathogen's identification and biology. These findings are crucial for developing targeted control measures and advancing our knowledge of fungal pathogenicity.

Rhamnolipids are recognized for their ability to effectively damage fungal mycelia, as confirmed by scanning electron microscopy analysis (Sen *et al*., 2016). The analysis revealed significant structural damage to the mycelia in treated samples compared to the control group.

In control fungal mycelia were intact while in treated the mycelia were ruptured and cell lysis can be observed. The surface of *B. theobromae* conidia displayed a rough texture with ridges and grooves. These structural details are critical for the identification of the fungus and differentiate it from other closely related species. The surface roughness and structural complexity observed in *B. theobromae* conidia may play a role in its adherence to host surfaces and resistance to environmental stresses.

Edible coating trial on banana

The biosurfactants coated banana showed less infected with the challenged *B. theobromae* pathogen (Fig 3). The mycelia of the *B. theobromae* were transferred by pricking the coated and non-coated surfaces using sterile tooth picks. After 6 days of incubation at ambient temperature and 50-60% relative humidity, the transverse plane cut were made at the point of fungal pricked surfaces. The non-coated banana showed the development of severe symptoms of the spoilage along the line of inoculation, whereas, in case of the RL coated banana showed negligible onset of the spoilage line.

At the same time, the liquefaction of the starch can also be observed in non-coated banana as compared to the RL coated banana. The degree of the starch hydrolysis is significantly low in case of the coated banana. The low starch liquefaction in case of coated banana also results in low acidity and better quality of the fruit. Applying coatings can slow down the ripening process, thereby reducing the rate of starch conversion. This helps maintain a firmer texture and a desirable taste for a longer period. Lower starch liquefaction typically leads to lower production of organic acids. As starches convert to sugars during ripening, acids can also be produced, impacting the fruit's acidity. By slowing down the ripening process, coatings can help preserve nutritional content, including vitamins and antioxidants (Bae and Lee, 2017).

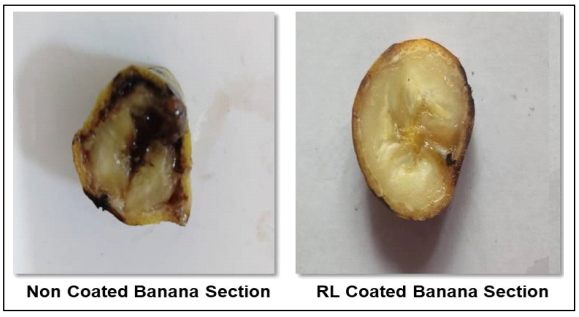


Fig 3: Effect of biosurfactant coating during challenged tests.

CONCLUSION

The antifungal activity of rhamnolipid, sophorolipid and surfactin alone and with aloe vera were evaluated against *B. theobromae*. Among them, the strongest antifungal effect was observed with rhamnolipid and rhamnolipid with aloe vera, rhamnolipid with aloe vera gave most satisfactory with lower concentration. The phytotoxicity test against *Brassica nigra* and *Triticum aestivium* results demonstrated that the seed germination, germination index and vigour index was high with rhamnolipid in comparison with SDS. Rhamnolipid gave antifungal effect on *B. theobromae* by generating lethal damage on its mycelial cells. This result turns rhamnolipid into a potential alternative for the biological control of *B. theobromae* under field conditions. Rhamnolipids can be used as edible coating for fruits on the basis of toxicity assessment with potential to minimize the post-harvest losses.

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Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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