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Anti-biofilm Potential of Biosurfactant Isolated from *Lactobacillus Spp.* Against Biofilms of *Candida Spp.*

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***Abstract*—**Work is focused to study the anti-biofilm activities possessed by various strains of *Lactobacillus spp.* over pathogenic *Candida spp.* Liquid-liquid extraction of the centrifuged *Lactobacillus spp.* in an equiratio of supernatant and ethyl acetate followed by evaporation of the solvent gives crude form of the biosurfactant. Pre-coating of the *Candida spp.* samples measure the percentage adhesion on the walls of 96-well microtiter plates at four different concentrations (0.05, 0.1, 0.125 and 0.2 g/ml) of the extracted biosurfactant. Findings reveal that the isolates inhibit (~75%) the adhesion of *Candida* cells. Conducting the pre-coating method at 0.2 g/mL of crude biosurfactant of the 10^{-5}Cb1 isolate reduced (~93%) the cell adhesion of *Candida spp.* However, few of the Lactobacillus isolates reduced (~90%) adhesion of *Candida* cells.

Keywords— Biosurfactant. Candida spp., Lactobacillus spp., Pre-coating method

I. INTRODUCTION

Lactic acid bacteria (LAB) express its peculiar anaerobic behaviour and structural variation through its diverse genetics and physiology [1,2]. The growth of rod-shaped Gram-positive and non-pigmented fermentative LAB produces a sufficient amount of lactic acid as a primary product in neutral or moderate pH (pH elevated to a maximum value of 7.5) [3]. A sophisticated molecular structure called a flagellum mediates the motility of these bacteria. Most often, *Lactobacillus* and *Bifidobacterium* are used as probiotics for the laboratories, and here in the experiment, *Lactobacillus* is considered [4]. Probiotics are products designed to deliver potentially beneficial bacterial cells to the microbiotic ecosystem of humans and other animals. Several *Lactobacilli spp.* is known to produce vital metabolites, among which bio-surfactants, in particular, have shown antimicrobial activity against several pathogens in the intestinal tract and female urogenital tract partly through interfering with biofilm formation and adhesion to the surfaces of the epithelial cells. Due to its proteinaceous nature, bio-surfactant produced by strains of *Lactobacillus* generally believed to be a surlactin type with high potential toward impeding pathogens adherence [2,3,4]. Researchers have recently focused on the anti-adhesive and antibiofilm properties of *Lactobacilli*-derived bio-surfactants [1,4,5]. Bio-surfactants are surface-active compounds synthesized by microorganisms with pronounced surface and emulsifying activities [6]. The increased interest in the bio-surfactant producing *Lactobacilli* is related to the well-known probiotic effects of these microorganisms that are natural components of human microbiota as well as the ability of such bacteria to inhibit pathogenic bacteria and fungi, to reduce adhesion of pathogenic microorganism to glass, silicone rubber, surgical implants, etc. [7]. Motivated from the above, authors studied the anti-biofilm activities possessed by various strains of *Lactobacillus spp*. over pathogenic *Candida spp.* We revealed the fact that every isolate of the *Lactobacillus spp*. has a specific concentration to show its activity against biofilm producers. Accordingly, it has revealed that the *Lactobacilli*-derived biosurfactants affect the interactions and reduce the fungal deposition on surfaces.

Remainder of the paper is sectioned in three major sections: Section II points out in the research work carried out in the field of biosurfactant and its mode of action in terms of inhibiting pathogens. Section III explains the isolation of *Lactobacillus spp.* and conducted microscopic physical observations and biochemical tests on the isolates. Section IV explains variation of adhesion in specific concentration and relative activities against biofilm producers.

II. RELATED WORK

According to certain literatures, the chemical structure of the biosurfactants produced by *Lactobacilli* was examined for different bacterial species: *Lactobacillus helveticus* derived biosurfactant is mainly constituted by lipid and sugar fractions; *Lactobacillus pentosus*, *Lactobacillus lactis* and *Lactobacillus paracasei* biosurfactants are glycoproteins or glycolipopeptides, while the *Lactobacillus plantarum* biosurfactants are of glycolipidic or glycoproteic in nature [7]. Furthermore, *Lactobacilli*

interfere with pathogens by competitive exclusion from receptors present on the surface of the epithelial cells and by co-aggregation with them, which contributes to create a barrier that prevents colonization by pathogens [8]. Although a few studies have been published on biofilm formation in *Coccidioides immitis* and *Cryptococcus neoformans*, most of the fungal biofilm work has focused on *Candida*. The *Candida* species (*C. albicans* and *C. glabrata* in particular) are major nosocomial pathogens and are responsible for various types of local and (often life-threatening) systemic infections [9]. Development of new technologies based on the control of the *Candida spp.* biofilm growth is, thus, foreseen as a major breakthrough in medicine and will have a strong impact in the clinical practice and preventive medicine. Many *Lactobacilli* are known to inhibit the growth of *Candida spp.* in different ways, such as competition for adhesion sites or production of different antagonistic metabolites which inhibit its growth however, the specific role of *Lactobacilli*-produced biosurfactant on *Candida spp.* biofilm has been rarely investigated [10]. Hence, in this study we have primarily focused on the action of *Lactobacillus*-derived biosurfactant on *Candida spp.* biofilms.

III. METHODOLOGY

Collection of Samples

Homemade curd samples from buffaloes and cows collected in sterile containers for the isolation of *Lactobacillus spp.* and *Candida spp.* collected from the Microbiology Laboratory, Down Town Hospital, Guwahati. All the samples were kept at 4°C to maintain the aseptic environment. Now, 1ml curd samples were added to sterile distilled water (9 ml), subjected to vortex for preparing a homogenous solution. Further, 1ml of sample was subsequently transferred to successive tubes and carried out till 10^{-6} dilutions, among which dilution 10^{-4}, 10^{-5}, and 10^{-6} were selected for inoculums [3].

Isolation of *Lactobacillus spp.*

Initially, the isolation process was started with the spread plate technique, continued with the streak plate technique on MRS agar (de-Mann Rogosa) media, and sterilized in an autoclave at 121°C for 15 minutes. A fixed amount of inoculum (i.e. 0.1 ml) has been placed at the center of the solidified media on the petri dish and evenly spread with a sterile glass rod spreader. Now, the plates incubated at 37°C for 24 hours [2, 3], and we could observe the bacterial colonies of different sizes, forms, colors, edges, elevations, margins, textures, and opacities (see Table 1). Later, selected colonies streaked on MRS agar plates for isolating the pure culture. Finally, after inoculation, these plates were incubated in an inverted position at 37°C for 24 hours [2, 3].

Microscopic Study and Biochemical Experiments

A microscopic study was conducted on bacteria by Gram staining, a method to distinguish bacterial species into two groups: Gram-positive and Gram-negative bacteria. A clean slide was taken on a staining tray with a heat-fixed smear, flooded with crystal violet, and gently rinsed with distilled water. Further, the slide was flooded with Gram's iodine and rinsed with distilled water. Now, 95% of ethyl alcohol was added to it until the alcohol run almost clear and immediately rinsed with water. Lastly, safranin was added to counterstain and gently rinsed with distilled water. Preparation was observed under a light microscope with three magnification zooms: 10x, 45x, and 100x [2, 11]. The endospore staining was used to identify the presence of endospores in a bacterial sample. Gently the smear was flooded with Malachite green and subjected to steam for 5 minutes. Further, the slide was then counterstained with safranin for 30 seconds and then washed with distilled water. Preparation was observed under a light microscope with three magnification zooms: 10x, 45x, and 100x [12]. Biochemical tests were conducted on 24 hours freshly cultured bacterial sample on MRS agar media and autoclaved at 121°C for 15 minutes at 15 psi. A catalase test was performed to identify the bacteria by transferring a small amount of bacterial colony to a surface of a clean glass slide in which a drop of 3% Hydrogen peroxide (H_2O_2) was placed. A positive result indicated by the production of oxygen bubbles in one minute [2, 12]. In another test, lactose broth base medium, and the phenol-red indicator used, the media was sterilized at 121°C for 15 minutes at 15 psi. Accordingly, a loopful of each isolate was inoculated into each test tube. Also, for the detection of gas, Durham tubes were filled with sterile media and inserted into the tubes in an inverted position. A positive test result turns the broth into yellow color at or below a pH of 6.8 due to the production of an organic acid, whereas the negative test result produces a red color at neutral pH [11]. The motility has been tested with the Hanging-drop wet method. Fresh broth cultures of the isolates were prepared and added on the convex side of the cavity slide and fixed with a coverslip with petroleum jelly. The slide was inverted and observed under a microscope of 40x magnification [11].

Harvesting cum Extraction of Biosurfactant

The oil spreading test was carried out in a Petri dish containing 50 ml of distilled water overlaid with 20µL of kerosene or vegetable oils (sunflower and olive oil). One drop of saline suspension of the isolate was added at the center of the plates and kept at room temperature for 1 hour. A clear zone was observed at the center after 1 hour [7, 13]. Accordingly, the isolates with positive oil spreading tests taken for biosurfactant production, 200 ml of MRS broth prepared by sterilizing for 15 minutes at 121°C. A loopful of the isolate was inoculated into the MRS broth and incubated for 24 hours at 37°C in static conditions. After 24 hours of incubation, the broth culture was centrifuged (REMI C24 BL) at 10,000 rpm for 30 minutes at 4°C. The supernatants were collected for further extraction procedure known as cell-free supernatant (CFS), which was acidified to pH 2.0 with 6N HCl and kept overnight at 4°C [13]. The acidified CFS was taken in an equal amount with the solvent ethyl acetate (1:1), and the liquid-liquid extraction process was carried out in a separating funnel. After the addition of liquids in the

separating funnel, the mixture was shaken vigorously for some time so that a visible separating layer differentiating both the organic layer (biosurfactant) as well as the solvent formed. Further, the organic layer is eluded in a beaker [10, 11]. The extracted liquid was then allowed to evaporate the remaining solvent in a rotary vacuum evaporator under low pressure at 40°C, and the remaining liquid was further dried on a hot plate at 55°C for 48 hours, and thus crude biosurfactant was obtained finally [13, 14].

Cultivation of Biofilm

The *Candida spp*. collected from Down Town Hospital was cultivated on Sabouraud Dextrose Agar (SDA) and stored in the same medium at 4°C [11]. To determine the best conditions for biofilm production by *Candida spp.*, the three different culture media viz. Yeast Nitrogen Base (YNB), Universal Medium for Yeast (UMY), and Sabouraud Dextrose Broth (SDB) media can be used, and the quantification was performed employing the crystal violet method. Yeast suspensions were made in the media mentioned above were incubated for 24h. For the *Candida* biofilm assay, 72 wells (6 rows with 12 wells) of flat-bottomed polystyrene 96-well microtiter plates (Tarsons) were inoculated with 150 µL of each yeast strain suspension, and 24 control wells were filled with each sterile medium and incubated at 37°C at 75 rpm in a shaker incubator. After overnight adhesion, for biofilm quantification and fixation, the supernatant removed, wells rinsed with 100 µL of saline, and 100 µL of 99% methanol added for 15 minutes. Then, after the supernatant removal, plates were air-dried. Further, a 100 µL of 2% crystal violet (CV) solution was added to all the wells. After 20 minutes, the excess CV was removed by washing the plates with distilled water, air-dried, and bound CV released by adding 150 µL of 33% acetic acid. The absorbance was measured in the ELISA reader at 630 nm. All steps were executed at room temperature. Absorbance values three times higher than the standard deviation of the sterile control indicated a good biofilm production; inversely, absorbance values three times lower indicate a lack of biofilm production [14].

Table 1. Cultural Characteristics of Lactobacillus isolates obtained from Curd.

Dilution	Plate	Colony Type	Colonies	Elevation
$B10^{-4}$	a b	3 2	128 87	Convex
$B10^{-5}$	a b	2 2	18 17	Flat
$B10^{-6}$	a b	2 3	55 34	
$C10^{-4}$	a b	3 2	64 110	Convex
$C10^{-5}$	a b	3 2	99 49	Flat
$C10^{-6}$	a b	2 3	30 14	Convex

Abbr.: Buffalo (B) and Cow (C)

All samples of cow and buffalo (Form: Round, Margin: Entire, Surface: Mucoid, Texture: Dry, Color: White-creamy, Opacity: Opaque)

Anti-biofilm Assay of Biosurfactant Extracted from *Lactobacillus spp.* against *Candida spp.* Biofilm Producers

The biofilm inhibition assay with the extracted crude biosurfactant can be performed through two different methods viz. pre-coating and co-incubation experiments. Briefly, in pre-coating experiments, flat-bottomed polystyrene 96-well microtiter plates were filled with 150 µL of different concentrations of biosurfactant (ranging from 0.1 g/mL to 1 g/mL) and incubated overnight at 37°C at 130 rpm. Control wells containing sterile water only were treated in the same way. Biosurfactant solutions were then removed, and the plates were carefully washed twice with saline pH 7.2 to remove non-adhering biosurfactants. Aliquots of 100 µL of *Candida spp.* suspension in Sabouraud Dextrose Broth was added to each well, and plates were incubated overnight at 37°C at 75 rpm. After this time, non-adherent cells were removed by gently washing twice the wells with 100 µL saline and then treated with 100 µL of 99% methanol to fix the adherent cells. The plates were kept for 15 minutes to achieve the adherence of cells properly. Furthermore, the plates were air-dried after removal of methanol, and 100 µL of 2% crystal violet solution was added to stain the fixed cells in the wells for 20 minutes. Later, the plates were washed with distilled water to remove the unwanted crystal violet stain, and the plates air-dried. Finally, 150 µL of 33% acetic acid solution was added to solubilize the stained cells in the wells, and the absorbance was measured [14]. In co-incubation experiments, 100 µL of 24 hours fresh broth cultures of *Candida spp.* were added to 50 wells of flat-bottomed polystyrene 96-well microtiter plates together with 150 µL different concentrations of the extracted biosurfactant, ranging from 160 µg/well to 2.5 µg/well (800 µg/mL to 17.5 µg/mL) and incubated as previously described. After this time, procedures were the same as for the pre-coating experiments except for the fact that each well was filled with fresh SDB added with the different biosurfactant concentrations. Incubation conditions were as above [14]. These have allowed us to estimate the percentage of microbial adhesion to the control wells, which were set at 100% indicating total cell adhesion in the absence of biosurfactant.

IV. RESULTS AND DISCUSSION

Results

The results obtained in the study entitled "Anti-biofilm potential of biosurfactant isolated from *Lactobacillus spp.* against biofilms of *Candida spp*." are summarized in this chapter with a brief explanation under different headings and sub-headings. Twenty-nine isolates of bacteria were obtained from both the curd samples (buffalo and cow). The cultural characteristics of the isolates on the MRS agar medium were studied and observed. Observations of the colonies were done according to the form, color, elevation, margin, texture, surface, and opacity. The isolates were identified as *Lactobacillus spp*. through various microscopic and biochemical studies. The summary of the cultural characteristics of the isolates is shown in table 1.

The microscopic studies of the bacterial colony were done by Gram staining and Endospore staining to determine whether the isolates were of *Lactobacillus spp. Lactobacilli* are Gram-positive and non-spore-forming. All the isolates obtained were observed as Gram-positive (stained purple) and endospore negative (stained pink), which indicate that the isolates to be *Lactobacillus spp*. A total of seventeen colonies were selected for identification purposes. The selected seventeen isolates of *Lactobacillus spp*. were taken for the test to determine their capability of producing biosurfactants by showing a positive oil-spreading test by forming a clear zone on the oily layer. The diameter of the dispersed oil by the biosurfactant of a particular isolate determined their potential of producing a higher valued biosurfactant. A larger clear area is correlated with higher surface activity. Out of seventeen *Lactobacillus* isolates, only two of the isolates were incapable of producing biosurfactants i.e. they showed a negative oil-spreading test. Hence, fifteen isolates of *Lactobacillus spp.* were considered for the biosurfactant production and extraction procedure. Fifteen bacterial isolates of *Lactobacillus* grown on MRS broth have given a better yield of biosurfactant in both CFS as well as crude biosurfactant. The cultivation of the isolates on 200 ml MRS broth gave approximately 150 ml of CFS. Further, the acidified CFS which was subjected to the extraction process with an equal volume of ethyl acetate gave 80 ml of the organic part containing the biosurfactant. Later on, when 80 ml of the liquid was dried by evaporating the remaining solvent on a rotary vacuum evaporator and the hot plate, finally gave 6.0 gm (approximately) of the crude biosurfactant from all the fifteen isolates. The capability to produce biofilm by human pathogenic *Candida spp.* was well observed by the crystal violet assay. The adherence and absorbance shown by the cells of *Candida spp.* were in the range of a good biofilm production species. The highest absorbance of *Candida spp*. was 1.209 at 630 nm wavelength, while the control tube containing only fresh media gave absorbance 0.111 at the same wavelength. Following is a plot (see Graph 1) showing the various absorbance of *Candida spp*. grown on SDB at 630 nm after an incubation period of 24 hours. The effect of pre-coating of the extracted crude biosurfactant on biofilm formation of *Candida spp.* strains are shown in Table 2. Four specific concentrations of the extracted crude biosurfactant from all the fifteen isolates were taken to experiment with the anti-biofilm assay with the crystal violet method. All the isolates were successful in inhibiting the adhesion of *Candida* cells by more than 75%. Results are expressed as a percentage of adhesion compared to control without biosurfactant. Pre-coating with a concentration of 0.2 g/mL of crude biosurfactant of 10^{-5}Cb1 isolates significantly reduced the percentage of cell adhesion of *Candida spp.* by 93%. There were certain isolates of *Lactobacillus* that have shown a reduction in the adhesion of *Candida* cells up to 90%. All the percentages have been depicted in the plot (see Figure 1) as well as Table 2.

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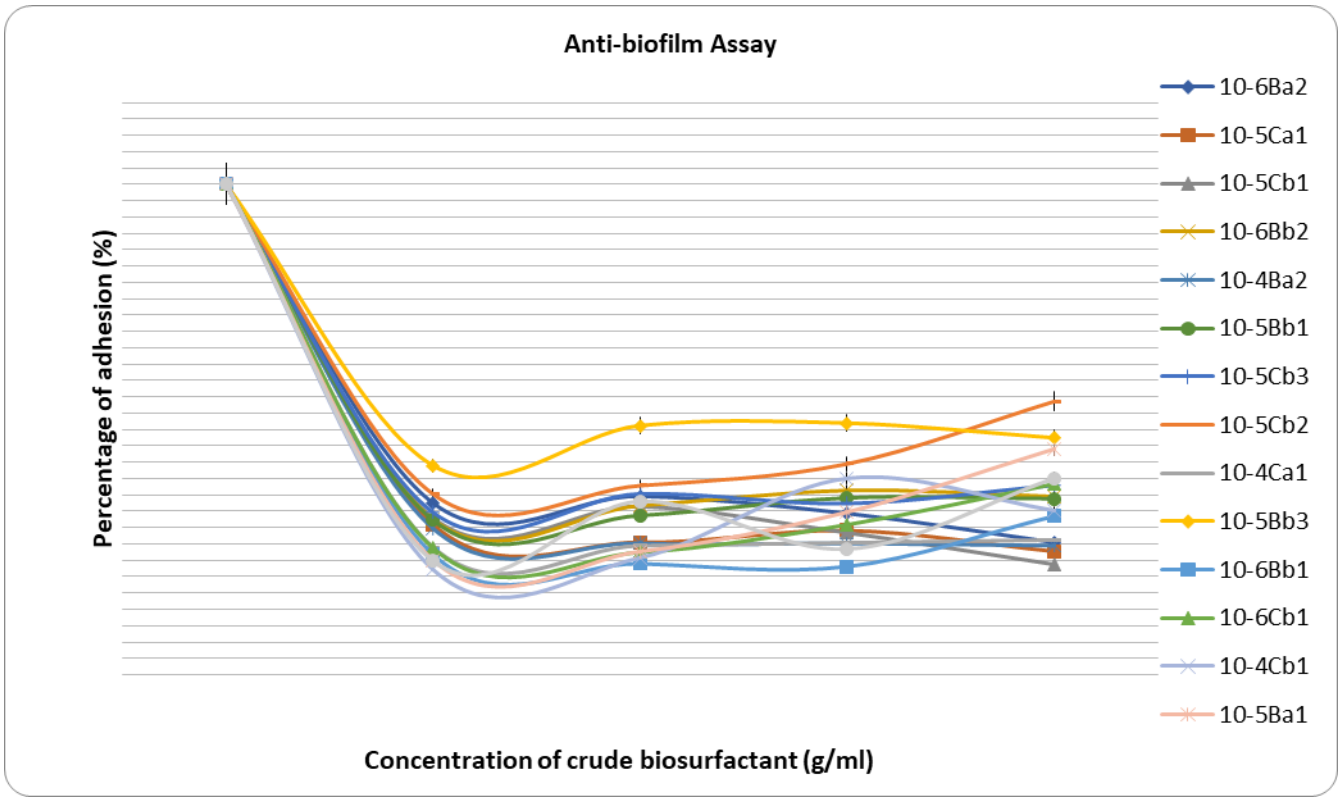

Figure 1. Graph showing the absorbance values of cells of *Candida spp.* after incubated with biosurfactant by the pre-coating method

Table 2. Percentage of Microbial Adhesion of *Candida spp.* with Crude Biosurfactant

Isolates	Concentration of Crude Biosurfactant (g/ml)							
	0.05		*0.1*		*0.125*		*0.2*	
	A(%)	*I(%)*	*A(%)*	*I(%)*	*A(%)*	*I(%)*	*A(%)*	*I(%)*
10^{-4} B a 2	15.79	84.21	12.18	87.82	12.15	87.85	11.6	88.4
10^{-5} B a 1	8.04	91.96	10.19	89.81	19.82	80.18	35.27	64.73
10^{-5} B b 1	17.8	82.2	19	81	23.3	76.7	23.1	76.9
10^{-5} B b 3	31.2	68.8	41	59	41.6	58.4	38	62
10^{-6} B a 1	7.84	92.16	22.44	77.56	10.78	89.22	28.12	71.88
10^{-6} B a 2	22	78	23.7	76.3	19.5	80.5	12.4	87.6
10^{-6} B b 1	9.41	90.59	7.18	92.82	6.48	93.52	18.83	81.17
10^{-6} B b 2	18	82	21.4	78.6	25.1	74.9	23.6	76.4
10^{-4} C a 1	11.2	88.8	11.6	88.4	12.3	87.7	13	87
10^{-4} C b 1	5.72	94.28	8.68	91.32	28.07	71.93	20.2	79.8
10^{-5} C a 1	16.7	83.3	12.4	87.6	15.3	84.7	10.2	89.8
10^{-5} C b 1	18.35	81.65	21.2	78.8	14.75	85.25	7	93
10^{-5} C b 2	24.2	75.8	26.3	73.7	31.6	68.4	46.9	53.1
10^{-5} C b 3	19.7	80.3	24.3	75.7	21.9	78.1	26.4	73.6
10^{-6} C b 1	10.99	89.01	9.99	90.01	16.73	83.27	26.7	73.3

Abbr.: Adhesion (A) and Inhibition (I)

10^{-4},10^{-5},10^{-6} are the dilutions; "B" stands for Buffalo sample; "C" stands for Cow sample; "a", "b" determines the plate number and numerical values "1", "2" and "3" determines the colony number of the respective plates.

Discussion

According to Cornea CP et.al., the oil spreading tests performed using kerosene or vegetable oils (sunflower oil and olive oil), the authors found ten strains of *Lactobacillus* giving a positive result. In the present study, out of 17 isolates, 15 isolates have shown positive results. These indicate that a higher number of isolates were capable of producing biosurfactants in comparison to the previous studies. The oil displacement test is an indirect measurement of surface activity of biosurfactants: a larger clear area is co-related with higher surface activity. When kerosene was used as a substrate, the diameters of the clear zones were larger than those with vegetable oils for all the strains. In literature, Surekha K. Satpute et.al., identified several *Lactobacilli spp*. which are known to produce important metabolites, among which biosurfactants, in particular, have shown antimicrobial activity against several pathogens in the intestinal tract and female urogenital tract partly through interfering with biofilm formation and adhesion to the surfaces of the epithelial cells. In the current study performed, all the 15 isolates of *Lactobacillus* were capable of inhibiting the adhesion of biofilm produced by human pathogenic *Candida spp*. by the extracted crude biosurfactant. Crude biosurfactant being more concentrated than the initially obtained cell-free supernatant gave better results in terms of inhibiting the flourishing range of any pathogenic strains. Jadye VV et.al, identified the antibiofilm activity of extracted bio-surfactants on *Candida albicans* and its antibiofilm activity was checked and enumerated by CFU assay. The extracted bio-surfactant exhibited 70% of efficiency. The study performed four specific concentrations (0.05, 0.1, 0.125, and 0.2 g/ml) of the extracted crude biosurfactant from all the fifteen isolates that were taken to experiment with the anti-biofilm assay with crystal violet method. All the isolates were successful in inhibiting the adhesion of *Candida* cells by more than 75%. Pre-coating with a concentration of 0.2 g/mL of crude biosurfactant of 10^{-5}Cb1 isolates significantly reduced the percentage of cell adhesion of *Candida spp*. by 93%. There are a few isolates of *Lactobacillus* that have shown a reduction in the adhesion of *Candida* cells up to 90%. However, it has been observed that on increasing the concentration of biosurfactants, the adhesion has decreased. On contrary, for some other isolates, the highest concentration of the biosurfactant gave the utmost inhibition. From this kind of result, it can be confirmed that every strain of the *Lactobacillus spp*. has a specific concentration for showing its activity against biofilm producers. The results support the opinion that *Lactobacilli*-derived biosurfactants remarkably affect these interactions and, as a result, the surface is made less supportive of fungal deposition.

V. CONCLUSION AND FUTURE SCOPE

The current study entitled, "Anti-biofilm potential of a biosurfactant isolated from *Lactobacillus spp*. against biofilms of *Candida spp*." was conducted to study the anti-biofilm activity possessed by various strains of *Lactobacillus spp.* over human pathogenic *Candida spp*. The study is completed successfully will remarkably establish an alternative way to retard the flourishing range of biofilm producers. This work will be beneficial in the field of medicine and therapeutics in inhibiting diseases such as Candidiasis. The anti-adhesive properties of the

Lactobacillus-derived biosurfactant against *Candida albicans* biofilm producers suggest its potential use as an anti-adhesive product on medical devices (catheters, prosthesis, stents) to prevent *Candida albicans* infections. In terms of the future prospect of the project, it can be further continued to specifically know the exact strain that will be best suited for inhibiting biofilm producers.

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