EXTRACTION & CHARACTERIZATION OF A BIOSURFACTANT FROM *Lactobacilli species*AND DETERMINATION OF IT'S ANTI-BIOFILM ACTIVITY ON URINARY TRACT INFECTION (UTI) CAUSING PATHOGENS

[1]Tafadzwa Kaurayi, [1]Lilian R Tomu, [1]Umang R Solanki, [1]Vinay M Tripathi, [2]Karishma Sawardekar
[1]Department of Biotechnology
[1]Chikitsak Samuha's S.S & L.S. Patkar College of Arts & Science, and V.P Varde College of Commerce & Economics, Mumbai, India

***Abstract*:**Biosurfactants (BS) are surface active agents produced by yeasts, bacteria and fungi. These biosurfactants are amphiphilic molecules consisting of hydrophilic and hydrophobic domain. In this study, bacterial strains with the potential of producing a biosurfactant were isolated from cheddar cheese obtained from a local supermarket in Goregaon West, Mumbai, India. The bacteria was identified as *Lactobacilli species*. The ability of isolated strains to produce a biosurfactant was determined by haemolysis test on blood agar. The activity of the biosurfactant obtained from bacteria was tested by standard qualitative methods. The biosurfactant was found to have both emulsifying activity and surface tension reduction. Based on biochemical and TLC tests, the extracted biosurfactant was found to be a lipopeptide. In-vitro analysis of antimicrobial, anti-adhesive andanti-biofilmabilities of the extracted biosurfactant was determined on six biofilm producing uropathogens which include *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis,Pseudomonas aeruginosa, Candida albicans* and *Staphylococcus species*. The extracted biosurfactant displayed anti-adhesive, anti-biofilm and antimicrobial abilities against all the above mentioned microorganisms. Highest anti-adhesive and antimicrobial activities were observed against *E. coli, Staphylococcus species*, *C. albicans* and *P. aeruginosa* however *K. pneumoniae* and *P. mirabilis* showed less susceptibility. Together, these capabilities may open up possibilities for biosurfactants as an alternative therapeutic approach for the prevention and treatment of Urinary Tract Infections.

Index Terms - Biofilm, Biosurfactant, anti-biofilm, antimicrobial, uropathogens

I.INTRODUCTION

Biosurfactants are biological surface-active compounds produced by microorganisms that can have some influence on interfaces. With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes (Lígia Rodrigues, 2015). Biosurfactants have also been reported to have various degrees of antimicrobial activity (Surekha K. et al.,2016). Bacteria are the main group of biosurfactant-producing microorganisms, although they are also produced by some yeasts and filamentous fungi. The best studied biosurfactants are those from *Bacillus* and *Pseudomonas* genera. However, a number of studies have reported the potential of *Lactobacilli* as biosurfactant producers. Biosurfactants isolated from several *Lactobacilli* have been characterized as multicomponentmixtures, consisting of protein and polysaccharides; in other cases, the surface-active compounds were identified as glycolipids.

The increased interest in the biosurfactant producing *Lactobacilli species* is related to the well-known probiotic effects of these microorganisms which are natural components of human microbiota as well as to the ability of such bacteria to inhibit pathogenic bacteria and fungi (C.P Cornea et al. 2016). Biosurfactants also reduce adhesion of pathogenic microorganisms to glass, silicone rubber, surgical implants etc. (Sharma. 2014).Studies have demonstrated that two strains of *L. plantarum* and one strain of *L. brevis* were able to produce cell-bound and excreted biosurfactants(C.P Cornea et al. 2016). Comparing with chemical surfactants, these compounds have several advantages such as lower toxicity, higher biodegradability, and effectiveness at extreme temperatures and pH values. Biosurfactants produced from *Lactobacilli species*also have antimicrobial and anti-adhesive property, thus can be used for inhibition of biofilm formation.

Biofilm is a complex structure adhering to surfaces and consists of colonies of bacteria. Yeast, fungi and protozoa secrete a mucilaginous protective coating in which they are encased. Biofilms can be formed on solid or liquid surfaces as well as on soft

tissue in living organism, and they are typically resistant to conventional methods of disinfections (S. Kaur. 2015).Biofilms are generally pathogenic in the body, causing diseases such as cystic fibrosis and otitis media. Adhesion is the first stage of biofilm formation and the best method of preventing biofilms is to apply anti-adhesive and anti-biofilm compounds. Adsorption of biosurfactants to a surface e.g. glass, polystyrene, silicone modifies its hydrophobicity, interfering with the microbial adhesion and desorption processes. Owing to the biofilm inhibition and antimicrobial property of biosurfactants, it can be utilized as a potential therapeutic molecule for numerous microbial infections (S. Kaur. 2015). In this study the aim was to study the effect of a biosurfactant on biofilms of UTI associated pathogens.

II. MATERIALS AND METHODS

Isolation of BS producing organisms

Cheddar cheese, amul cheese& curd sample was collected from a local supermarket. De Man, Rogosa and Sharpe agar (MRS) was used to isolate the organism from given sample. Characterization of isolated microorganism was done by Gram staining and biochemical tests and results were compared with that of Bergey's manual. The ability of the isolate 3 to produce a BS was determined by haemolysis test on blood agar&was used to produce BS. (Lígia Rodrigues, 2015).

Production of BS

Enrichment was done by inoculating 25 ml of culturesuspension of isolated organisms (OD 0.1 at 520 nm)in 400 ml of MRS broth, incubated at 37^0C for 24 hours under static conditions. The mixture was centrifuged at 3000 rpm for 30 minutes. A thick culture suspension was prepared by re suspending the pellet in 8 ml of sterile saline. BS production was done by inoculating 8 ml of the thick culture suspension into 400 ml of MRS broth, incubated at 37^0C for 48 hours under shaker conditions (100 rpm). The mixture was centrifuged at 6000 rpm for 30 minutes at 4°C.The obtained pellet was re suspended in 100 ml of Phosphate Buffer Saline (PBS) of pH 7.0 and it was incubated at room temperature for 2 hours on shaker. This mixture was centrifuged at 6000 rpm for 10 minutes at 4^0C. Supernatant was used as crude biosurfactant (Sharma, 2014)

Partial purification

Biosurfactant was partially purified by dialysis method. The efficiency of dialysis was monitored by phosphate and chloride tests.

Characterization of BS

Emulsification Test: 2 ml of Kerosine/Motor oil was added in a test tube containing 2 ml of the extracted BS. Forstandard 2 ml of kerosene was added in test tube containing 2ml of SDS. Mixture was vortexed for 5 minutes and then incubated at RT for 24 hours (C.P Cornea et al, 2016).

Emulsification activity was calculated as follows:

$$\text{Emulsification index } (EI_{24}) = \frac{\text{Height of the emulsified layer}}{\text{Height of the total liquid column}} \times 100$$

Oil spread test: 10 µl of Kerosine was added on the surface of 50 ml of distilled water in a big petri dish followed by addition of 3 drops of crystal violet.10µl of the extracted BS was dropped at the center of the oil layer. The displaced diameter of oil was measured after 30 seconds (Sumaiya. M, 2017).

Quantitative analysis of proteins and carbohydrates was carried out using Folin Lowry's test and Anthrone test respectively.

Thin Layer Chromatography (TLC): To determine if the extracted BS is lipopeptide or glycolipid in nature TLC was carried out (Sharma, 2014). Silica gel plates were prepared. A spot of the extracted sample was placed at the center of the baseline marked 1cm from the edge of the plate. Mobile phase used was chloroform: methanol: water in the ratio 90:10:5 respectively. The plates were sprayed with ninhydrin and anthrone reagent.

Anti-biofilm activity of BS

Biofilms of microorganisms were formed in-vitro by following Christen et al. Crystal Violet Tube Assay (Triveda, 2016). Effects of biosurfactant on the biofilm formation of *E. coli*, *Pseudomonas*, *Proteus mirabilis, C. albicans*, *Staphylococcus species* and *Klebsiellapneumonia* was determined by co-inoculating 250 µl of the biosurfactant with each of the test cultures adjusted to 0.1 OD at 530nm in 5 ml of Tryptic Soy Broth and incubating the culture tubes for different time periods (24 hours, 48 hours, 72 hours, 96 hours and 120 hours). Controls were maintained containing Tryptic Soy Broth inoculated with culture without BS. After each time interval the growth medium was discarded and tubes were washed with sterile PBS to remove unbound bacteria .The remaining bacteria were fixed with 5ml of 99% methanol for 10 minutes. Tubes were emptied and dried. The attached film was

stained for 5 minutes with 5 ml of 2% Crystal violet. The tubes were emptied again and the dye attached to the cells was dissolved in 5 ml of 33% Glacial acetic acid. Quantitative estimation of produced biofilms was done by colorimetric method at 620 nm.

Antimicrobial activity

The produced BS was tested for antimicrobial activity by disc diffusion method on *E.coli*, *Pseudomonas*, *Proteus,C albicans*, *Staphylococcus* and *Klebsiella*. 10mcg Gentamycin standard antibiotic discs were used as positive control and 1% PBS as negative control.

III. RESULTS

Isolation of BS producing organisms

Table 1: Characterization of isolates

Morphological Characteristics	ISOLATE 1	ISOLATE 2	ISOLATE 3	ISOLATE 4	Lactobacillus Characteristics (Bergey's Manual)
Color	Cream-white	Cream-white	Cream-white	Cream-white	Cream-white
Shape	circular	circular	Circular	circular	Circular
Size	0.6µm	0.9µm	0.5µm	10µm	0.5µm-0.8µm
Motility	Non motile	Non motile	Non-motile	Non motile	Non motile
Gram Nature	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive
Shape	Spherical	Spherical	Bacilli	Spherical	Bacilli

Isolate 1 and 2 were obtained from Amul cheese, isolate 3 from Cheddar cheese and isolate 4 from curd. Biochemical tests were done only for isolate 3 since it shows all similar characteristics as that of *lactobacillus* and also shows positive hemolysis activity on blood agar confirming its BS producing capabilities

Figure 1: Hemolysis activity

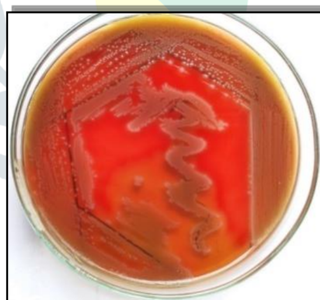


Table 2: Biochemical Characterization of Isolate 3

Biochemical Characterization	ISOLATE 3	Lactobacillus Characteristics (Bergey's Manual)
Indole	-	-
Methyl red	-	-
Voges proskauer	-	-
Citrate	-	-
TSI	-	-
Catalase	-	-
Sugar fermentation		

dextrose	+	+
fructose	+	+
Lactose	-	+
Xylose	-	-

Key: + Positive Result
- Negative Result

Characterization of BS

In oil drop test, anoil displacement measuring 28cm was observed showing biosurfactant activity of the extracted sample.

TLC: Two orange spots developed upon spraying the plate with ninhydrin reagent & no spot development was observed when sprayed with anthrone reagent. The amount of proteins in the BS was found to be higher i.e. 110µg/ml compared to carbohydrates i.e. 13µg/ml. This suggests that theBS may be lipopeptide in nature (Sharma, 2014)

Emulsification index was calculated to be 60.8% and this value is above 50% hence the extracted BS has surfactant activity.

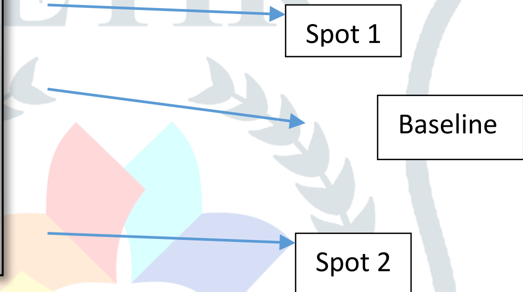
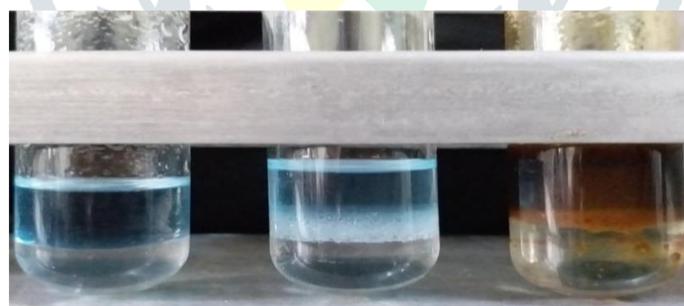


Figure 2: Oil spread test Figure 3: TLC Plate

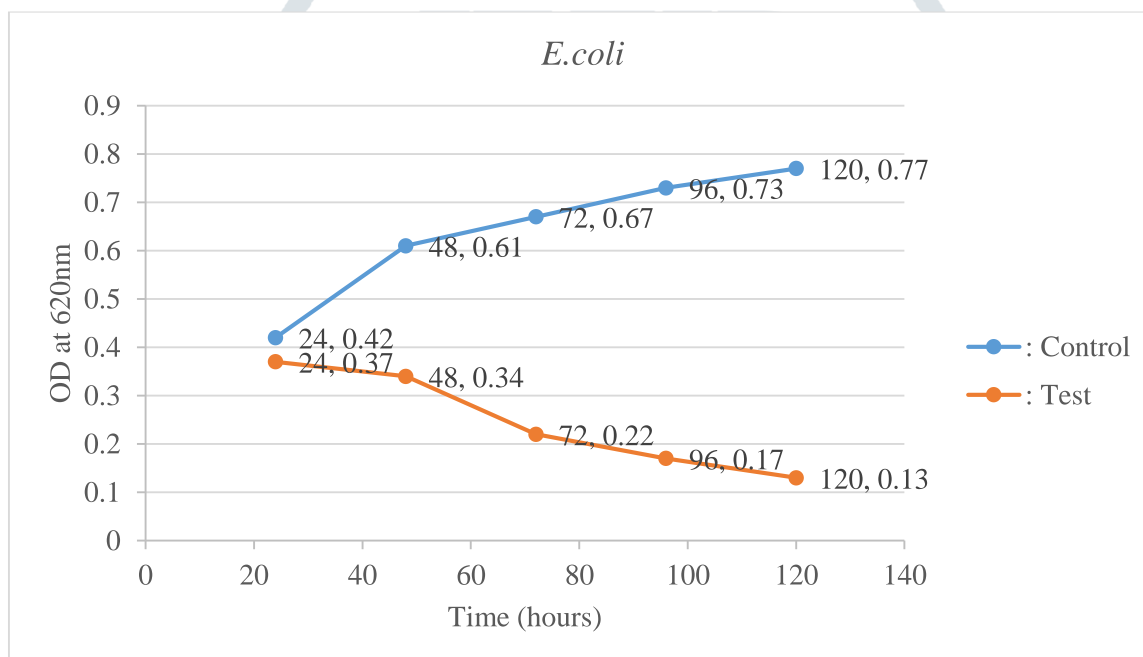


BS on Kerosine SDS on Kerosine BS on Motor oil

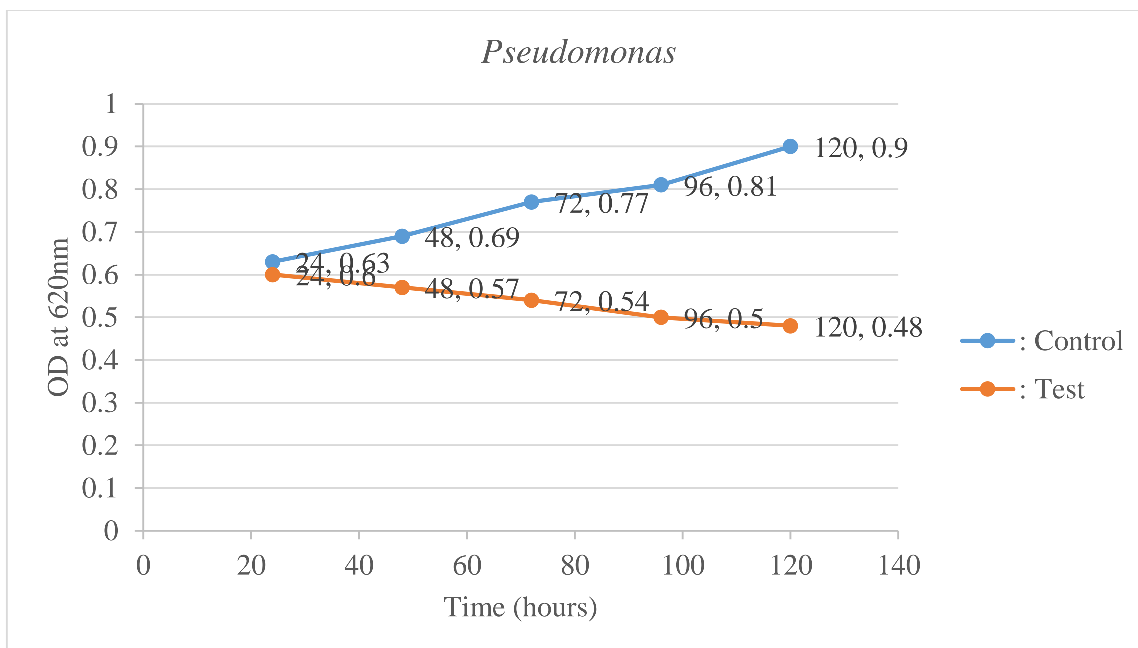
Figure 4: Emulsification Test

Figure 5: Biofilm formation Figure 6: CV tube assay

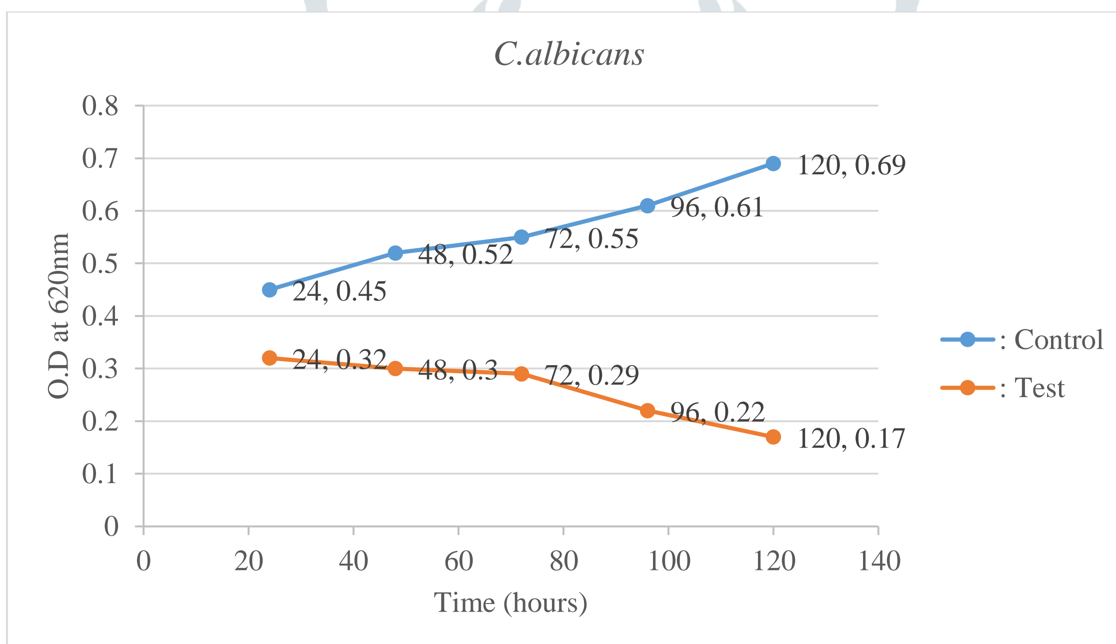
Anti-Biofilm activity of BS on test organisms by Crystal violet tube assay



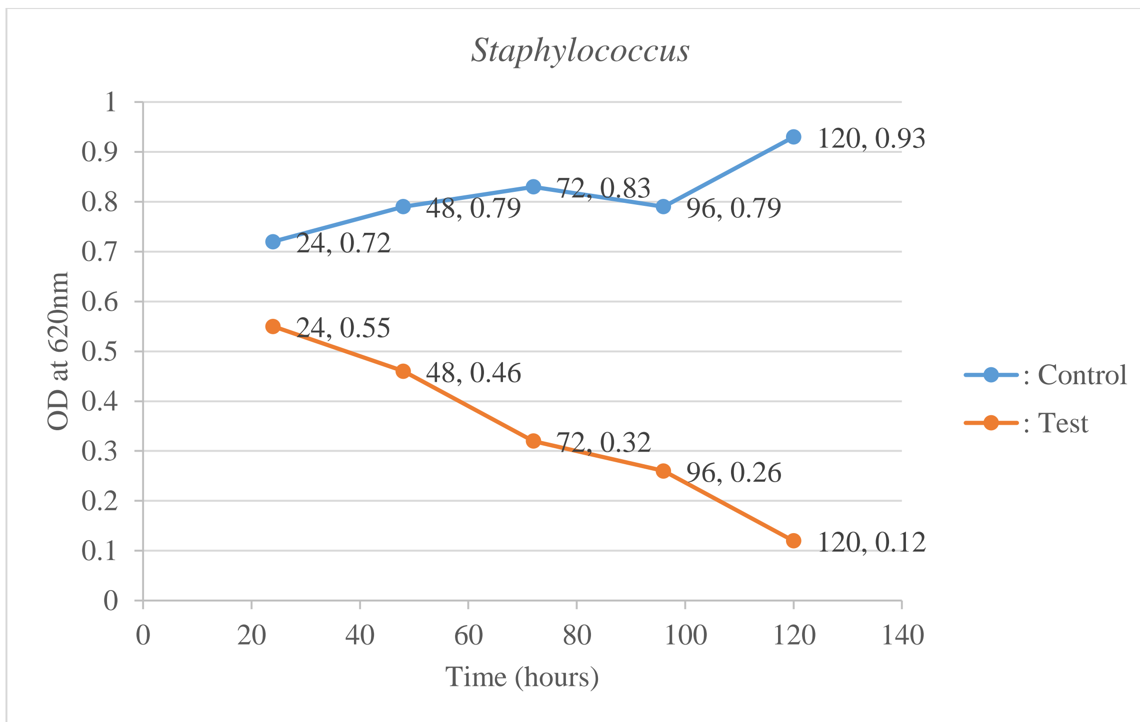
Graph 1: Effect on *E. coli* biofilm formation in the presence & absence of BS over 120 hours of incubation.



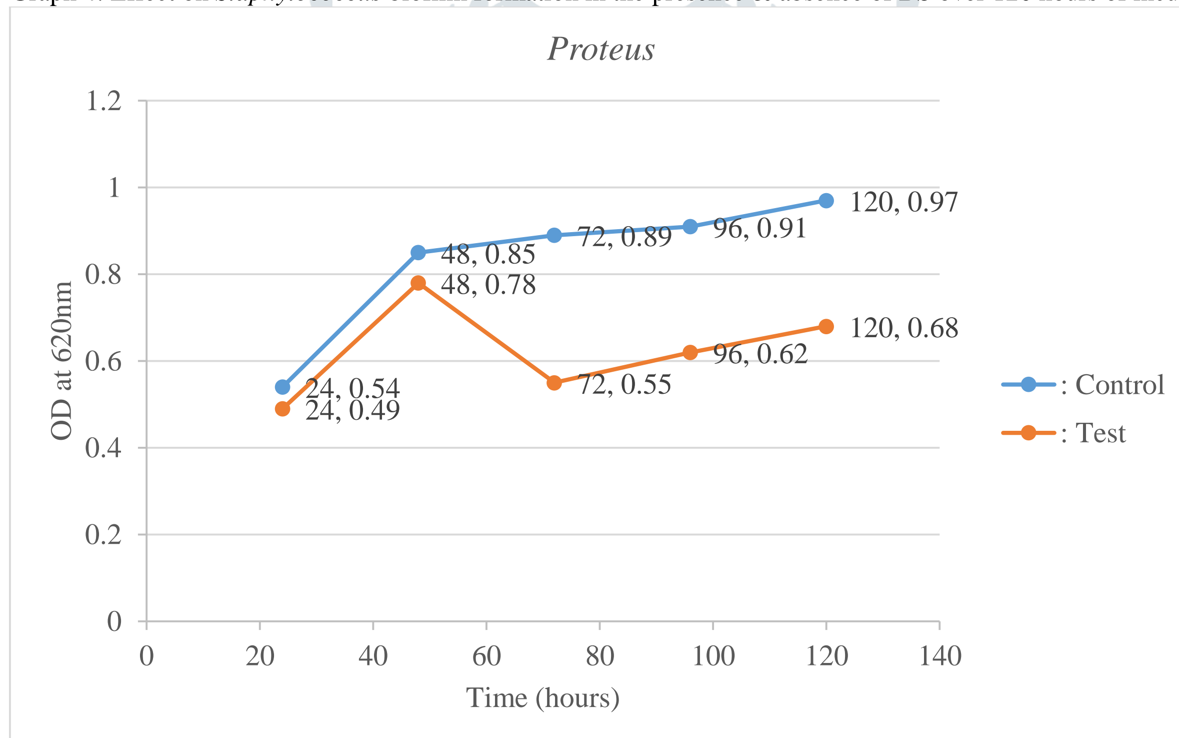
Graph 2: Effect on *Pseudomonas* biofilm formation in the presence & absence of BS over 120 hours of incubation.



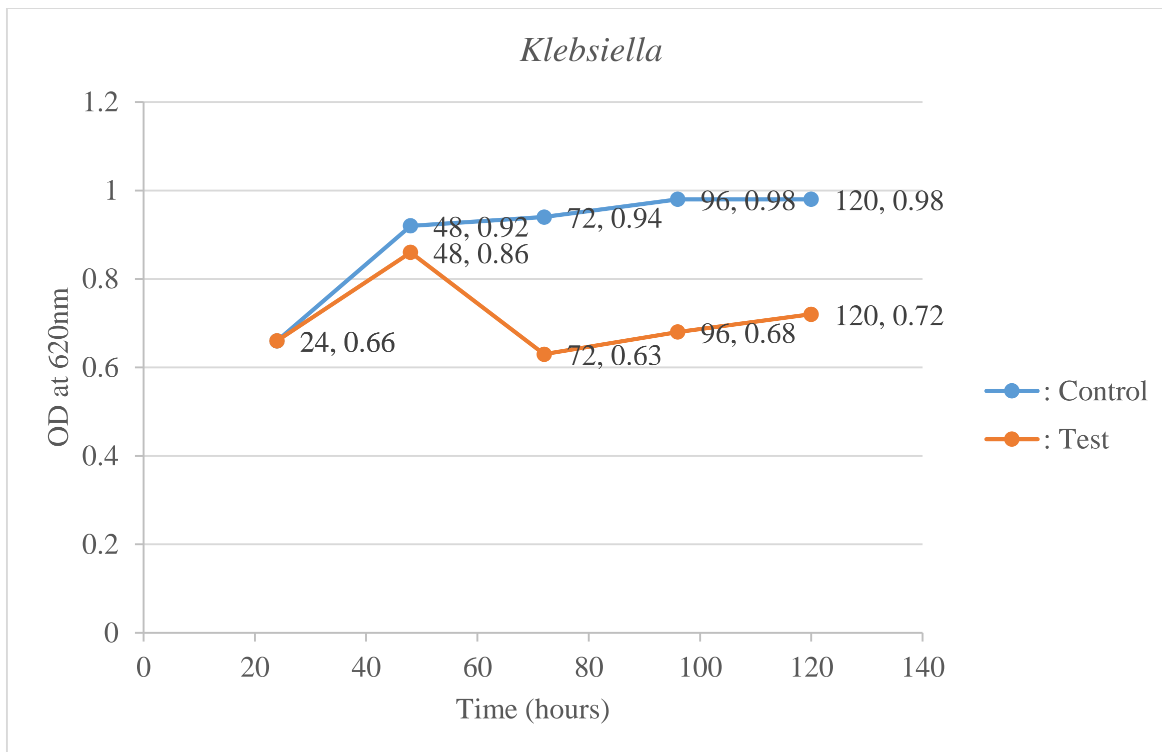
Graph 3: Effect on *C. albicans* biofilm formation in the presence & absence of BS over 120 hours of incubation.



Graph 4: Effect on *Staphylococcus* biofilm formation in the presence & absence of BS over 120 hours of incubation.



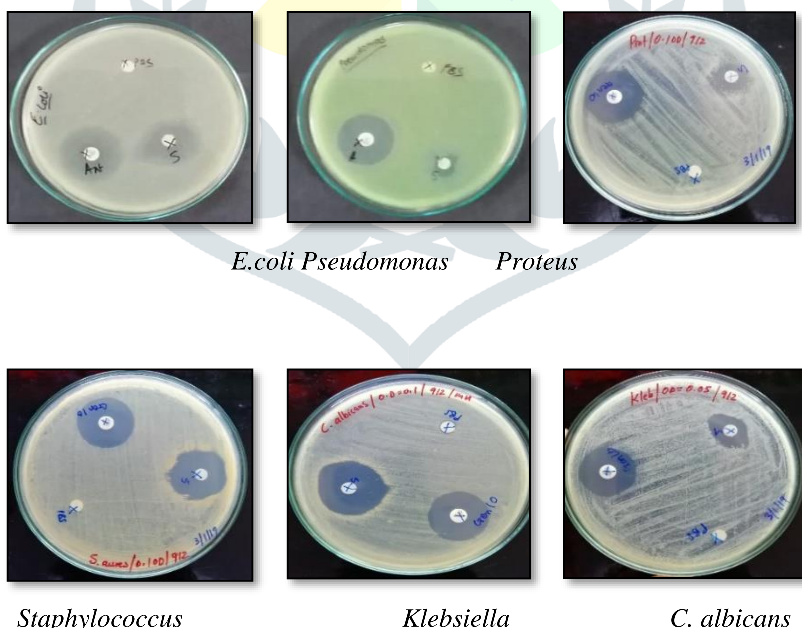
Graph 5: Effect on *Proteus* biofilm formation in the presence & absence of BS over 120 hours of incubation.



Graph 6: Effect on *Klebsiella* biofilm formation in the presence & absence of BS over 120 hours of incubation.

In Graph 1, 2, 3 and 4 O.D of Test decreases sharply with increase in incubation time & the O.D of test is less than that of control. Thus, BS shows an anti-biofilm activity towards *E. coli, Pseudomonas, Staphylococcus*&*C. albicans*. While in Graph 5 and 6 O.D of Test decreases after 48 hours and slightly increases after 72 hours of incubation. However, O.D of test seen to be less than that of control. Thus, BS might be less effective towards *Proteus*&*Klebsiella*.

Antimicrobial Sensitivity Tests (AST) of BS against test organisms



E.coli Pseudomonas *Proteus*

Staphylococcus *Klebsiella* *C. albicans*

Figure 7: Zones of inhibition observed on AST plates.

Table 3:The diameter of the zones of inhibition observed on the AST plates

Test organism	Diameter of the zone of inhibition (mm)	
	Positive control	Test (BS)

	(gentamycin)	
E. coli	25	17
Proteus	22	10
Pseudomonas	23	8
Klebsiella	17	11
Staphylococcus	26	22
C. albicans	24	18

IV. DISCUSSION

Urinary tract infections are in general nosocomial, affecting compromised patients who receive broad-spectrum antibiotic therapy and often with indwelling urinary catheters and endotracheal tubes. One of the main problems associated with UTI infections is increasing resistance against a great number of antibiotics through biofilms formation. Increasing problems of resistance to synthetic antimicrobials have encouraged the researchers to focus on alternative natural products such as probiotic bacteria to combat resistance of pathogens toward antibiotic therapy (S. Kaur. 2015). Biosurfactants are one of the natural products, on which many studies have been carried out to determine their potential as antimicrobials and anti-biofilms. Microorganisms such as lactic acid bacteria have been found to be biosurfactant- producing strains.

Biosurfactants are amphiphilicmolecules produced by microbes mostly on their cell surface or secreted extracellularly, and can reduce the surface or interfacial tension in liquids as they contain both hydrophobic and hydrophilic moieties (J. Arutchelvi. 2009). Biosurfactants can also change the surface tension and bacterial cell-wall charge (Ibrahim M. Banat et al. 2010). These factors are very important in overcoming the initial electrostatic repulsion barrier between the microorganism cell surface and its substrate.

This theory can be supported by the results of this study. As illustrated by graph 1, 2, 3 and 4, the adhesion of *E. coli, C. albicans, Staphylococcus* and *Pseudomonas* to glass tube could be reduced by co-inoculating with BS. From table 3 & figure 7, it can be stated that BS also showed antimicrobial effect against all the test cultures. A zone of inhibition was observed with respective to *E. coli, Pseudomonas, Staphylococcus* and *C. albicans* (O.D = 0.1)and for *Klebsiella* and *Proteus* a zone was observed at (O.D = 0.05) as at 0.1 OD no zone of inhibition was seen for these two organisms. The resistance of *Klebsiella* can be explained by the polysaccharide capsule and the inner layer of the capsule is formed by a palisade of thick and dense bundles of the fibers standing at right angles on the surface of the outer membrane. In the outer layer these thick bundles of fibers loosened into fine fibers which spread over the bacterial surface, forming a fine network structure.This may confer some form of resistance to the BS hence more concentration of the BS may be required to show antibacterial action against *Klebsiella.* The mechanism of antifungal activity of biosurfactant from probiotic bacteria is due to the cell permeabilizing and cellular damaging ability of the BS. This may explain the high susceptibility of *C. albicans* to the extracted BS (P. Saravanakumari et al. 2015).

V. CONCLUSION

The isolates from the cheddar cheese were identified to be *Lactobacilli species*by various tests and comparing them with Bergeys Manual and its BS producing capability was confirmed by haemolysis activity seen on blood agar. Extracted biosurfactant was characterized by determining its emulsification index and oil spreading capabilities. The BS was found to be a lipopeptide in nature by TLC. The anti-biofilm activity of the extracted biosurfactants was examined on UTI causing pathogens: *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus species* and *Candida albicans.* The results showed that co-inoculation of cultures with biosurfactants was effective in preventing biofilm formation of culture. The BS was more effective on *E. coli, Staphylococcus, Pseudomonas* and *Candida.* Whilewas less effective in preventing biofilm formation of *Proteus and Klebsiella*. BS also showed antimicrobial properties. Therefore, the extracted BS might have both anti-adhesive and antimicrobial properties towards test culture. Since biosurfactants proved inhibitory, there is a potential of *Lactobacilli* species, producing biosurfactants, to prevent biofilm formation of *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus species* and *Candida albicans* on medical devices such as catheters and hence reduce the number of biofilm forming uropathogens that are nosocomial in nature.As a future prospective, FTIR can be done to determine the molecular and atomic composition of the extracted BS. Further purification by advanced chromatographic techniques such as UPLC needs to be done. The anti-adhesive properties of the BS can be used in combination with small concentrations of antibiotics to produce a synergistic effect against nosocomial infections. Application of the BS as a component of toothpaste to prevent tooth decay causing bacteria from adhering to the teeth enamel. Further studies can be done to incorporate the BS in cosmeceuticals as a preservative agent. The BS can be used as an anti-bio fouling agent.

REFERENCES

[1] A. Sharma . (2014). A Study on biosurfactant production in *Lactobacillus* and *Bacillus sp*. International Journal of Current Microbiology and Applied science, 723-733.

[2] C.P Cornea . (2016). Biosurfactant production by *Lactobacillus spp*. Romanian Biotechnological Letters, 1-2.

[3] Chowdhury A, Hossain Md.N, Mostazir NJ, Fakruddin Md, Md. Billah M. (2012). Screening of *Lactobacillus spp*. from Buffalo Yoghurt for Probiotic and Antibacterial Activity. J Bacteriol Parasitol 3:156. doi:10, 4172/2155-9597.

[4] Croes, S., Deurenberg, R. H., Boumans, M-L. L., Beisser, P. S., Neef, C., & Stobberingh, E. E. (2009). *Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S.aureus* lineage. BMC Microbiology, 9, [229]. DOI: 10, 186/1471-2180-9-229.

[5] Deepansh Sharma, Baljeet Singh Saharan, Nikhil Chauhan, Suresh Procha and Sohan Lal. (2015). Isolation and functional characterization of novel biosurfactant produced by *Enterococcus faecium*. SpringerPlus, 1-14.

[6] Ibrahim M. Banat . (2010). Microbial biosurfactants production, applications. Applied Microbiology and Biotechnology, 427-444.

[7] J. Arutchelvi . (2009). Production and Characterization of Biosurfactant from *Bacillus subtilis YB7*. Journal of Applied Sciences, 3151-3155.

[8] Kalyani A.L.T, Naga Sireesha G, Aditya A.K.G, Girija Sankar G. (2014). Isolation and antimicrobial activity of rhamnolipid (biosurfactant) from oil-contaminated soil sample using humic-acid salts-vitamin agar. IJRET: International Journal of Research in Engineering and Technology, 2321-7308.

[9] Kavitha, P., D. Sindhuja and Banumathi, M. . (2016). Isolation and Biochemical Characterization of *Lactobacillus spp* from Dahi. Int.J.Curr.Microbiol.App.Sci. 5(4, 1042-1049.

[10] Lígia Rodrigues . (2015). Modeling of Biosurfactant Production by *Lactobacillus*. Researchgate, 1.

[11] Mohammad Irfan . (2015). In Vitro Synergistic Effect of Biosurfactant Produced by *Bacillus subtilis.MTCC 441* against drug resistant Staphylococcus aureus . Journal of Applied Pharmaceutical Science.113-116.

[12] Nermeen Mahmoud Ahmed Abdallah, Shereen Bendary Elsayed, Manal Mohamed Yassin. (2011). Biofilm forming bacteria isolated from urinary tract infection, relation to catheterization and susceptibility to antibiotics. International Journal for Biotechnology and Molecular Biology Research Vol. 2(10), 172-178.

[13] P. Saravanakumari . (2015). Mechanisims of control of *C. albicans* by biosurfactants puried from *Lactococcus lactis*. International Journal of current Microbilogy and Applied Sciences 4 (2), 529-545.

[14] Parvaneh Mesgari and Masoud Hosseinzadeh. (2016). Isolation and Identification of *Lactobacillus acidophilus* and *Bifidobacterium f*rom Different Types of (Traditional) Cheese and Study Their Antibacterial Properties . International Journal of Advanced Biotechnology and Research (IJBR), 275-281.

[15] S. Kaur . (2015). In vitro biosurfactant production and biofilm inhibition. International Journal of Probiotics and Prebiotics, 17-22.

[16] S. Vijayakumar and V. Saravanan. (2010). Biosurfactants-Types, Sources and Applications. Research Journal of Microbiology 10 (5), 1-18.

[17] Salta, M. (2013). Anti-Biofilm Performance of Three Natural Products against Initial Bacterial Attachment. Int. J.Mol. Sci, 21757-21780.

[18] Shokouhfard M, Kasra Kermanshahi R, Vahedi Shahandashti R, Feizabadi MM, Teimourian Sh. (2015). The Inhibitory effect of a *Lactobacillus acidophilus* derived biosurfactant on biofilm producer *Serratia marcescens.*Iran J Basic Med Sci, 1001-1007.

[19] Su¨nje Johanna Pamp and Tim Tolker-Nielsen. (2007). Multiple Roles of Biosurfactants in Structural Biofilm Development by *Pseudomonas aeruginosa*. Journal of bacteriology Vol. 189, No. 6, 2531–2539.

[20] Sukirtha TH, Usharani MV. (2013). Production and Qualitative Analysis of Biosurfactant and Biodegradation of the Organophosphate by *Nocardia mediterranie*. J Bioremed Biodeg 4 :198, 4172/2155-6199.

[21] Sumaiya.M, Anchana Devi .C & Leela K. (2017). A Study on Biosurfactant Production from Marine Bacteria. . International Journal of Scientific and Research Publications, Volume 7, Issue 12, 2250-3153.

[22] Surekha K. (2010). Methods for investigating Biosurfactants and Bioemulsifiers: a review. Critical reviews in Biotechnology, 1–18.

[23] Surekha K. (2016). Biosurfactant/s from Lactobacilli species. Journal of Basic Microbilogy, 1-2.