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Inhibitory effect of biosurfactant purified from probiotic yeast against biofilm producers

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Abstract:*Extracellular surface active agents are produced by microorganisms by reutilizing the accumulated waste or by the assimilation of low cost carbon sources or other renewable resources. Isolation, identification, and screening of probiotic yeast Saccharomyces cerevisiae to check for its ability to produce microbial surfactants was the primary angle in the present work. The second angle of the work was to study the inhibitory action of the purified biosurfactant against Candida and Bacillus biofilms or aggregates of microorganism that can adhere to inanimate objects, resulting in the spread of many infections.*
Keywords: *Microbial surfactants, Anti-adhesion, Saccharomyces cerevisiae, Biofilm producers*

I. Introduction

Microbial surfactants possess the potential to reduce the number of biofilm producers, and its anti-adhesive activity becomes a promising strategy for protection against the harmful effects of every biofilm communities (1). They are promising compounds often showing antimicrobial and anti adhesive properties, sometimes penetrating and removing mature biofilms (2). Adsorption of these surface active agents to a surface e.g. glass, polystyrene, silicone modifies its hydrophobicity, interfering with the microbial adhesion and desorption processes. Despite the advantages of microbial surfactants, the industrial use is limited due to the high cost production. Many research studies emphasize on the usage of renewable sources as substrates for cost reduction in its production. Many studies have been carried out using low-cost feedstock or agricultural byproducts as substrates for microbial surfactant production. Low-cost carbon sources, used include sludge palm oil, cassava waste water, dairy waste, vegetable oil refinery waste and more. Waste disposal is a growing problem which depicts the increasing interest in waste valorization through microbial transformation. Reutilization of these accumulated waste decreases the cost of carbon source for microbial surfactant production, thus reducing the pollution caused by waste disposal in landfills. Factors that influence microbial surfactant production are nitrogen and carbon sources, pH, temperature, agitation, and aeration. The optimization of the production process is a key factor to improve the yield and reduce production costs (3). Adhesion is the first stage of biofilm formation and the best moment for the action of inhibitory and anti-biofilm compounds Biofilm is an aggregate of microorganisms in which cells adhere to each other on the surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPS). Its formation on the surface of inanimate objects used in hospitals, food processing industries, and other sectors, bring in infections that can harm millions of humans, biofouling, and microbial-induced corrosion. Prolonged administration of antimicrobial agents to patients is undesirable because of the danger of inducing resistance (4). Microbial surfactants are surface active agents derived from biological sources, usually extracellular, produced by bacteria, fungi, or yeast. Research studies on microbial surfactants have taken a significant production in the industry due to its certain properties when compared to synthetic compounds. Its properties include biodegradability, low toxicity, diversity of applications, and functionality under extreme conditions. Majority of these microbial surfactant producers are pathogenic in nature that restricts the wide application of these compounds in many varied industries (5). The study of microbial surfactant production by yeast has been growing in importance due to its non-toxic or non-pathogenic status, thus allowing its magic in many food, healthcare, and pharmaceutical sectors (6). The present study aims in the production of microbial surfactant from probiotic yeast species with the usage of renewable carbon sources. It also throws light on the inhibitory effect of produced microbial surfactant on biofilm producing microorganisms.

II. Materials And Methods

1.1 Collection and processing of samples for the study

Dairy wastewater sample used in this study was obtained from a local dairy farm (Tamil Nadu, India) and stored at 4^0C until analysis. All other chemicals used in the present study were of highest purity grade, produced by Sigma-Aldrich (USA). The collected sample was inoculated into Yeast Extract Peptone Dextrose medium and incubated at 30^0C for 48 hours and later maintained for more than three days at room temperature. After incubation, yeast like cells were subcultured and maintained in YEPD. The isolates were then cultured in MSM medium containing 4% glycerol.

1.2 **Isolation and Identification of yeast strains**

Isolated strains were identified based on the microscopic and biochemical techniques. The pure culture obtained was maintained on YEPD medium at 4°C used for the study.

1.3 **Screening for the production of microbial biosurfactant**

Isolates were grown in 500 ml Erlenmeyer flasks, each containing 100 ml medium of wastewater, and mineral salt medium supplemented with industrial waste as sole carbon source, and incubated at 37^0C. Inoculums of screened yeast isolate was poured in the MSM medium with pH 7.2 and incubated at 250 C on shaker at 160 rpm. Biomass was determined in triplicate by centrifuging of 10 ml samples (culture liquid) at 5500 rpm for 25 minutes at room temperature. The cell pellets was washed with distilled water, dried at 105°C and then weighed. The supernatant was used for the estimation of surface or interfacial tension and biosurfactant concentration. Biomass was determined directly by taking the optical density of culture liquid at 545ŋm at UV-Vis spectrometer (7).

1.4 **Estimation of microbial biosurfactant activity**

The estimation of biosurfactant activity is done by oil spread technique (8). The biosurfactant property was also confirmed by emulsification activity (modified from Cooper and Goldenberg). Emulsifying activity was determined using a modified turbidometric method (9) and expressed as turbidity at 620 nm. The surfactant (1 mg) was introduced into a 50 ml flask with distilled water to make 10 ml; 0.1 ml of each hydrophobic liquid was added. The mixtures were incubated at 30°C with reciprocal shaking for 1h and allowed to stand for 10 min. The lower phase was taken and its turbidity measured at 610 nm. Soybean oil and crude oil were used as the hydrophobic liquids. A non-surfactant reaction was used as the control. Emulsification index (E24) was calculated by taking equal volume of culture broth (1ml) and n-paraffin (1ml) and allowed to stand for 24 hours. After 24 hours, emulsified layer was measured in centimeters (10).

E24 was calculated using the formulae:

E24 = Height of the emulsified layer (cms) / Total Height of the liquid column (cm) X 100

1.5 **Purification of biosurfactant**

The supernatant was acidified by using 2N HCL solution to pH 2 and incubated at 0^oC, overnight. It was extracted by adding equal volume of acetone to the supernatant and incubated at 40^oC, overnight. Weights of biosurfactants obtained were quantified by subtracting weight of emptied tubes after centrifugation (post-weight) by pre-weight (weight of empty tube before centrifugation).

1.6 **Inhibitory activity of biosurfactants against biofilm producers**

The action of various biosurfactants, as well as chemicals: CTAB (cetyl trimethyl ammonium bromide) and SDS (Sodium dodecyl sulphate) were analyzed against *Candida albicans* and *Bacillus subtilis*. The assay was performed in microtitre plates. Both organisms were pre-loaded in two different plates in an increasing order of concentration (1µg/10µl to 10µg/10µl) in wells 1-10 respectively. Wells 11 and 12 were taken as control and added 100µl of distilled water. Later equal volumes of chemicals and different biosurfactants were added separately in all wells of a row. Kept for incubation at room temperature for three days and measured OD at 600nm using an ELISA reader. The microbial inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as: % reduction in adherence = [(A control) - (A sample)] / A control × 100

III. Results And Discussion

The present study was carried out to produce microbial surfactants from probiotic yeast isolated from dairy effluents and to study the inhibitory effect of microbial surfactants against biofilm producers. Biofilms are populations of microorganisms that accumulate at interfaces and are typically surrounded by a matrix of extracellular polymeric substances (EPS). Attachment of microorganisms to surfaces and the development of microbial biofilms at phase boundaries are frequently encountered in natural, technical and medical environments. They are generally formed by bacterial species such as *Bacillus subtilis* and also by some yeast like fungi *Candida albicans*. The biofilms can be destructed by adding chemicals like CTAB and SDS, or by the use of surfactants. Though there are many chemical surfactants, there is an increase in demand for microbial surfactants or biosurfactants. Comparing with chemical surfactants, these compounds have several advantages such as lower toxicity, higher biodegradability, and effectiveness at extreme temperatures and pH values. Moreover, biosurfactants can be tailor-made to suit different applications by changing the production conditions, or by modifying the genes involved in their biosynthesis. Dairy effluent is one of the cheap and viable substrate for the production of biosurfactants. It contains valuable nutrients including proteins, peptides, amino acids, and lipids that can support good microbial growth. Daniel *et al*. used dairy wastes as substrates and

achieved production of high concentrations of sophorolipids using two-stage cultivation process for the yeast *Cryptococcus curvatus* ATCC 20509. Whey, buttermilk, and their derivatives are some of the byproducts from dairy industries. *Candida* species, *Cryptococcus* species, and *Pichia* like yeast species can be isolated from these effluents. Dairy waste samples were collected for the study as it is a good source of probiotic fungal species. It was placed on YPD medium with peptone and glucose nutrient sources. All isolates were incubated on mineral salts medium supplemented with yeast extract and 2% paraffin/crude oil as carbon source. Biosurfactant activity of the collected culture filtrates were analyzed by emulsification activity and emulsification index.

Among the isolated strains D1, D2, and D3, D2 exhibited the collapse of drop within 10 seconds for screening the production of biosurfactant (Figure 4). Based on the microscopic techniques and biochemical results, D2 was identified as *Saccharomyces cerevisiae* which are large oval shaped budding yeast cells (Fig 1 and 2).

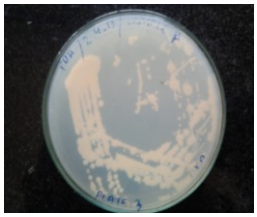

Figure 1 and 2: *Saccharomyces cerevisiae*

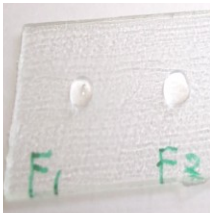

Figure 3 and 4: Screening for biosurfactant activity

The height of emulsification activity of biosurfactant measured at 610 nm was 0.05 and emulsification index calculated as 70%, along with controls (sodium dodecyl sulphate) (Figure 3). A white, snow like sedimentation was obtained during the purification of microbial biosurfactant which weighed 0.1g/10 ml (Table 1).

Table: 1 Results showing biosurfactant activity of isolates

Sample No.	Emulsification activity (610 nm)	Emulsification index (%)
Negative Control	0.00	0.00
Positive Control (SDS)	0.06	84
D-1	0.04	66
D-2	0.05	70
D-3	0.03	57

Among the three isolates, the probiotic yeast *Saccharomyces cerevisiae* depicts a higher emulsification activity than others. During the purification process, formation of a white color precipitate indicates the presence of biosurfactants. Surfactants may affect the development of flagella, suggesting changes in the attachment capability of bacteria (12). In consequence, the microbial adhesion is mediated by specific interactions between cells surface structures and molecular mass on the substratum surface, or by non-specific interaction forces, including electrostatic forces, acid-base interactions and Vander Waals forces (13) during exponential forces during exponential growth, presumably as a result of increased cell wall hydrophobicity during this growth phase. Mannoprotein in the cell wall of *Saccharomyces cerevisiae* was extracted by autoclaving in a citrate buffer (pH 7.0), and purified. The yield of emulsifier extracted from a commercial strain of *Saccharomyces cerevisiae* was 80-100 g/kg.

The Mannoprotein consisted of approximately 380-410 g/kg protein and 210 g/kg carbohydrate (14). Cell surface hydrophobicity was assessed by microbial adhesion to the hydrocarbon method (MATH) according to Rosenberg et al. (1980). Several studies with plant derived oils have shown that they can act as effective and cheap raw materials for biosurfactants production, for example, rapeseed oil (41), Babassu oil and Corn oil (16, 17). Similarly vegetable oils such as sunflower and soy-bean oils (18, 19) were used for the production of rhamnolipids, sophorolipids, and mannosylerythritol lipid biosurfactants by various microorganisms.

Recently in 2010, L. Fracchia *et al*., had proposed that in co-incubation assays, biofilm formation of candida strains of CA-2894 and DSMZ 11225 was inhibited by 70% at 160.5 µg/well and by 81% at 19.95 µg/well, respectively. No inhibition of both *C. albicans* planktonic cells was observed, thus indicating that the biosurfactant displayed anti-biofilm formation. The probiotic strain of *Saccharomyces cerevisiae* at 2µg/well has effectively controlled the growth of both fungal and bacterial pathogens. This has paved new pathway in the development of drug for control of these pathogens. When *Candida albicans* grows on a surface, it triggers mechanisms like biofilm formation and invasion (21). In addition to the diseases caused by *Candida*, biofilm formation can also cause device failure (22).

Inhibitory activity purified biosurfactants against *Candida albicans* (Table 2) and *Bacillus subtilis* (Table 3) were analyzed using ELISA reader. Both pathogens were loaded on two different microtitre plates in an increasing concentration of 1µg/10µl to 10µg/10µl in wells 1-10 respectively. Wells 11 and 12 were taken as

control and filled with distilled water. The results were read at 600 nm. The results observed indicate that biosurfactant with varying concentrations, purified from *Saccharomyces cerevisiae* (D-2) showed the highest inhibitory activity against biofilm producing *Candida albicans* (Table 2) and *Bacillus subtilis* (Table 3). The microtitre-plate antiadhesion assay estimates the percentage of microbial adhesion reduction in relation to the control wells, which were set at 0% to indicate the absence of biosurfactant and therefore of its anti-adhesion properties.

The microtitre-plate anti-adhesion assay allows the estimation of the crude biosurfactant concentrations that are effective in decreasing adhesion of the microorganisms studied (23). *Candida* cells treated with biosurfactant were characterized by chromatin condensation and cell shrinkage in the early stage, and then the nucleus and cytoplasm fragment, forming membrane-bound apoptotic bodies which can be engulfed by phagocytes. The result is that many cells can be deleted from tissues in a relatively short time with little to show for it in conventional microscopic sections. The process is called apoptosis, a tightly regulated form of cell death, also called the programmed cell death.

Chemicals and biosurfactants were added in the order: (Figure 5 and 6).

Row A: SDS (Sodium dodecyl sulphate) – (positive control)

Row B: Negative control

Row C: D-3

Row D: D-2

Row E: D-1

Figure 5 : *Candida albicans*

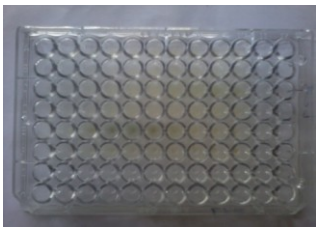

Figure 6: *Bacillus subtilis*

Table: 2 – Inhibitory (Anti-adhesive) activity of biosurfactant against *Candida albicans*.

←----------concentration----------->

		1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	0%	0%
		1	2	3	4	5	6	7	8	9	10	11	12
Positive control (SDS)	A	0.3	0.3	0.28	0.2	0.1	0.01	0.01	0.01	0.01	0.01	0.3	0.3
Negative Control	B	0.0	0.0	0.00	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.0
D-1	C	0.25	0.25	0.26	0.1	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
D-2	D	0.3	0.3	0.3	0.22	0.01	0.01	0.01	0.01	0.01	0.01	0.3	0.3
D-3	E	0.2	0.2	0.1	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Table: 3 – Inhibitory (Anti-adhesive) activity of biosurfactant against *Bacillus subtilis.*

←----------concentration----------->

		1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	0%	0%
		1	2	3	4	5	6	7	8	9	10	11	12
Positive control (SDS)	A	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.01	0.3	0.3
Negative Control	B	0.0	0.0	0.0	0.0	0.0	0.3	0.00	0.00	0.00	0.0	0.0	0.0
D-1	C	0.3	0.3	0.3	0.3	0.3	0.3	0.28	0.25	0.3	0.01	0.2	0.2
D-2	D	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.3	0.3
D-3	E	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.3

IV. Conclusion

Biosurfactant controls the microbial growth by reducing surface adhesion property of pathogen or by cellular leakage by cell membrane damage. Biosurfactants have also been reported to have various degrees of antimicrobial activity. Several biosurfactants have strong antibacterial, antifungal and antiviral activity (24). The high production costs for biosurfactants currently prohibits their large-scale utilization, although organisms involved in their production can use a wide range of cheap substrates including industrial (oils / fats) and agricultural waste products (25,26). To delineate, the present study evaluates the potential of biosurfactant produced by *Saccharomyces cerevisiae* and its action to control the establishment of *Candida albicans* and *Bacillus subtilis* on epithelial surface by anti adhesive activity or cellular leakage. The diversity of biosurfactants makes them an attractive group of compounds for use in a wide variety of industrial and biotechnological applications such as agriculture, food production, chemistry, cosmetics, and pharmaceutics.

They can be easily isolated from our surroundings including contaminated soil, water, effluent samples were used.

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