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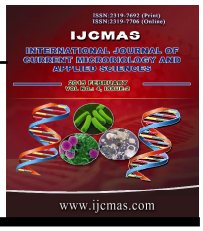

Original Research Article

Mechanism of control of *Candida albicans* by biosurfactant purified from *Lactococcus lactis*

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K e y w o r d s

Biosurfactant , Anti-adhesive, Cellular leakage, *Candida albicans*

A B S T R A C T

The multidrug resistant, opportunistic pathogen *Candida albicans* pose an important threat in immunocompromised individuals. Drug of choice has also become scarce. Recently biosurfactants has been found to be a promising remedy for this problem. The present study evaluates the molecular mechanism of action of biosurfactant against *C. albicans*. Biosurfactant producing *Lactococcus lactis* was isolated and 0.05 mg biosurfactant/ml of production medium was purified. *Candida albicans* was isolated from vaginal swab showed resistance to itraconazole and clotrimazole and was controlled by biosurfactant purified from *L. lactis* from 4.5 mg/ml concentration. Inhibitory activity of purified biosurfactant showed 50% greater activity than ketaconazole. It controlled the growth of pathogen by lowering the interfacial tension, it actively removed the pathogen and also by cellular damage to *C. albicans*. Biosurfactant recorded to have 6% more permeabilizing ability than the efficient antifungal drugs fluconazole and ketoconazole on candidal cells. IC_{50} of biosurfactant against *C. albicans* was recorded as 5 mg/ml and induced cell apotopsis. So, the present study suggests that mechanism of antifungal activity of biosurfactant produced by *L. lactis* is due to the cell permeabilizing and cellular damaging ability than anti-adhesive activity. Development of resistance towards such bifunctional lipopeptides is impossible by the pathogens. So, similar to other antifungal agents, biosurfactants can also be used as drug for treatment of infections caused by *C. albicans* safely.

Introduction

Microorganisms are having a large surface-to-volume ratio and produce a variety of surface-active agents (biosurfactants) that adsorb to and alter the conditions prevailing at interfaces. Biosurfactants are the structurally and chemically diverse group of surface-active molecules synthesized by wide group of microorganisms. Surfactants concentrate at interfaces because they are amphipathic, and are divided into low molecular weight molecules that efficiently reduce surface and interfacial tensions and high molecular weight polymers that bind tightly to surfaces.

The most important advantages of biosurfactants compared to the synthetic surfactants are their ecological acceptance owing to their low toxicity and biodegradable nature (Karanth *et al*., 1999). Another advantage of biosurfactants is that they can be modified by biotransformation to generate new products for specific requirements (Deleu *et. al*., 2004). Modified forms are commercially used in pharmaceutical, cosmetic, food and petroleum industries. Increasing environmental concern had led to consider biological surfactants as alternative to chemically manufactured compounds preferably in pharmaceutical industry. During the last few decades, investigations on various aspects of biosurfactants such as their role in bacterial adhesion (Neu, 1996) and in growth of bacteria on hydrocarbons (Rosenberg and Ron, 1996), surface active polymers from the genus *Acinetobacter* (Rosenberg and Ron, 1998), biochemistry of surfactin (Peypoux *et al.*, 1999), microbial surfactants (Rosenberg and Ron, 1999), biosurfactant assay (Lin, 1996), production (Wang and Wand, 1990), molecular genetics (Sullivan, 1998), and commercial applications including enhanced oil recovery (Fiechter, 1992; Banat, 1995) bioemulsifiers (Rosenberg and Ron, 1997) and sophorolipid as anticancer drugs (Ron and Rosenberg, 2001; Hu and Ju. 2001) were undertaken.

Since their chemical structures and surface properties are so different, it is likely that each group of biosurfactants will have a specific use (Desai *et al*., 1997). Dairy *Streptococcus thermophilus* strains, can produce biosurfactants that cause their own desorption (Busscher *et al*., 1991). *Lactobacillus* and *Streptococcus* species have been shown to be able to displace adhering uropathogenic *Enterococcus faecalis* from hydrophobic and hydrophilic substrata in a parallel-plate flow chamber through biosurfactant production (Velraeds *et al*., 1996).

There is anecdotal evidence among patients that the consumption of buttermilk, which contains antimycotic-releasing *Lactococcus lactis* (Batish *et al*., 1990) prolongs the lifetime of indwelling voice prostheses disrupted by the adhesion of yeast to the silicone rubber. Experiments had proven that biosurfactant produced by the probiotic microorganisms are responsible for antiadhesive activity. Nowadays concept of use of biosurfactant having potential antimicrobial activity from probiotic origin as drug is under consideration (Saravanakumari and Mani, 2010). In this view, mechanism of action of antimicrobial activity of biosurfactant purified from probiotic bacteria *L. lactis* against the drug resistant, opportunistic pathogen *Candida* sp., analysed in the present study.

Candida albicans is a normal residence of the digestive tract, mouth, genital region and skin of man (Eggiman, *et al.*, 2003). The organism assumes a pathogenic role whenever the normal defense mechanisms are interrupted or in patients of diabetes mellitus and iatrogenic factors like antibiotic use, indwelling devices, intravenous drug use, and hyperailmentation fluids. *C. albicans* causes a wide variety of disorders in such cases, which include thrush (Grigoriu *et al*., 1987), candidal enteritis (Muller, 1993), vulvovaginitis and urinary tract candidiasis (David *et al*., 1992), mucocutaneous candidiasis (Filler *et al*., 1993), and invasive candidiasis (Ashley *et al*., 1997; Bikandi *et al*., 1998). Nosocomial bloodstream infections due to *Candida* species are associated with a 40% crude (total) mortality rate in the United States. A recent review of candidemia identified crude mortality rates of 44%–46% in Switzerland,

44%–45% in Spain, 59% in Taiwan, and 30%–52% in Canada (Eggiman, *et al.*, 2003). Thus *Candida* species is associated with a high crude mortality rate globally. Increase in mortality rate is due to more use of intensive antibiotics, corticosteroid and immunosuppressive therapy, invasive diagnostic and pressure monitoring devices and longer survival rates in critical in ill patients.

Under normal circumstances, beneficial bacteria control levels of *Candida* sp., infection. However, if the bacteria-fungus balance is upset by the use of antibiotics or if the immune system is compromised, an overgrowth of *Candida* sp., occurs, resulting in infection (Braunwald, *et al.*, 2001). A rise in the incidence of antifungal resistance to *Candida* sp., has also been reported over the past decade (Sojakova *et al.*, 2004; Skrodeniene *et al.*, 2006). There are several reports on azole resistance, specifically in *C. albicans* and *C. tropicalis* (Brun *et al.*, 2004; Yang *et al.*, 2004; Vermitsky and Edlind, 2004). Sanglard and Odds in 2002 described precise mechanisms responsible for drug resistance in *Candida* species is due to over expression of *CtERG11* gene associated missense mutation. Currently, the most significant form of azole resistance is that seen between *Candida* and commonly used potential antifungal drug fluconazole. So, there is an urgent need for the development of new antifungal agent to provide additional tools for treating refractory infections.

Recent researchers have reported that biosurfactant controls the microbial growth by reducing surface adhesion property of pathogen or by cellular leakage by damaging of cell membrane. To delineate, the present study evaluates the potential of biosurfactant produced by *L. lactis* and its mechanism of action to control the establishment of *Candida albicans* on epithelial surface by anti-adhesive activity or cellular leakage.

Materials and Methods

Culture conditions

Lactoccous lactis was isolated from fermented milk (curd) on De Man Rogosa Sharpe Agar (MRS). Fungal pathogen *Candida albicans* was isolated from vaginal sample on Saboraud's dextrose agar and confirmed. Stock cultures were maintained at 4^{o}C.

Determination of biosurfactant activity

The isolated *L.lactis* was inoculated in 100 ml of mineral salt medium with 2% paraffin (Francy *et al*., 1991) as carbon source and was incubated at room temperature for 48 h. After incubation, the culture broth was centrifuged at 5000 rpm for 20 min and cell free supernatant was used for the test. Uninoculated mineral salt medium was used as control. The 25 µl of cell free culture supernatant were pipette out as droplet onto a parafilm surface. Collapsing of the drops was recorded after 1 min (Kim *et al.*, 2004). Emulsification index and emulsification activities of the culture broth were determined according to the procedure of Bodour *et al.*, in 2004. The ability of the biosurfactant produced by the isolate to reduce surface tension of the liquids was determined by burette method and surface tension was expressed in N/m (Newton's per minute).

Purification of biosurfactant

Equal volume of culture supernatant was mixed with acetone and incubated overnight at 4ºC. After incubation, precipitate was collected by centrifugation at 6000 rpm for 15 min at 4ºC. Precipitate was weighed and extracted with 250 µl of chilled acetone.

Minimal inhibitory activity of biosurfactant

The Muller Hinton agar plates were swabbed with cell suspension of *Candida* sp., at a turbidity of 0.5 McFarland Standard. Standard volume of (20µl) of biosurfactant suspension concentrations ranging from 2 to 5 mg /ml were added to appropriately labelled wells in agar plates and were incubated at 37^{o}C for 24 hrs. After incubation, zone of clearance around the wells were measured.

Anti-adhesive assay of biosurfactant (Heinemann *et al*., 2000)

The anti-adhesive activity of the crude biosurfactant against *Candida* sp., was quantified according to the procedure described by Heinemann *et al*., in 2000. The microbial inhibition percentages at 2-5 mg / ml of biosurfactant concentrations were calculated using the formula. Triton – X, Ketaconazole and clotrimazole at 5 mg/ml concentration were used for comparison.

% reduction in adherence = $[(A_{control}) - (A_{sample})] / A_{control} \times 100$

Where, A_{sample} represents the absorbance of the well with a biosurfactant concentration and $A_{control}$ the absorbance of the control well.

Analysis of cell viability (mtt assay)

Cell viability of *Candida* sp., cells on exposure to biosurfactant was determined by a colorimetric 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay discussed by Nakagawa *et al*., in 2000. The 50% inhibitory concentration (IC_{50}) of biosurfactant against the cells was thus calculated.

Cellular apoptosis assay

Microscopic examinations of cellular interaction between biosurfactant and *Candida* sp., was analysed by apoptosis assay. Aliquotes of *Candida* sp., cells were treated with 1 mg of biosurfactant, 1 ppm of triton X-100 as positive control and water as negative control. After incubation at 37 °C for 24 h, the reaction mixtures were stained using eosin and methylene blue staining solution. The apoptosised cells were visualized under light microscope.

Cellular damage of biosurfactant on *Candida* sp., cells were assayed by suspending cells in 10 ml of PBS and added with different concentrations of biosurfactant (2 to 5 mg/ml), 1% triton x 100 and two antifungal drugs such as amphotericin B and nystatin were taken in separate tubes. Optical density (A_{260}) of the suspension was measured at 260 nm before and after 2 h of incubation at 37°C. The cellular leakage value was calculated as A_{260} (after incubation) - A_{260} (before incubation). The samples were subjected to agarose gel electrophoresis for confirmation of leakage of DNA and SDS - PAGE for external release of cellular proteins.

Results and Discussion

Measurement of biosurfactant activity

Turbidity in the mineral salt media inoculated with curd sample indicated the growth of biosurfactant producer. As paraffin was used as the carbon source in the media biosurfactant enabled to utilize it as carbon source. By biochemical tests, the isolate was identified as *Lactococcus lactis*.

The culture filtrate from the isolate showed positive to biosurfactant production by collapsing the drop within one minute. Emulsifying activity (EA) of isolate was recorded as 0.04 OD, surface tension was calculated as 0.067 Nm^{-1} and emulsification

index (EI) was 66% (Plate 1). Pure white colour precipitate of 0.05 mg of biosurfactant / ml of medium was obtained.

Assay of drug resistance pattern of *candida albicans*

Drug resistance pattern test confirmed that isolated *Candida albicans* was sensitive to amphotericin B, nystatin, fluconazole and ketaconazole and was resistant to commonly used antifungal drugs clotrimazole and itraconazole (Plate 2). Measured values of zone of inhibitions were tabulated in table 1.

Biosurfactant controlled the growth of pathogen and minimum inhibitory activity of biosurfactant against *Candida albicans* was determined as 4.5 mg / ml.

Anti-adhesive assay of biosurfactant

Biosurfactant was very effective in controlling the attachment of *Candida albicans* on the well surface from 4 mg / ml concentration. Compared to chemical surfactants, Triton X and anti-fungal drugs ketaconazole and fluconazole, biosurfactant showed 7% increased activity. The anti-adhesive activity of the purified biosurfactant against *Candida albicans* was expressed in terms of reduction in cell adherence and the results were tabulated (TABLE 2).

Analsis of cell viability (mtt assay)

The maximum reduction in cell viability (IC_{50}) against biosurfactant was obtained as 19% at 5 mg / ml concentration. Even an effective chemical surfactant Triton X showed IC_{50} as 20%. IC_{50} of biosurfactant was 50%, which was greater than the activity of other effective antifungal agent Ketaconazole, which showed IC_{50} as 9%. So, compared to ketaconazole, purified biosurfactants are 50% more effective in inhibiting the growth of the *Candida albicans*. The calculated inhibitory concentration of biosurfactant against *Candida albicans* cells were tabulated (Table 3).

Cellular apoptosis assay by biosurfactant

The interaction between biosurfactant and *Candida albicans* was visualised microscopically. After 24 h of biosurfactant interacted with *Candida albicans* cells, resulted in apoptosis to the cells. Apoptosis is refers to as the cell shrinks and detaches from neighbouring cells and the nucleus is broken down. The nuclear fragments and organelles condense and are ultimately packaged in membrane-bound vesicles, exocytosed and ingested by surrounding cells. The photographs of this process were taken and recorded. Control (biosurfactant untreated) cells showed a clear red colour delimiting membrane and blue colour condensed DNA at the centre of the cell. In biosurfactant treated cells, the scattered broken red colour stanined membrane and blue coloured granules confirmed the damage of cell membrane and DNA.

Cell injury analysis by biosurfactant

The drug treated samples maximum OD values of 1 after incubation. The drug untreated control showed decrease in fungal growth that is 0.07 OD value. So, all the drugs are effective in controlling candidal cell growth by causing cell injury on the cell membrane and damage to the cellular components. It was again concurred by reading absorbance maximum of proteins and DNA at 280 nm and 260 nm respectively to the drug treated culture supernatants (Plate 3). The calculated values of cell injury analysis were tabulated (Table 4).

Biophotometer results showed that biosurfactant are more effective in damaging the cells and releases proteins and DNA. Biosurfactant at 5 mg/ml concentration released 40 μg of DNA / ml of supernatant and increased the turbidity of the suspension to 0.772 OD value by the release of proteins. Compared to efficient anti-fungal drugs fluconazole and ketoconazole, biosurfactant showed 6% increased damage to the cells (TABLE. 5).

Effective break down of the genomic DNA by all the test compounds at \different concentrations were recorded in agarose gel electrophoresis. The study confirmed that biosurfactants more than functioning as an anti-adhesive agent, controls the growth of *Candida* sp. by cellular damage.

Control of multi-drug resistant pathogens, *Candida albicans* is major goal of current work. Studies were focused to develop efficient, safe and economic drug. Instead of using chemicals, natural drugs that are harmless to human and functioning effectively at prevailing biological conditions are of major prerequisite. Growing knowledge of functions of biosurfactant has attracted to use it as drug against multidrug resistant pathogen, especially *Candida albicans*. Rotrigues. L *et al.,* in 2006, proposed that biosurfactant produced by *Lactococcus lactis* effectively controlled the growth of several bacterial and yeast strains isolated from explanted voice prostheses even at lower concentrations by its anti-adhesive activity. Meanwhile, Sotirova A. V *et al.* in 2007, recognized that rhamnolipid biosurfactant exert a disruptive action on *B. subtilis* at lower concentrations through cell permiablizing and cell membrane leakage. Thereby biosurfactants were declared as potential candidate for application in biomedicine but mechanism of action is under controversy. In this study, the mechanism of action of biosurfactant as antifungal agent against *Candida albicans* was thoroughly analysed, so that effective means of administration of biosurfactant as a drug and concentration required for treatment can be recommended. There are different types of organisms such as *Pseudomonas* sp., and *Bacillus* sp., produces biosurfactants for industrial purposes. Under safety consideration, it is highly insisted to use biosurfactant from probiotic bacteria such as *Lactococcus lactis* for biomedical applications. So biosurfactant from *L. lactis* used for this study.

The fungal pathogen *Candida albicans* was opted in this study to analyse the mechanism of action of biosurfactant, because; experimental, clinical and molecular similarity studies have proven that the most threatening fungal candidemia in human is due to gastrointestinal infection caused by *C. albicans* (Nucci, *et al.*, 2001). *C. albicans* is amongst the most common fungal causative agent in superficial and deep seated candidiasis (Gullo, 2009). The serious effect of opportunistic candidemia leads to 40 –50% of death among patients.

For the isolation of biosurfactant producing *L. lactis*, fermented dairy product was enriched in a hydrocarbon, paraffin containing media and then sub-cultured on selective MRS agar. The isolated microorganism was further confirmed as *Lactococcus lactis* by fermentation reactions. Turbidity in mineral salt media inoculated with paraffin showed growth and production of biosurfactant. Collapsing of a drop culture of filtrate placed on a paraffin layer within a minute was due to the reduction in interfacial tension between water and oil layer because of presence of biosurfactant in culture filtrate. Quantitative measurement of emulsification activities

determines produced biosurfactant activities. Concentration of biosurfactant and its activities are directly proportional to each other. Biosurfactant produced by *L. lactis* emulsified the paraffin and increased the turbidity of the oil in water mixture to 0.04 OD at 610 nm and emulsification index was determined as 66%. Reduction in surface tension of culture media was recorded from 0.074 N/m to 0.067 N/m. By organic solvent extraction method 0.05 mg of biosurfactant / ml of minimal salt medium was extracted from *L. lactis* inoculated broth.

Candida albicans was isolated from vaginal swab produced large opaque, cream colonies on sabourad's dextrose agar. The appearance of small filaments projecting from the cell surface confirmed the formation of germ tubes. Braude *et al.* in 1986 also reported the association of virulence of *C. albicans* with ability to produce germ tubes. Antifungal drug resistance pattern of isolated pathogen, *C. albicans* was studied against with six antifungal drugs; amphotericin B, clotrimazole, fluconazole, itraconazole, ketaconazole and nystatin and the zone of inhibition were 17 mm, 10 mm, 38 mm, 11 mm, 25 mm and 21 mm respectively. In this test, *C. albicans* showed greater sensitivity to fluconazole (38 mm) and lower to clotrimazole (10 mm). Sensitivity to fluconazole by *C. albicans* already described by Rex *et al.* in 1994 and Anaissie *et al.* in 1996.

Antifungal activity of the biosurfactant at different concentrations was measured using agar well diffusion method. Biosurfactants exhibited antifungal activities from the concentration of 4.5 mg/ml (10 mm) and 5 mg/ml (11 mm). The antifungal activity increased with increasing concentration of biosurfactant. Sandrin *et al.* in 1990 has described antifungal activity of lipopeptide biosurfactant produced by *B. subtilis.* In another study Jenny *et al.* in 1991 have reported the significant antimicrobial activity of biosurfactant produced by *B. licheniformis*.

The anti-adhesive activity of biosurfactant was evaluated against *C. albicans*. Different concentrations of biosurfactant showed anti-adhesive activity against *C. albicans*. The concentration of biosurfactants 4.0, 4.5 and 5 mg/ml exhibited higher reduction in adherence (20.90%) compared with positive control Triton X 100 (13.43%). So compared to chemical surfactant Triton X, biosurfactant produced by *L. lactis* showed 7% increased activity. Even highly active clotrimazole showed 14.93% of reduction in adhered cells. Pratt-Terpstra *et al.* in 1989, also evidently proved that the biosurfactant released by *Streptococcus mitis* was found to reduce the adhesion of *Streptococcus mutans*. Falagas and Makris, in 2009 have proposed that the application of biosurfactants purified from probiotic bacteria to patient care equipments such as catheters and other medical insertional devices in hospitals, with the aim of decreasing colonization by microorganisms responsible for nosocomial infections.

Lethality of cells on exposure to biosurfactant and various azole drugs were measured using MTT assay at different concentrations of biosurfactant and the cytotoxic effect on candidal cells was confirmed. The fifty percentage of reduction in cell viability (IC_{50}) was calculated and tabulated (Table 3). Compared to 9% of reduction in cell viability by the antifungal agent ketaconazole, biosurfactant reduced the cell viability to 18.63% at 5 mg /ml concentration. IC_{50} of biosurfactant is 50% greater than the activity of effective antifungal agent ketaconazole. So, compared to ketaconazole, biosurfactant 50% more effectively inhibited the growth of *Candida*

albicans. Xiao-Hong Cao *et al*., in 2009 reported that a lipopeptides biosurfactant from *Bacillus natto* is able to suppress the viability of K562 and BEL-7402 tumour cells in a dose-dependent manner. The IC_{50} for K562 and BEL-7402 cells at 48 hrs were 19.1 mg/l and 30.2 mg/l, respectively. In addition, the effects of biosurfactant on the survival rate of normal cell lines including BRL and HEK293 at 48 hrs were 105.4 mg/l and 93.8 mg/l, respectively. Compared to our result, biosurfactant from *L. lactis* can be safely used with normal cells at 5 mg/ml concentrations.

The biosurfactant interaction with *Candida* sp., was visualised by light microscopy. Eosin stained the cell membrane and other apoptic bodies whereas, methylene blue stained the DNA. Biosurfactant shrinks the candidal cells, breaks the nucleus and detaches from the neighbouring cells. It was determined by the absence of continuous eosin stained, delimiting membrane around the candidal cells. In biosurfactant treated cells, the scattered broken red colour stanined membrane and blue coloured granules confirmed the damage of cell membrane and DNA.

In cell injury analysis, all drug treated candidal cells showed maximum OD values of 1 after 2 h of incubation. The drug untreated control showed fungal growth increased by 0.07 OD value. So, all the drugs are effective in controlling candidal cell growth by causing cell injury on the cell membrane and damage to the cellular components. It was again concurred by reading absorbance maximum of proteins and DNA at 280 nm and 260 nm respectively to the drug treated culture supernatants using Biophotometer. Biosurfactant at 5 mg/ml concentration released 40 μg of DNA / ml of supernatant and increased the turbidity of the suspension to 0.772 OD value by the release of proteins. Compared to efficient antifungal drugs fluconazole and ketoconazole, biosurfactant showed 6% increased damage to the cells.

Cell injury leads to leakage of cellular content such as proteins and broken DNA into the culture media. So, after cell injury analysis, the sample was subjected to agarose gel electrophoresis and SDS-PAGE for the confirmation of DNA and proteins released by cellular damage. In agarose gel, the candidal cells treated with biosurfactant and other drugs were produced DNA bands were observed near the well. The molecular weight was found to be greater than 1000 bp. No bands were demonstrated in sample, candidal cells which was not treated with biosurfactant), suggests that there was no cellular damage.

In SDS PAGE gel, biosurfactant and other drugs treated cells produced more than 3 bands each and the molecular weight was found to be ranging between 14 kDa to 45kDa. Control, untreated *C. albicans* cells produced only three bands at the prevailing conditions. Thereby, the study concluded that biosurfactant produced by *L. lactis* have permeabilizing ability into the candidal cells and caused cell membrane injury, cellular leakage of DNA and protein. Permeabilization behaviour induced leaks and stabilizes them by covering their hydrophobic edges. These data resolve that detergent-like effects of antibiotic peptides on membranes. The results are compared with published parameters characterizing the antibacterial activity. Heerklotz *et al*. in 2007, explained the similar phenomena using antimicrobial, lipopeptide biosurfactant surfactin, produced by *Bacillus subtilis*. Surfactin was proved to cause cell membrane leakage and lysis of vesicles, membrane leakage starts at a surfactin-to-lipid ratio in the membrane. The transient,

graded nature of leakage and the apparent coupling with surfactin translocation is due to a bilayer-couple mechanism.

The present study revealed that mechanism of antifungal activity of biosurfactant produced by *L. lactis* is due to the cell permeabilizing and cellular damage than compared to the anti-adhesive activity on *Candida albicans.* So, similar to other antifungal agents biosurfactants are also can be used as drug for treatment of gastroinstetinal infections caused by *C. albicans.* As the produced biosurfactant is having both anti-adhesive and cellular damaging acti vities it can effectively control the candidal cell growth.

Biosurfactant producers were enriched in a hydrocarbon paraffin containing media and then subcultured on mineral salt agar media with paraffin. The isolated organism was confirmed as *Lactococcus lactis* by morphological and biochemical tests. Biosurfactant production was determined by emulsification activities and surfactant activity. *L. lactis* produced 30 mg of biosurfactant/50 ml of the medium.

The fungal pathogen *Candida albicans* was isolated from vaginal swab and substantiated by formation of germ tubes. Isolated *C. albicans* was resistance to itraconazole and clotrimazole and growth was controlled by only fluconazole and ketaconazole. Purified biosurfactants effectively controlled the growth of *C. albicans* from 4.5 mg/ml concentration. Inhibitory activity was 50% greater than the commonly used antifungal agent ketaconazole. First mechanism of biosurfactant to control the establishment of pathogen was resolved as antiadhesive activity. By lowering the interfacial tension, it actively removed the pathogen. Next, similar to other antifungal agents, biosurfactant caused cellular damage to *C. albicans*. Biosurfactant penetrated the cell wall of *C. albicans* and caused cell membrane leakage, which was confirmed by the presence of damaged cellular proteins and DNA in electrophoretic gel. Biosurfactant recorded to have 6% more permeabilizing ability than the efficient antifungal drugs fluconazole and ketoconazole on candidal cells. IC_{50} of biosurfactant against *C. albicans* was recorded as 5 mg/ml and induced cell apotopsis. The present study suggests that mechanism of antifungal activity of lipopeptide biosurfactant produced by *L. lactis* is due to the cell permeabilizing and cellular damaging ability than anti-adhesive activity. Permeabilization behaviour induced leaks and stabilized broken cell membrane by covering their hydrophobic edges. Development of resistance towards such bifunctional lipopeptides is impossible by the pathogen. Similar to other antifungal agents, biosurfactants can also be used as drug for treatment of gastrointestinal infections caused by *C. albicans* safely.

Table.1 Assay of drug resistance of *Candida albicans*

ANTIFUNGAL DRUG	ZONE OF INHIBITION (mm)	DRUG SENSITIVITY
Amphotericin B	**17**	**S**
Clotrimazole	10	R
Fluconazole	38	S
Itraconazole	11	R
Ketoconazole	25	S
Nystatin	21	S
R = resistant to drug; S = sensitive to drug		

Table.2 Antiadhessive activity of biosurfactant

Anti-adhesive agent		Mean OD value at 630 nm	Mean % of reduction in adherence
Biosurfactant concentration (mg/ml)	2	0.061	8.96
	2.5	0.059	11.94
	3	0.057	14.93
	3.5	0.055	17.91
	4	0.053	20.90
	4.5	0.053	20.90
	5	0.053	20.90
Triton X 100 (5 mg/ml)		0.058	13.43
Ketaconazole (5 mg/ml)		0.057	14.93
Fluconazole (5 mg/ml)		0.057	14.93

Table.3 Results of cell viability analysis test

Samples		Mean OD value at 630 nm	Mean % of reduction in cell viability (IC_{50})
Biosurfactant concentration (mg/ml)	2	0.162	0.0003
	2.5	0.146	0.0047
	3	0.134	8.39
	3.5	0.132	9.01
	4	0.125	11.18
	4.5	0.120	12.73
	5	0.101	18.63
Control		0.161	0.0
Triton X 100 (5 mg/ml)		0.098	19.57
Amphotericin B (5 mg/ml)		0.083	24.22
Ketoconazole (5 mg/ml)		0.133	8.70
Fluconazole (5 mg/ml)		0.162	0.0003
Nystatin (5 mg/ml)		0.093	21.12

Table.4 Results of cell injury analysis by biosurfactant

Samples	Concentration (mg/ml)	Before incubation (260 nm)	After incubation (260 nm)
Biosurfactant	1.0	0.6	1
	2.0	0.6	1
	2.5	0.6	1
	3.0	0.6	1
	3.5	0.587	1
	4.0	0.6	1
	4.5	0.6	1
	5.0	0.6	1
Triton X 100	10 (µl/ml)	0.6	1
Amphotericin	5. 0	0.6	1
Ketoconazole	5. 0	0.6	1
Fluconazole	5. 0	0.6	1
Nystatin	5. 0	0.6	1
Control	0.0	0.6	0.67

Table.5 Biophotometer result for cell injury analysis

Samples	Concentration (mg/ml)	Values for DNA		OD at 280 nm for protein
		µg / ml	purity	
Biosurfactant	2.0	14.3	1.17	0.612
	2.5	12.9	1.11	0.243
	3.0	35.2	1.12	0.723
	3.5	31.3	1.28	0.746
	4.0	37.3	1.65	0.785
	4.5	41.0	1.25	0.768
	5.0	40.2	1.25	0.772
Triton X 100	10 µl / ml	28.3	0.35	0.662
Amphotericin	5. 0	1.7	0.39	0.129
Ketoconazole	5. 0	32.7	0. 38	0.726
Fluconazole	5. 0	34.4	0.39	0.760

Plate.1 Emulsification activity

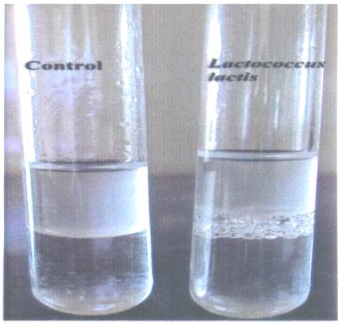
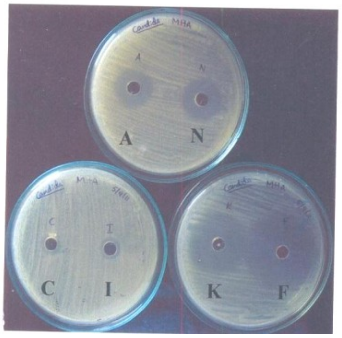


Plate.2 Anbiogram pattern of isolated *Candida albicans* of isolate



A → Amphotericin B

N→ Nystatin

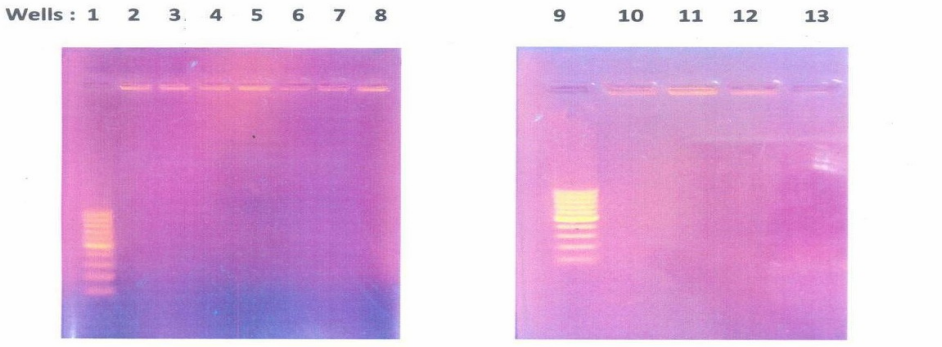
C→ Clotrimazole

I→ Itraconazole

K→ Ketaconazole

F→ Fluconazole

Plate.3 Analysis of release of DNA from lysed *Candida albicans* cell by biosurfactant



Well 1: 100bp DNA ladder

Well 2: 2 mg/ml (biosurfactants treated with *Candida*)

Well 3: 2.5 mg/ml

Well4: 3mg/ml

Well 5: 3.5mg/ml

Well 6: 4 mg/ml

Well 7: 4.5mg/ml

Well8: 5mg/ml

Well 9: 100 bp DNA ladder

Well 10: Triton X 100

Well 11: Amphotericin B

Well 12: Nystatin

Well 13: Untreated *Candida albicans*

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