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EXTRACTION, CHARACTERIZATION AND FORMULATION OF *LACTOBACILLUS* DERIVED BIO-SURFACTANT INHIBITING BIOFILM FORMATION OF HUMAN OPPORTUNISTIC *CANDIDA ALBICANS*

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ABSTRACT

Aim of this study is to avoid the biofilm formation of Candida albicans with the application of formulated toothpowder containing Lactobacillus derived bio-surfactant. Isolation of Lactobacillus was carried out using dairy products. Total 17 isolates were obtained, and isolates were screened by using oil spread assay and isolates showing positive emulsification activity were selected for bio-surfactant production. Extraction of bio-surfactant was carried by using ethyl acetate. Isolation of Candida albicans was carried out using SAB plates; sample used were mouth swab of patient suffering from candidiasis. Total 6 isolates were obtained, and the isolates were characterized by germ tube test and colony coloration on CHROM agar. Antibiofilm activity of extracted bio-surfactant was performed and percentage inhibition was found out by CFU assay followed by adhesion assay. Bio-surfactant obtained from isolate RM4 was efficient anti-biofilm agent, followed by the formulation of toothpowder showing 71% of efficiency in inhibiting biofilm formation of Candida albicans. On the basis of above studies, it concludes that use of Lactobacillusderived bio-surfactant and use of formulation can be effective weapon against colonising opportunistic Candida albicans and can be applied in oral care sectors.

KEY WORDS

Bio-surfactant, Candida albicans, anti-biofilm activity, oral care sectors

INTRODUCTION:

*Lactobacillus*is a genus of Gram positive, rod shaped, facultative anaerobic, microaerophilic organism. *Lactobacillus* spp. is well known probiotic, having positive effect on the maintenance of human health. They can be used in many different areas and fields. They are able to produce different kinds of agents such as bio-surfactant. Bio-surfactants are surface active agents with different chemical and physical properties where as they are amphiphilic in nature and they have recently became an important product of biotechnology for industrial and medicinal applications. Nowadays, bio-surfactants are used in industries as a cosmetic and special chemical substance, food, pharmaceutics, agriculture, cleansers, enhanced oil recovery and bioremediation of oil-contaminated environments and can be effectively used in numerous processes, including enhanced oil recovery, transportation in pipelines, cleaning of oil storage tanks, refining and product formulation.

In a healthy human being oral micro flora is consist of many types of organisms in which *Candida albicans*

plays a major role but in immune compromised patient, *Candidaalbicans* becomes opportunistic and will be responsible to form a biofilm and colonise in many different areas it causes oral health issues such as candidiasis. Pathogenesis is initiated by the formation of biofilm and it's becoming a major concern.

The formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. It is thought that the first colonist bacteria of a biofilm adhere to the surface initially through weak, reversible adhesion via van der Waals forces and hydrophobic effects. During surface colonization bacteria cells are able to communicate using quorum sensing (QS) products such as *N*-acyl homo-serine lactone (AHL). Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. Polysaccharide matrices typically enclose bacterial biofilms. The final stage of biofilm formation is known as dispersion and is the stage in which the biofilm is established and may only change in shape and size.

In vitro experiments have shown biofilm development to occur in a series of sequential steps over a period of 24–48 hrs. The initial step consists of the adhesion of single fungal yeast cells to the substrate forming a foundation of a basal yeast cell layer (adherence step). This is followed by a phase of cell proliferation across the surface and filamentation where cells form elongated projections that continue to grow into the filamentous hyphal forms (initiation step). The production of hyphae is a hallmark of the initiation of biofilm formation followed by the accumulation of an extracellular polysaccharide matrix as the biofilm matures (maturation step). Finally, in the last step, non-adherent yeast cells are released from the biofilm into the surroundings where they can colonize other surfaces (dispersal step). Dispersion of biofilm-associated cells carries great clinical significance as released cells can initiate formation of new biofilms or disseminate into host tissues and therefore, they are associated with Candidiasis and disseminated invasive disease

The aim of this study, therefore, is to evaluate the effect of *Lactobacillus* derived bio-surfactant on the *Candida albicans* biofilm development during the adhesion, initial colonization, and maturation phases of the yeast and also to formulate a toothpowder and evaluation of the effect of formulated toothpowder on the biofilm formation.

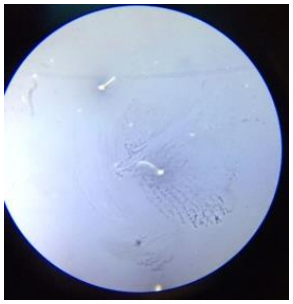
RESULT AND CONCLUSION:

1. Characterization of *Lactobacillus* producing bio-surfactant and Extraction of bio-surfactant

Oil spread assay was done by emulsification activity and observed that 4 isolates were positive oil spread assay. Those 4 isolates have been further selected for the production of bio-surfactant. Ethyl acetate extraction method was used for all the four extracted bio-surfactants viz., RM4, PM5, RM1, and RM3.

2. Isolation and confirmation of *Candida albicans*

Mouth swab of immune compromised patients has been used and spread plate technique was followed. Media used was SDA.Total 7 isolates has been obtained and characterized and Confirmed by using germ tube test,all the isolates were able to produce germ tube. On CHROM agar isolates were able to give green colored colonies. Form 7 isolates 3 were selected for further research.



3. Characterization and Antibiofilm activity of extracted bio-surfactant against *Candida albicans*

Antibiofilm activity of extracted bio-surfactants has been determined on *Candida albicans* and its antibiofilm activity was checked and enumerated by CFU assay. Extracted bio-surfactant exhibited 70% of efficiency. Efficient RM4 has been characterised by ^{13}C NMR and was compared with standard bio-surfactant surlactin showing 90% of similarity.

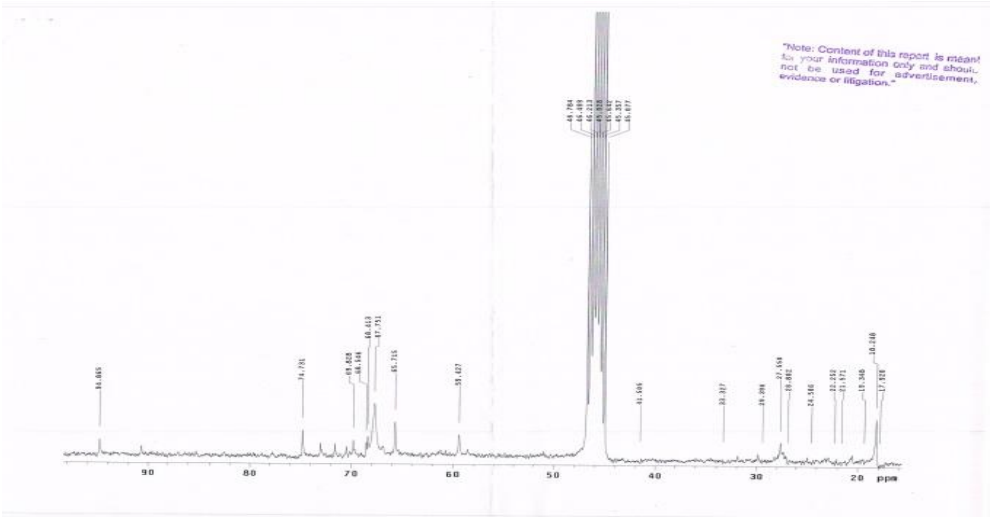


Figure 1: 13 C NMR of extracted, efficient bio-surfactant (RM4)

4.Formulation of toothpowder and Antibiofilm activity

Toothpowder formulation is done by using extracted efficient bio-surfactant and herbal products and followed by Antibiofilm activity. Antibiofilm activity observed by formulated toothpowder is 71%.

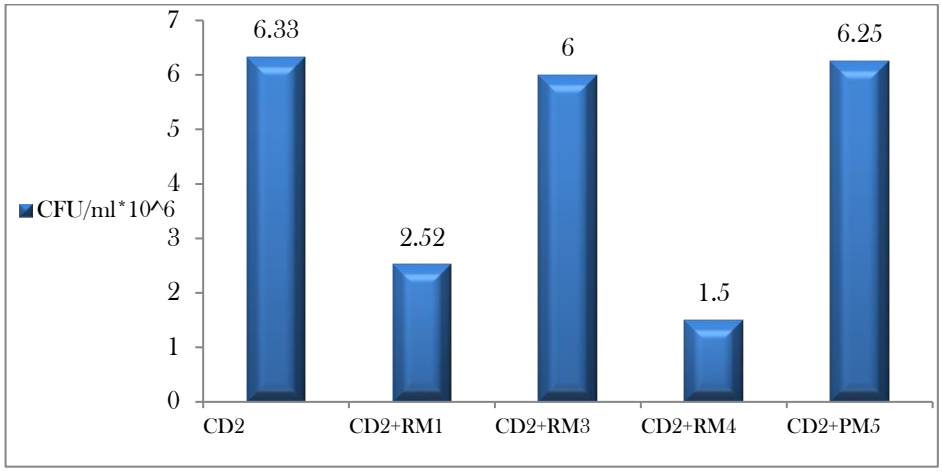


Figure 2: Antibiofilm activity of Bio-surfactants.

Antibiofilm activity of individual bio-surfactants viz., RM1, RM3, RM4, PM5 has been checked and enumerated by CFU assay. RM4 showed more efficiency i.e RM4 was found to be 70% efficient against biofilm formation of *Candida albicans.*

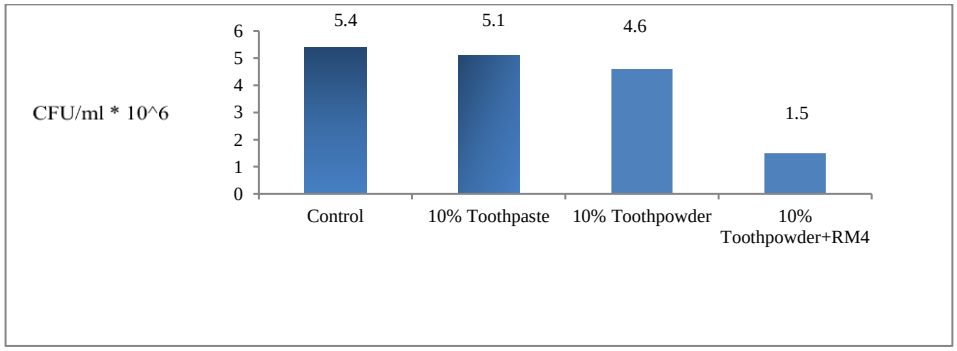


Figure 3: Antibiofilm activity of Bio-surfactants.

Antibiofilm activity of formulated toothpowder with bio-surfactant(RM4) has been checked and confirmed by comparing with toothpaste and toothpowder without bio-surfactant and enumerated by CFU assay. Efficiency of formulated toothpowder with RM4 was found to be 71% against biofilm formation of *Candida albicans.*

Methods:

1.Extraction and Characterization of *Lactobacillus* producing bio-surfactant and production of bio-surfactant

Clean and empty Petri plates were taken and filled with 20ml of water.Each plate containing water was covered with 15 µL crude oil layer. One drop of a saline suspension of isolate was added at the centre of plates.Plates were kept at room temperature for 1 hr.The Clear zone was observed at the centre after 1 hr. Positive Oil spread assay was shown by 4 isolates.

Isolate which will be positive in Oil spread assay were taken for the bio-surfactant production. 50 ml of Sterile MRS broth was taken and 1 ml of a saline suspension of isolates which was adjusted at 0.1 OD was added in to it. Inoculated broths were incubated at 37°C for 96 hrs. After 96 hrs of incubation, the broth was centrifuged at 20°C under 12,500 rpm. Biomass was separated out and supernatant was taken for the further procedure. pH of taken supernatant was adjusted by adding 1 ml of 6N HCl. Supernatant was taken and an equal amount (35ml) of Ethyl Acetate was added in it. Broth was vigorously shaken and kept to settle down.White colored and oily layer i.e. organic phase was separated out and collected by using separating funnel.The extracted organic layer will be kept under 55°C for 72 hrs.

1. Isolation and confirmation of *Candida albicans*

An oral swab from gums and palate of HIV positive patient was taken by using St. Cotton Swab. The immediately collected sample was swabbed on St. Sabouraud Dextrose agar (SDA). Swabbed plates were kept at 37°C for 48 hrs. After 48 hrs colonies with typical morphological characteristics were selected from plates. Isolates were characterized by germ tube test and for the confirmation streaked on CHROM agar. Isolated colonies of *Candida albicans* was added in 15 ml of Sterile Trypticase Soy broth. Broth was incubated at 37°C for 3 hrs.After 3 hrs of incubation loopful of Trypticase Soy broth was taken on a glass slide and Nigrosin stain was added (Negative Staining). After drying slide were observed under 45X with a compound microscope for germ tube formation.

2. Characterization and Antibiofilm activity of extracted bio-surfactant against *Candida albicans*

Candidal adhesion assays was performed in Sterile Eppendorf tubes.500 µL of Sterile Brain Heart Infusion was added to each well of pre-sterilised, Eppendorf tubes.100 µL of a saline suspension of *Candida albicans* was adjusted at 0.6 O. D. After the completion of adhesion assay in each tube 100 µL of extracted bio-surfactants has been added and Candidal adhesion assay will be performed. Following the 90 min adhesion phase, each tube was washed twice with 1000 µL of Sterile Saline to remove loosely adherent cells.

After completion of Adhesion assay, 500 µL of Sterile BHI medium was pipetted into each of the washed tubes and incubated at 37°C for 96 hrs. The growth medium was replenished for every 24 hours. After 96 hrs of incubation, all tubes were washed off with sterile saline. In empty tubes, 100 µL of Sterile saline was added forcefully and it was used for further serial tenfold dilutions. Tenfold dilution was performed by sterile saline till 10^6 dilutions. 100 µL of diluted sample from last three dilutions viz., 10^4, 10^5 and 10^6 were used for the spread plate technique. After spreading plates were incubated at 37°C for 48 hrs. After 48 hrs of incubation colony forming unit per ml and average colony forming unit was calculated

Efficient bio-surfactant which was screened by using Antibiofilm formation assay was selected for Nuclear Magnetic Resonance Spectroscopy.13C NMR has been performed and solvent used was deuterated methanol with the help of SAIF (Sophisticated Analytical Instrument Facility) IIT Bombay.

3. Formulation of toothpowder and its Antibiofilm activity against *Candida albicans*

According to the standard composition, tooth powder was prepared in which Haritaki, Alum, Ajwain, Camphor, White cumin, Clove oil, Mint flower, Nilgiri Oil; Wood charcoal was added in a specified quantity. 1gm of extracted bio-surfactant was added in a prepared toothpowder. Adhesion assay is followed by Biofilm formation and Antibiofilm activity is checked and confirmed

DISCUSSION

Infection caused by *Candida albicans* is very common in immunocompromised patients. There are high chances of oral candidiasis in patients suffering from different kinds of cancers and HIV.

The International Diabetes Federation has stated that 387 million people throughout the world have diabetes mellitus in 2014, which will rise to 592 million in 2035 The World Health Organization reported that there were 14.1 million new cases and 8.2 million deaths in 2012 globally. Interestingly, there is now considerable

professional evidence indicating that T2DM is associated with an increased risk of several cancer types, such as liver, pancreatic, colon, breast, and bladder cancers.

Oral candidiasis is a common opportunistic infection of the oral cavity caused by an overgrowth of Candida species, the commonest being *Candida albicans*. The incidence varies depending on age and certain predisposing factors. There are three broad groupings consisting of acute candidiasis, chronic candidiasis, and angular cheilitis.

Oral candidiasis is an opportunistic infection of the oral cavity. It is common and underdiagnosed among the elderly, particularly in those who wear dentures and in many cases is avoidable with a good mouth care regimen. It can also be a mark of systemic disease, such as diabetes mellitus and is a common problem among the immunocompromised. Oral candidiasis is caused by an overgrowth or infection of the oral cavity by a yeast-like fungus, candida. The important ones are *C. albicans, C. tropicalis, C. glabrata, C. pseudotropicalis, C. guillierimondii, C. krusei, C. lusitaniae, C. parapsilosis, and C. stellatoidea, C. glabrata,* and *C. tropicalis* represent more than 80% of isolates from clinical infection.3 Oral candidiasis is the most common human fungal infection especially in early and later life. In the general population, carriage rates have been reported to range from 20% to 75%4 without any symptoms. The incidence of *C. albicans* isolated from the oral cavity has been reported to be 45% in neonates, 45%–65% of healthy children, 30%–45% of healthy adults, 50%–65% of people who wear removable dentures, 65%–88% in those residing in acute and long-term care facilities, 90% of patients with acute leukaemia undergoing chemotherapy, and 95% of patients with HIV.

Well known probiotic organism *Lactobacillus* has the ability to produce bio-surfactant which is antimicrobial in nature and can be used in several ways. Extracted bio-surfactant from Lactobacillus has been characterised by ^{13}C NMR and comparison with standard bio-surfactant surlactin is carried out. It was able to show 90 % of similarity with surlactin. Extracted and efficient bio-surfactant is used for the production of toothpowder.

Any current registered toothpaste is not able to show the Antibiofilm activity against *Candida albicans*. Hence, in future *Lactobacillus* derived bio-surfactant can be the easily used to combat with the biofilm forming strain *Candida albicans*.

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