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Characterization of an exopolysaccharide from probiont *Enterobacter faecalis* MSI12 and its effect on the disruption of *Candida albicans* biofilm

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Abstract

Biofilm-forming pathogens are potential threat to indwelling medical devices and infectious diseases. Management of device-associated *Candida* infections remains to be challenging to the existing drug discovery platforms. The available antifungal drugs are effective on the control of free-living pathogens but not effective on the biofilm-film forming pathogens. Thus antifungal drug synergized with antibiofilm agent would be an effective strategy to treat *Candida* biofilm. The mature *C. albicans* biofilms are anchored by complex architecture in terms of distribution of fungal cells stabilized by exocellular polymeric substances. Findings of the present study bring out a new insight on the possible development of enterococci probiotics and/or its expolysaccharide (EPS) as synergistic with existing antifungal drugs to treat biofilm infections. The probiont *Enterococcus faecalis* MSI12 was picked from 142 seawater isolates screened for EPS production using congo red plate assay. The probiotic characteristics of the isolate MSI12 was evaluated based on the temperature, pH, acid tolerance, autoaggregation, hydrophobicity and antioxidant activity. The biofilm disruption ability of the lyophilized EPS was determined in microtitre plate assay using fluconazole as reference drug. The scanning electron microscope and confocal laser scanning microscope images were used for analysis of antibiofilm activity. The cell viability of *E. faecalis*MSI12 was very high at higher temperature, acidic pH, bile salt and salt concentration when compared to the reference strain *Lactobacillus plantarum*. Therefore the strain MSI12 might survive in the niche like human gut without prebiotics. The EPS from *Enterococcus* sp. MSI12 showed significant reduction of treated *Candida* biofilm. The antibiofilm potential of EPS was very stronger than standard antifungal drug fluconazole. This study revealed that biofilm disruption/control using a probiontEPS could deliver a synergistic

approach as the probiotic strain can colonize in the host to prevent the formation of *candida* biofilms.

Key words: *Candida albicans*, biofilm, biofilm-disruption, antibiofilm activity, probiotic enterococcus, expolysaccharide (EPS), antifungal

1 Introduction

Biofilms are formed by the biomass of bacterial cells housed in exo-polymeric substances on biotic and abiotic surfaces. Biofilm-forming pathogens are a major threat to human health and challenge to existing drug discovery platforms. It was confirmed that up to 80% of clinical infections are progressed to biofilm and formation of biofilm on indwelling medical devices are responsible for a large proportion of nosocomial infections.[1] The pathogenic species of the genus *Candida* have gained recognition as nosocomial agents in recent years which may be attributed to specific risk factors associated with modern therapeutics like immunosuppressive agents, cytotoxic drugs, and broad-spectrum antibiotics that suppress the normal bacterial microbiota. Candidiasis is normally associated with indwelling medical devices such as dental implants,[2] catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints, central nervous system shunts etc. which can act as abiotic substrates for biofilm growth.[3] Even with current antifungal therapy, mortality of patients with invasive candidiasis can be as high as 40%.[3,4] The biofilm is a shield of progressive pathogenic *Candida* cells that facilitate escape from the host immune system and dramatically reduced susceptibility to clinically used antifungal drugs like fluconazole, nystatin, amphotericin B, and chlorhexidine.[3] The available antifungal drugs are effective on the control of *Candida* pathogens but not effective on their biofilm as the drugs cannot cross biofilm boundary to target the cells. The exo-polymer matrix in the biofilm can restrict the access to the antimicrobial agents or the microbial cells present deep in the colony escape the drug access or changes in the membrane sterol composition during biofilm development makes the microbial cells resistant to antibiotics.[5] Thus antifungal agent synergized with antibiofilm agents would be an effective strategy to treat *Candida* biofilm.

The genus *Enterococcus* belongs to lactic acid bacteria (LAB), a prominent group of probiotics in human and animal healthcare. This genus was typically characterized as non-spore forming Gram-positive bacteria which appear as cocci, diplococci or short chains, and produce lactic acid as the major end product of glucose fermentation. [4] This genus was proven group of probiotics and third-largest genus of LAB after the genera *Lactobacillus* and *Streptococcus*.[5] Among the LAB, the enterococci can be designated as robust probiotics due to their highest salt, temperature and pH tolerance. The well-characterized and commercial probiotics in the genus enterococci was *E. faecium* SF68 that is being used in the treatment of diarrhoea.[7] Literature showed that enterococci probiotics was effective in the prevention of antibiotic-associated diarrhoea.[6] But their potential on the disruption/prevention of pathogenic biofilm has not been reported. The enterococci genus occurred in diverse habitats including soil, surface waters, ocean water, sewage, on plants and gastrointestinal tract.[6] In this study, the isolation was performed from seawater to isolate enterococci genus with high salt, pH and temperature tolerance. Marine ecosystems represent a large and as yet largely under explored reservoir of biodiversity with respect to industrially useful enzymes, bioactive molecules and biosurfactants. These ecosystems can range from coastal environments to deep-sea hydrothermal vents with high hydrostatic pressure and temperatures as high as approximately 400 °C.[8]

Exopolysaccharides (EPSs) are carbohydrate polymers that make up a substantial component of the extracellular polymers surrounding most microbial cells in the marine environment.[9] The EPS support the microbial communities to survive extremes of temperature, salinity, and nutrient limitation. The EPS can be used as bioflocculants for synthesis of silver nanoparticles, bioabsorbents, encapsulating materials, heavy metal removing agents, drug delivery agents, a

natural immunomodulator, antioxidant and antibiofilm agent.[10,11,12] It was reported that mature *C. albicans* biofilms have a highly complex architecture in terms of distribution of fungal cells in extracellular material.[3] The common antifungal drugs are not effective to treat established (biofilm) *Candida* infections. We hypothesized that the probiotics with antibiofilm potential would be a synergistic approach to treat established pathogens. Findings of the present study bring out a new insight on the possible development of enterococci probiotics and it's EPS as synergistic to the antifungal drugs being used for antiinfective therapy. In this study, we report the EPS from a probiotic strain *Enterococcus faecalis* MSI12 isolated from the marine environment and its potential as antibiofilm agent and/or disrupting the pre-established biofilms of the most prominent human pathogen *Candida albicans*. This is a first report of probiotic EPS produced by marine *E. faecalis* disrupting the pathogenic biofilm of *C. albicans*.

2. Materials and Methods

2.1 Isolation and identification of EPS producing marine bacterium

Seawater samples were collected aseptically at 150 m depth from the coastal region of Pondicherry, India (11° 52' 56 N"; 11° 59' 53" E). Bacterial isolation was performed on Zobell marine agar (Himedia). Based on the stability in sub-culturing steps, 142 isolates were screened for EPS production. The congo red plate assay was performed for screening of EPS producing isolates. The stain congo red bind to the extracellular matrix such as polysaccharides and cellulose to give a dark-red stained background around the colony.[13] Among the 11 positive isolates, the maximum EPS producing isolate MSI12 was selected for further characterization. The selected isolate MSI12 was identified by 16S rRNA gene sequence analysis. Briefly,

genomic DNA from strain MSI12 was extracted with suitable modification in the previously described method.[14] The 16S rRNA gene was amplified using universal primers 8F (5′- AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′- GGT TAC CTT GTT ACG ACT T-3′) and the gene product was cloned using the TOPO TA cloning kit according to the manufacturer's instructions. The 16S rRNA sequence of the isolate was then analyzed using the megaBLAST algorithm of GenBank (http://www.ncbi.nlm.nih.gov/genbank). Sequences of the isolate MSI12 was confirmed with seqmatch program of RDPII (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). Multiple alignments of these sequences were performed using Clustal W 1.83 version of EBI (http://www.ebi.ac.uk/cgi-bin/clustalw/) with 0.5 transition weight. Phylogenetic trees were constructed using MEGA 6.0 version (http://www.megasoftware.net) with maximum parsimony algorithm. The maximum parsimony bootstrapping was performed with 1,000 replicates with pseudorandom number generators. Nucleotide composition of each aligned sequence was predicted by BioEdit software. The nucleotide sequences were deposited in the genebank with an accession number KR559728.

2.2 Production and quantification of EPS

The bacterium MSI12 was inoculated in 250 ml of Zobell marine broth and incubated at 30°C for 72 h at 200 rpm on a shaking incubator (Orbitek). After incubation the culture was centrifuged at 15,000 *g* for 20 min at 4 °C. The proteins and nucleic acids were precipitated out with 20% trichloro acetic acid at 4 ºC for 30 min. Two volume of ice cold acetone was added to the supernatant and allowed for precipitation of the EPS at 4 °C for 24 h. The precipitated EPS was separated by using a filter membrane 0.45 µm (Millipore) and dialyzed against deionized water for 24 h. The separated EPS was lyophilized (Yamato) and stored at 30^{o}C. The

biochemical composition of the EPS was determined for carbohydrates [15], uronic acids[16] and protein.[17]

2.3 Antimicrobial activity

The antimicrobial effect of the EPS on *Candida albicans* was performed in plate assay. The *C. albicans* strain was collected from microbial type culture collection (MTCC) with accession number 227. The lyophilised EPS from MSI12 was aliquoted in sterilized distilled water to obtain different concentration of EPS (5µg-20µg/ml). The inoculums was prepared from 48 h culture of *C. albicans* containing $1x10^6$ cells and was uniformly spread over Sabouraud dextrose agar (SDA) plate using a sterile cotton swab (HiMedia). Absorption of excess moisture was allowed to occur for 10 min. Wells with a diameter of 4 or 7 mm were then punched in the swabbed plates and loaded with different concentration of the EPS aliquot.The drug fluconazole was also used at the concentration of 10-150 mg/ml to determine the MIC. The plates were incubated at 37 °C and were observed for zone of inhibition after 48h.

2.4 Evaluation of the strain MSI12 for probiotic characteristics

1. Stress tolerance assay

Heat stress tolerance of the isolate MSI12 was determined as per Stacket al.[18] with necessary modifications. The isolate *Enterococcus* sp. MSI12 was allowed to grow overnight in a 250ml conical flask at 30ºC with shaking at 200 rpm. Then 1 ml of culture was centrifuged at 5,000 × *g* for 10 min, the supernatant was removed, and the pellet was resuspended in 1 ml of preheated 58°C nutrient broth and incubated at 58°C with agitation for different time intervals (10 min to 1 h). The incubated broth on various time intervals were serially diluted and plated on nutrient agar

plates and incubated at 37°C for 48h. For acid stress assay 1 ml of overnight culture of MSI12 was centrifuged at 5,000 × *g* for 10 min and the pellet obtained was resuspended in 2 ml of nutrient broth of pH 2.0 preadjusted with 1 M HCl. The $OD_{600\,nm}$ was recorded every 30 min interval to 5 h. For salt stress assay the cell pellet was resuspended in nutrient broth containing 5 M NaCl and incubated with agitation at 37°C. Bile tolerance was determined using the cell pellet resuspended in 2 ml nutrient broth with 1% of bovine bile (Sigma-Aldrich). The $OD_{600\,nm}$ was recorded every 30 min interval for 5 h. For all the above assays, *L. plantarum* MTCC 2621 were used as control and maintained under similar assay conditions except the medium (MRS broth). All the assays were performed in triplicates and each value presented is the mean value± standard deviation.

2. Survival of MSI12 in a simulated gastric environment

Simulated gastric juice was prepared as per Corcoran et al[19]. The ability of survival of MSI12 in gastric juice was compared with the control *L. plantarum*. Overnight grown cultures of MSI12 (NB) and *L. plantarum* (MRS) were centrifuged at 10,000 × *g* at 4°C for 10 min. The pellet was washed in equal volume of cold 0.25× Ringer's solution. Pellets were then resuspended in an equal volume of simulated gastric juice and incubated at 37ºC in a shaker incubator (Orbitek) at 150 rpm for 90 min. Samples were taken at frequent time intervals 0, 10, 30, 60, and 90 min and plated on respective media and incubated at 37°C for 72 h.

3. Auto aggregation assay

Auto aggregation assay was performed with minor modification of the method described by Del Re et al.[20] Briefly, 48 h culture of *C. albicans* was centrifuged at 13,000 x g for 5 min at 4°C. The pellet thus obtained were washed and resuspended in sterile phosphate-buffered saline (PBS,

pH 7.2) to obtain OD_{600} of 0.5. The absorbance was measured at OD_{600} after 1 h of incubation. The percentage of auto aggregation was expressed as: $1-(A_t/A_0)x\ 100$, where A_t represents the absorbance at 1 h and A_0 was the absorbance at 0 h.

2.5 Hydrophobicity and Antioxidant assay

Bacterial adhesion to hydrocarbon was performed as per the procedure described by Rosenberg[21] and Kiran et al.[22] Microbial hydrophobicity was measured in exponential growth phase of strain MSI12. Phosphate urea magnesium sulfate buffer (PUM) ($g\ l^{-1}$): 19.7 K_2HPO_4, 7.26 KH_2PO_4, 1.8 urea and 0.2 $MgSO_4 \cdot 7H_2O$ was used for the hydrophobicity test. After centrifugation, cells were washed twice with PUM buffer, resuspended in the PUM buffer to get an optical density of ca. 1.0 (A_0). Optical density was measured at 600 nm on a UV–vis Spectrophotometer (PG Instruments, UK). Then, 400 µl of hydrocarbons such as xylene or toluene was added to 2 ml of microbial suspension and vortexed for 2 min. After 10 min, the optical density of the aqueous phase was measured (A_1). The degree of hydrophobicity is calculated as $[1 - (A_1/A_0)] \times 100$ [%]. The antioxidant activity of EPS was measured by DPPH radical scavenging activity.[23] The percentage of DPPH scavenging ability was calculated as $1-(A_{sample} - A_{blank})A_{control}] \times 100\%$. All these assay condition were performed in triplicates.

2.6 Characterization of EPS

Functional groups of EPS was determined using Fourier transformed infrared (FTIR) spectroscopy analysis. The dialysed EPS was thoroughly mixed with KBr and dried. The dried sample was subjected to IR spectral analysis using Fourier Transform Infrared Spectrophotometer (Thermo Nicolet Model:6700) with spectral range of 4000–400 cm^{-1}. The lyophilized EPS was subjected to Gas chromatography (GC) (JEOL GCMATE II GC-MS system) analysis to detect the alditol-acetate derivative as described in Kavita et al.[24] The peaks

of the GC were subjected to mass-spectral analysis and the spectra were analyzed by using the NIST database (version 2.0). The ^{1}H NMR spectra was acquired by dissolving the EPS in deuterated water at a concentration of 20 mg/ml and analyzed on a Bruker Model: Avance-II at 22°C.

2.7 Thermal gravimetric (TG) and differential scanning calorimetric (DSC) analysis

Thermal stability and weight loss of EPS was performed and determined using TA instruments, Q600 SDT and DSC. Approximately 7–8 mg of sample was placed in standard 70 µl aluminium pans. The analysis was carried out over the temperature range from 0°C to 500°C at a rise in temperature of 10°C/min. The flow rate of the gas was 50 ml/min. The weight loss is recorded as a function of temperature.[25]

2.8 Antibiofilm activity of EPS

The biofilm disruption ability of lyophilized EPS was determined in microtitre plate assay. The 48 h old *C. albicans* culture was inoculated in 96 well polystyrene microtiter plates using a sterilized 96-pin replicator. The well plate was prepared with aliquoted EPS of varying concentration (50–250 µg/ml) in Sabourauds dextrose (SD) broth. The biofilm formed by *C. albicans* in the absence of EPS was used as the control. The plate was incubated for 48 h, and then the medium was aspirated. The wells were gently rinsed with phosphate buffer (50 mM, pH 7.0), air-dried, and the biofilms were quantified using the crystal violet assay.[22, 25] Fluconazole (50–250 µg/ml) was used as standard antifungal drug. The data related to these experiments are depicted as average values of triplicate observations and error bars indicate standard deviation.

The results are expressed in terms of percent biofilm formed in the presence of EPS compared to untreated wells (indicating 100% biofilm coverage).

2.9 Disruption of pre-formed *C. albicans* biofilm using the EPS

The *C. albicans* biofilm was developed in a cover glass immersed in a 50-mL Erlenmeyer flask containing SD broth and incubated at 28 °C for 48 h. To study the biofilm disruption, the mature preformed biofilm was treated with varying concentration of EPS and fluconazole ranging from 50-250 µg/ml and incubated for 24 h.The biofilm developed by the *C. albicans* in the absence of EPS was used as the control. The exposed cover slip was washed, dried and stained with a 0.4% crystal violet solution (w/v) and observed under a phase-contrast microscope (Optica) at × 40 magnifications.

3.0 Confocal Laser Scanning Microscopy (CLSM) and SEM analysis

To prepare culture for confocal imaging, the cells were grown in SD broth at 37 °C for 48 h. This fresh culture was used to form biofilm on sterilized glass slides and the pre-formed biofilm was treated for 24 h with EPS and fluconazole of varying concentration ranging from 50-250 µg/ml. Untreated biofilms were used as controls and the biofilm coverage thus formed on glass slides were stained with 0.1% acridine orange and subjected to visualization in a CLSM (LSM 710, Carl Zeiss). The 488-nm Ar laser and 500–640 nm band pass emission filter were used to excite and detect the stained cells. Multiple (20) images were scanned and processed using Zen 2009 image software. Effective disrupted concentration of EPS was selected for SEM and confocal biofilm analysis with *BacLight* Live/Dead stain (Molecular Probes, Eugene). The cell viability was observed and the images were recorded using CLSM (Carl Zeiss).

4. Results

4.1 Identification of probiont MSI12

Isolation of bacteria from seawater was performed on Zobell marine agar and 142 stable isolates were picked for screening. Of the 11 positive isolates (S1), the strain designated as MSI12 produced maximum EPS and was selected for further evaluation. The production of EPS by MSI12 was 580µg/ml after 72 h of incubation at 30°C. The isolate was characterized as Gram positive, facultative anaerobe, non-motile, catalase negative and oxidase positive. The megaBLAST analysis of 16S rRNA gene sequence of MSI12 showed similarity of 98% with strains of *Enterococcus faecalis* (Fig. 1). Based on the biochemical characteristics and phylogenetic analysis, the isolate MSI12 was identified as *Enterococcus faecalis*. The RDPII analysis confirmed the isolate had closet match with *E. faecalis*.

4.2 Probiotic characteristics *E. faecalis* MSI12

The strain MSI12 was evaluated for probiotic potential along with *L. plantarum* as reference strains (control). After 10 min exposure at 58 ºC, a significant increase in viability was observed for MSI12 over the control strains. The increase in viability of MSI12 was observed at various exposure periods of 20, 30, 40, and 50 min respectively. At 40 min, a 50-fold difference in survival of MSI12 was observed when compared to the control. The control strain was reduced to 4.4 x 10^3 CFU/ml from the initial biomass of 8.5 x 10^8 CFU/ml whereas the strain MSI12 was reduced to 4 x 10^7 CFU/ml from 2 x 10^9 CFU/ml. Even after 60 min exposure to 58^{o}C the cell viability of MSI12 was significantly higher over the controls.

The isolate MSI12 showed increased acid tolerance over the control strain *L. plantarum* and survived at pH values as low as 2.0 for 5 h. The result showed the survival rate of *E. faecalis* was

60% in 1% bile acids when compared to the control *L. plantarum* (40%) after 5 h. The strain MSI12 showed high tolerance to salt concentration upto 5 M NaCl. Auto aggregation was investigated on the basis of their sedimentation characteristics after 1 h. The strain MSI12 showed strong aggregation (81.2%) and was strongly hydrophobic with 61.47% adhesion to xylene, and 60% to toluene (Table 1). The DPPH scavenging activity of EPS from *E. faecalis* was 39.24 ± 4.25% at a concentration of 200 µg/ml which was higher than the standard ascorbic acid (37.54 ± 4.55%) at a concentration of 250µg/ml (Fig. 2a). We also observed the scavenging activity increases with the increase in EPS concentration.The survival of probiotic strain MSI12 in simulated gastric juice was compared with the control *L. plantarum* from 0 to 90 min. Decrease in growth was observed in both the strains at 90 min of incubation. Comparatively, the MSI12 has better survival in gastric juice of more than 70% at 90 min of incubation suggesting MSI12 can be a potential probiotic strain (Fig. 2b). The control has lesser survival of 40% in gastric juice.

4.3 Chemical characterization of EPS

Chemical analysis showed that the purified EPS composed of 76.6% (w/w) total sugar and 12.8% (w/w) total protein and 5% uronic acids indicating that MSI12 derived EPS was mainly composed of polysaccharide. FTIR spectrum of MSI12-EPS showed characteristic functional groups as shown in Fig.3. The strong absorption band at 3449 cm^{-1} corresponds to the presence of hydrogen bonded –OH functional group. The peaks at 2926 and 2962 cm^{-1} corresponds to the different types of C-H stretching frequencies. The weak absorption at 1708 cm^{-1} showed the presence of carbohydrate containing carbonyl group. The medium absorption at 1657cm^{-1} showed the stretching frequency of carbohydrates such as glucose and mannose. The C-C stretching frequency was appeared in the range of 1445 cm^{-1} and C-O stretching frequencies

were appeared in the ranges of 1000-1300 cm^{-1}. GC-MS analysis of the EPS exhibited the presence of sugars like glucose and mannose with retention time of 16.7 and 15.2 min respectively.

The well resolved ^{1}H signal appeared in the range of δ4.4 –δ5.4 ppm, reveals the presence of carbohydrate anomeric protons. The tertiary carbon hydrogen (R-C**H**-OR') was observed as a multiplet at δ4.3-3.6 ppm. The sharp single peak at δ3.5 ppm reveals the presence of methoxy (–OCH_3) functional group. The acid functional group –OH proton and carbon attached with –OH proton was appeared in the ranges of δ3.2 ppm and δ2.8 ppm respectively. The sharp single peak at δ2.1 ppm showed the presence of carbonyl group attached with CH_3. The alkyl -CH_2- protons were appeared in between δ1.8 to 1.3 ppm as a multiplet. The singlet peak was observed at δ1.1 ppm, this reveals the presence of -CH_3 group was attached with quaternary carbon (Fig. 4a). GC-MS analysis of the EPS showed the presence of sugars like D- mannose, D-galactose, glucose, D-arabinose with retention time of 5.32, 5.93, 6.61, and 10.67 min respectively. The molar percentage of glucose (47.4%) was highest followed by galactose, mannose and arabinose (Fig. 4b).

4.4 Thermal stability of EPS

Degradation of EPS took place in two steps (S2). In the first step, weight loss of (19.24%) was observed at 110°C due to the loss of moisture molecules in the polymer. In the second step, depolymerisation occurred up to 240ºC and a weight loss of about 27.48% was observed. The thermal transition of the EPS was studied by differential scanning calorimetric analysis. The thermogram showed exothermic peak of EPS with crystallization temperature (T_c) of 83.06°C accompanied with 196.4 J/g latent energy. The melting transitions started at 253.27°C (Supplementary file).

4.5 Antifungal and antibiofilm activity of EPS

The EPS of *E. faecalis* MSI12 extracted after 72 h of incubation showed significant inhibition pattern on *C. albicans* MTCC226 when compared to the control. The crude and the purified EPS showed concentration dependent activity (Fig. 5). The *C. albicans* was more susceptible to the crude EPS at the MIC of 50 µg/ml and purified EPS at MIC of 10 µg/ml. There is no growth inhibition of *C. albicans* was observed at micromolar concentration of fluconazole (150 µg/ml). But raising the fluconazole concentration from micromolar to milimolar, growth inhibition was noticed at 100 mg/ml. The effect of EPS and fluconazole on biofilm formation of *C.albicans* was evaluated using microtitre plate assay and phase contrast microscopy observations. The control biofilms showed 100% biofilm coverage. In the presence of EPS (50 µg) showed significant decrease (78%) in biofilm formation by *C. albicans* when compared to fluconazole (50 mg) showed the biofilm coverage was decrease to 43%. The reduction of biofilm (98%) was achieved with EPS at concentration of 250 µg whereas the reference drug fluconazole (250 mg) was effective on the reduction of biofilm coverage to 83% (Fig. 6). Phase contrast images (Fig. 7) showed 200 and 250 µg of EPS was effective in the disruption of *C.albicans* biofilm when compared to fluconazole. Comparatively the EPS was found to be effective in reducing the survival/ biofilm of *C. albicans* and it also proves the drug fluconazole was not effective in inhibiting the biofilm of *C.albicans.*

4.6 CLSM observations

The biofilms of *C.albicans* formed on glass slides was treated with EPS of 50 µg to 250 µg and fluconazole of varying concentration ranging from 50 to 250 mg. The image anaylsis (Fig. 8) lucidly vizualized that the EPS was potentially effective on the disruption of *C. albicans* biofilm

over the referenece drug. The effect of various dilutions of EPS on the biofilm disruption potential was concentartion dependent. Data revealed that at lower concentration of EPS (50µg) the biofilm disruption was not significant when compared to 100 µg and above concentration. SEM images (Fig. 9) showed the representative image of the control biofilm and Figure 8b depicts the biofilm disruption potential of EPS (150µg) on *C.albicans*. The Bac/light staining method showed live and dead cells of *C. albicans* treated with EPS. The CLSM images showed the cells were stained in red colour indicating dead cells of *C. albicans* treated with 150 µg EPS whereas control live cells were stained green (Fig. 10). The live-dead differential staining procedure proven the potential effect of MSI12-EPS on the disruption/control of biofilm forming *Candida* infections.

5. Discussion

Probiotics in human health care is now emerging as a proven microbial therapeutics and/or as functional foods. The health benefits of probiotics was well-established including potential antagonists of infectious diarrhea,[26] immune stimulation,[27] treatment of inflammatory bowel diseases, reduction in allergic responses[28, 29] and lowering serum cholesterol.[18] Literature showed that probiotics is an effective method of treating diarrheal infection particularly and all type of bacterial infection in general. The existing probiotic-based anti-infective treatments are reactive and less explored their potential on the treatment of established pathogens. This investigation would impart a new dimension on the treatment of established pathogens like biofilms. Based on the present findings the potential implications of probiotics could be envisaged as preventive / control method to treat biofilm formation in implant medical devices and/or alternatively as synergistic to the existing antibiotics to disrupt *Candida* biofilm. The increasing number of

indwelling medical implants is facing potential threat due to device-associated biofilm infections caused by *Candida* spp.[30] Management of device-associated *Candida* infections remains to be challenging. In this context the present finding are promising to develop effective probiotic strategy to maintain the implants free from *Candida* biofilm.

In principle, a probiotic strain can colonize, aggregate and adhere to the host. The stability and longevity of probiotic establishment is depends on their tolerance potential in host. In this study, we established the probiotic strain *E. faecalis* MSI12 was growing and tolerating to the niche conditions like human gut. Most of the prominent probiotic LAB strains are heat tolerant, but their viability at 60°C and above was constraint in their utilization as technologically feasible. The isolate *E. feacalis* MSI12 survived and showed high tolerance to acid even after 5h of incubation at pH 2.0. An approximate concentration of 0.3-0.5% bile level is found in the human gastrointestinal tract. The strain *E. feacalis* MSI12 showed 60% survival on treatment with 1% bovine bile. The EPS was used as shield and adhesive by the producing microorganisms to protect the cells against heat and other stress. In this study, the EPS produced from *E. faecalis* MSI12 exhibited high antioxidant activity, increased auto-aggregation (81.2%) and hydrophobicity which evident the isolate MSI12 as a potential probiotic strain. The isolate MSI12 exhibited stronger auto-aggregation of 81.2% in 1 h and the percentage of aggregation was higher when compared to *Lactobacillus* 63.1% after 5 h as reported by Anwar et al.[31] Del Re et al.[20] reported auto-aggregation as an important factor in the adhesion of bacterial cells to intestinal epithelium and its maintenance in the gastrointestinal tract. To improve their heat tolerance, the LAB was treated or formulated with prebiotics. For e.g. gum acacia treatment offered protection to *Lactobacillus paracasei* NFBC 338 during heat, bile, and H_2O_2 stress[30]. Dixit et al.[32] examined 3 strains of *L. acidophilus* and found that the survival of strain NCIM

2285 was reduced by 50% and the strain NCIM 2903 by 21% after 4 h of incubation at pH 2.5. Previous report evidences *E. faecium and E. faecalis* strains are used as probiotics and are ingested in high numbers to treat diarrhoea, antibiotic-associated diarrhoea or irritable bowel syndrome, to lower cholesterol levels or to improve host immunity.[33] The MSI12 was established as potential probiotic strain compared to *L. plantarum.* The cell viability was very high at higher temperature, acidic pH, bile salt,survival in simulated gastric juice and salt concentration and therefore the strain MSI12 can survive gut like niche without prebiotics. The tolerance at higher temperature, acidic pH, bile salt, gastric juice and salt concentration are the prerequisite characteristics of a probiotic strain. The overall tolerance potential envisages that the strain MSI12 can colonize on any surfaces like gastro intestinal tract, vaginal, dental and mucosal surfaces.

The possible mechanism of destabilizing the EPS of biofilm includes breaking the bonds that connect polysaccharide residues in the EPS. This could be an effective means of dispersing biofilms and making the subjacent bacteria more susceptible to treatment by antibiotics.[34] Enterococci were known for secretion of bacteriocins[35] and therefore the EPS of *E. faecalis* MSI12 was expected to be synergized with antimicrobial secretions.This study revealed that biofilm disruption/control using a probiont could deliver a synergistic approach as the probiotic strain can colonize in the host to prevent the formation of *candida* biofilms.The EPS from *Enterococcus* sp. MSI12 showed significant reduction of treated *Candida* biofilm. The antibiofilm potential of EPS was very stronger than standard antifungal drug fluconazole.The strain *C. albicans* MTCC227 used in this study is resistant to fluconazole. Previously it was reported that even 3000 $\mu g/ml^{-1}$ of fluconazole was not effective in inhibiting the growth of C. albicans MTCC 227.[36] The EPS produced by the probiotic *E. faecalis* MSI12 showed 100%

disruption of *C.albicans* biofilm when compared with the drug flucanazole (80%). Literature showed fluconazole is still used as drug in patients with candidemia or suspected invasive candidiasis.[37] Fluconazole has excellent *in vitro* activity against *C. albicans*. Fluconazole can also be effective against non-*albicans Candida* species, including *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata*, although higher doses may be required.[38,39] Usage of fluconazole has side effects and may cause gastrointestinal tract disorders like nausea, abdominal discomfort, vomiting, diarrhea. The exopolysaccharides released by the biofilm of *E. faecalis* act as effective antifungal as well as biofilm inhibitory agent. This study is the first report on *E. faecalis* probiotics from marine environment as effective antifungal/ antibiofilm agent.

The production of EPS was beneficial to the human health as well as EPS was exploited in the food industry as viscosifiers, stabilizers, emulsifiers, or gelling agent.[18] Based on the available reports probiotic mediated disruption of *candida* biofilm is new and this report illustrates MSI12-EPS an effective antibiofilm agent to contain *candida* infections. Thus a probiotic strain secrets EPS to prevent/control *candida* biofilm is a novel, effective and safe method of developing antibiofilm therapy. This study brings out a new safest way of *candida* biofilm control. The EPS from *Oceanobacillus iheyensis* was effective on the disruption of biofilm formed by *S. aureus.*[40] Biosurfactant and rhamnolipids were showed effective on the disruption of pathogenic biofilms of *Candida.*[22,41,42] Carbohydrates consumed in the diet are the primary and preferred nutrient sources for *Candida albicans*[43,44,45] Based on the present findings the *E. faecalis* was a probiont can establish an EPS assisted biofilm which in turn potential antagonistic to the *Candida* cells as well as inhibitory to the formation of biofilm and/or disruption of established biofilm during

Candida infections, thereby innovative approaches to contain *Candida* infection was demonstrated.

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Figure and Table legends

Fig.1 Phylogenetic tree of *Enterococcus faecalis* MSI12.Maxium parsimony phylogenetic tree based on 16S rRNA gene sequence of *Enterococcus faecalis* MSI12 showing representatives of other related taxa. The analysis was performed with bootstrap values of 1000 replications greater than 50% are shown at branching points.

Fig. 2a Antioxidant activity of EPS. Ascorbic acid was used as standard in the DPPH scavenging activity of EPS from *E. faecalis* MSI12. The activity (39.24 ± 4.25%) of EPS (200 µg/ml) was equal to the standard (37.54 ± 4.55% at a concentration of 250 µg/ml). **Fig. 2b. Survival rate of MSI12 in the simulated gastric juice**. The survival rate of MSI12 in the simulated gastric juice was compared with the control *L. plantarum*. The analysis showed the survival rate of the MSI12 was better than the control *L. plantarum.*

Fig. 3 FT-IR spectra of EPS from *E. faecalis* MSI12. Absorption band at 3448.72 cm^{-1} corresponds to the presence of hydrogen bonded –OH functional group, peak at 2926 and 2962 cm^{-1} corresponds to C-H stretching frequencies, carbohydrate containing carbonyl group shown in absorption at 1708 cm^{-1}.

Fig. 4 ^{1}HNMR specrta of MSI12-EPS. The well resolved ^{1}H signal appeared in the range of δ4.4 –δ5.4 ppm, reveals the presence of carbohydrate anomeric protons. The tertiary carbon hydrogen (R-C**H**-OR') was observed as a multiplet at δ4.3-3.6 ppm. The sharp single peak at δ3.5 ppm reveals the presence of methoxy (–OCH_3) functional group. The acid functional group –OH proton and carbon attached with –OH proton was appeared in the ranges of δ3.2 ppm and δ2.8 ppm respectively. The sharp single peak was appeared at δ2.1 ppm showed the presence of carbonyl group attached with CH_3. The alkyl -CH_2- protons were appeared in between δ1.8 to 1.3 ppm as a multiplet. The singlet peak was observed at δ1.1 ppm, this reveals the presence of -CH_3 group was attached with quaternary carbon. **Fig.4b. GC-MS spectra of the EPS.** The spectra showed the presence of sugars like D- mannose, D-galactose, glucose, D-arabinose with retention time of 5.32, 5.93, 6.61, and 10.67 min respectively.

Fig. 5 Antifungal activity of MSI12-EPS on *C. albicans*. The well diffusion assay was performed using crude and purified EPS. A is 5 µg/ml of crude EPS, B is 25 µg/ml of crude EPS, C is 50 µg/ml of crude EPS, D is 5 µg/ml of purufied EPS and E is 10 µg/ml of purified EPS.

Fig. 6 Antibiofilm potential of MSI12-EPS. Antibiofilm activity of MSI12-EPS on *C. albicans* biofilm was assayed in 96-microtitre plate with varying concentration of EPS and flucanazole. Each concentration was set as triplicates. The reduction of biofilm significantly high (98%) with 250 µg of EPS whereas the reference drug fluconazole (250 µg) showed reduction of biofilm to 83%.

Fig. 7 Phase contrast micrographs showing biofilm disruption potential of MSI12-EPS on *C. albicans*. The *C. albicans* biofilm was developed on a cover glass and then it was treated with varying concentration of EPS and fluconazole ranging from 50-250µg. The treated cover glass was stained with crystal violet and observed under a phase-contrast microscope (Optica) at × 40 magnifications. A - control biofilm, B1 – 50 mg fluconazole, B2 – 50 µg MSI12-EPS, C1 – 100 mg fluconazole, C2 – 100 µg MSI12-EPS, D1 – 150 mg fluconazole, D2 – 150 µg MSI12-EPS, E1 – 200 mg fluconazole, E2 – 200 µg MSI12-EPS and F1 – 250 mg fluconazole, F2 – 250 µg MSI12-EPS.

Fig. 8 Confocal laser scanning micrographs showing biofilm disruption potential of MSI12-EPS on *C. albicans*. The pre-formed biofilm was treated for 24h with EPS and fluconazole of varying concentration ranging from 50-250 µg. Untreated biofilms were used as controls and the biofilm coverage thus formed on glass slides were stained with 0.1% acridine orange and subjected to visualization in a CLSM (LSM 710, Carl Zeiss). A - control biofilm, B1 – 50 mg fluconazole, B2 – 50 µg MSI12-EPS, C1 – 100 mg fluconazole, C2 – 100 µg MSI12-EPS, D1 –

150 mg fluconazole, D2 – 150 µg MSI12-EPS, E1 – 200 mg fluconazole, E2 – 200 µg MSI12-EPS and F1 – 250 mg fluconazole, F2 – 250 µg MSI12-EPS.

Fig. 9 Scanning Electron Microscope images showing antibiofilm potential of MSI12-EPS on *C.albicans*. A.Control biofilm and B. Disrupted by EPS.

Fig.10. CLSM analysis with *BacLight*live/dead stain.A. Control biofilms of *C.albicans* B. EPS treated biofilms stained using Bac/light kit. Based on this analysis the red colour staining shows the EPS effectively kills the *C. albicans* cells.

Table 1 The results represents the mean value of triplicate experiments on acid, bile, autoaggregation after 5h of incubation and hydrophobicity assays. The experiment was compared with the standard probiotic *L.plantarum*, and all the results indicated the isolate MSI12 has increased resistance than control.

Strain	A600nmHydrophobicity (%)					
	NB (pH 2.0, 5hr)	NB+bile (5hr)	NB+salt	Auto-aggregation (%)	Xylene	Toluene
Lactobacillus plantarum MSI 12	6.69 ± 0.21 7.42 ± 0.10	5.74 ± 0.33 6.48 ± 0.23	6.36 ± 0.31 7.42 ± 0.12	55.11 ± 0.32 81.20 ± 0.19	38.22 ± 0.14 61.47 ± 0.09	20.92 ± 0.43 60.01 ± 0.29

Table 1

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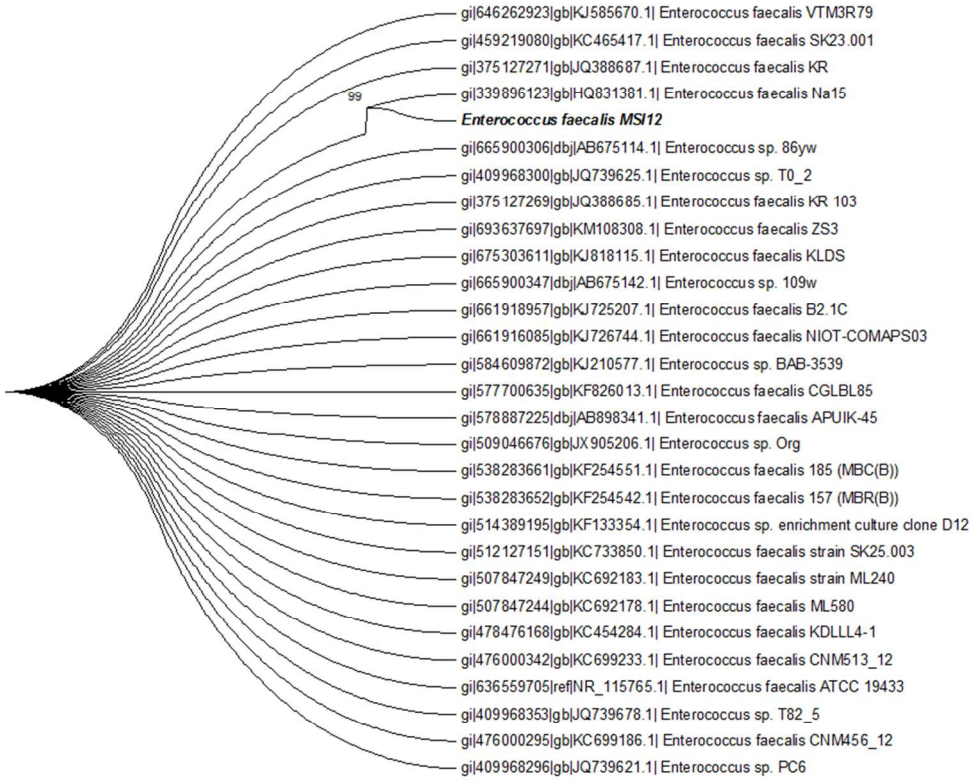

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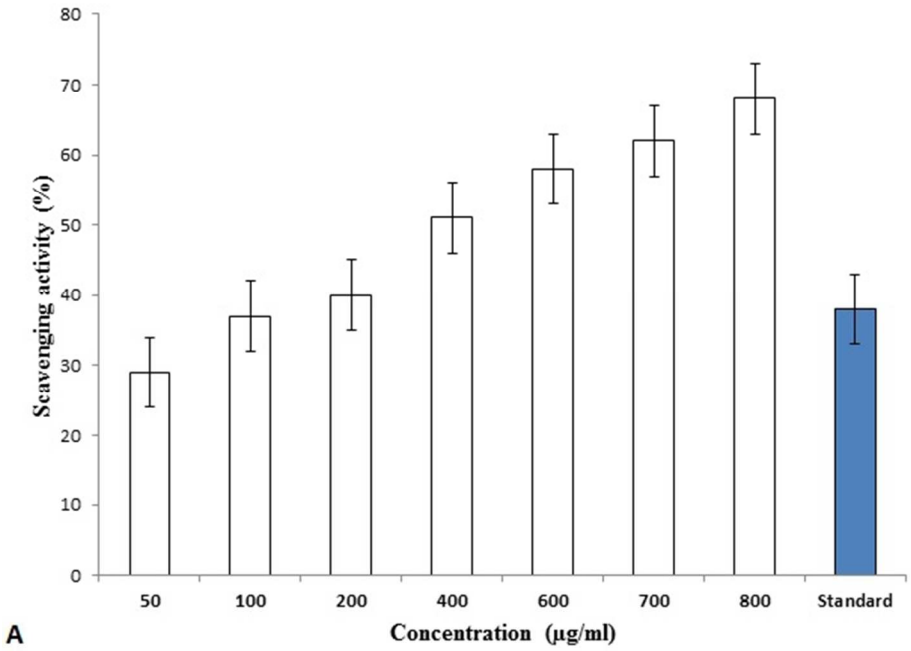

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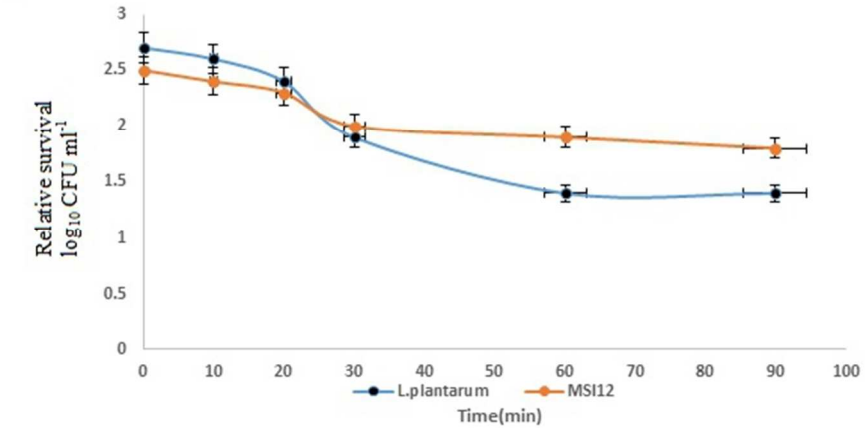

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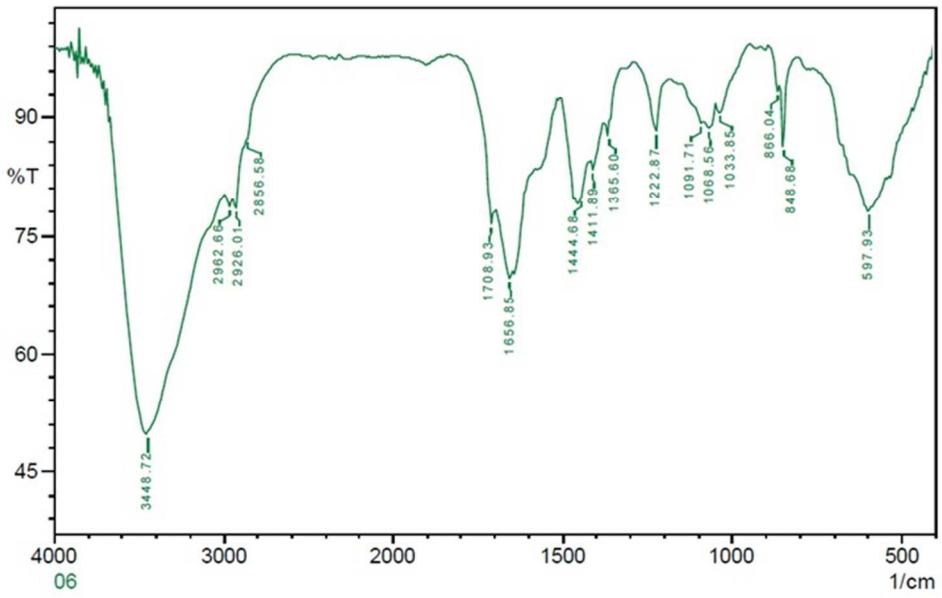

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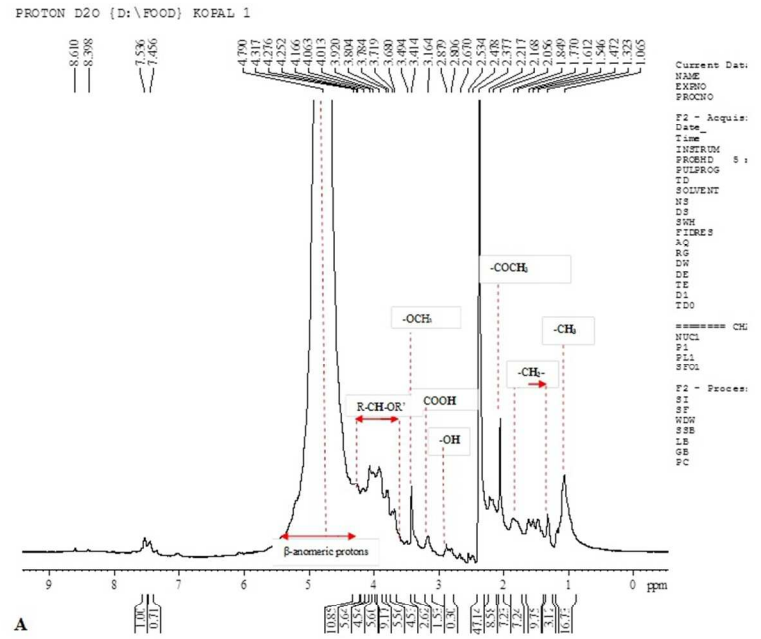

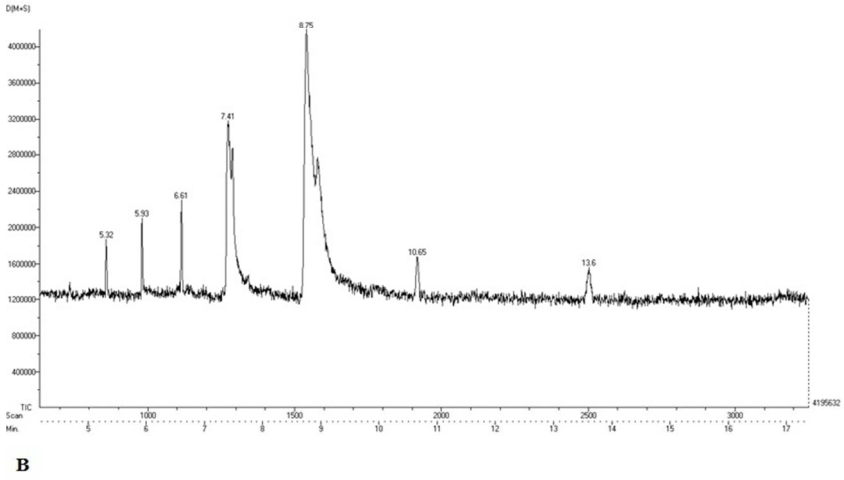

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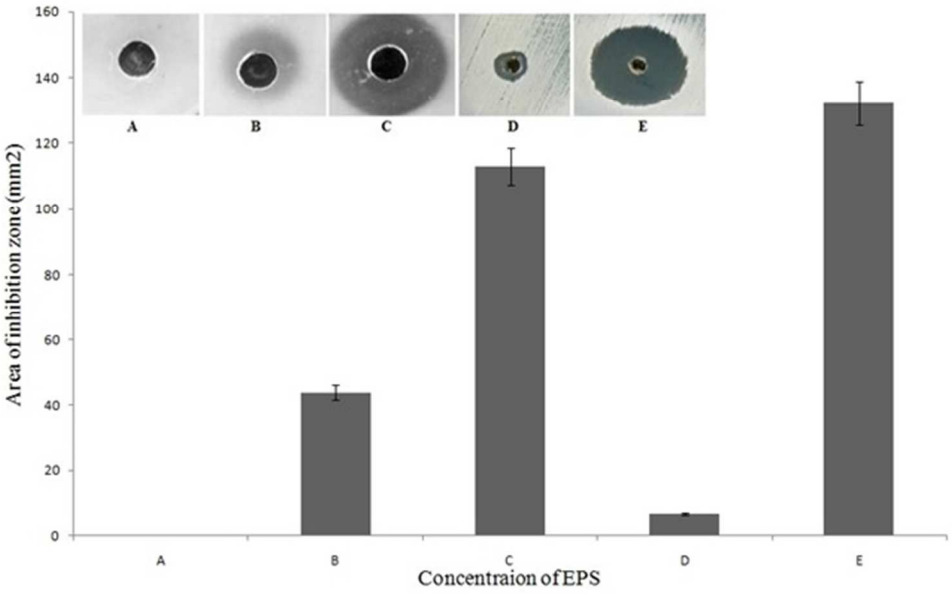

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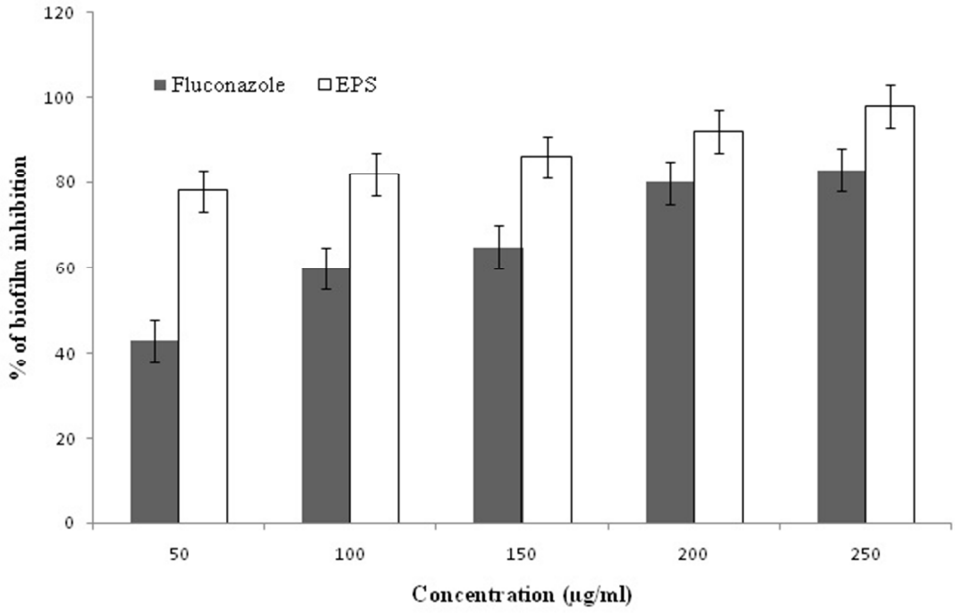

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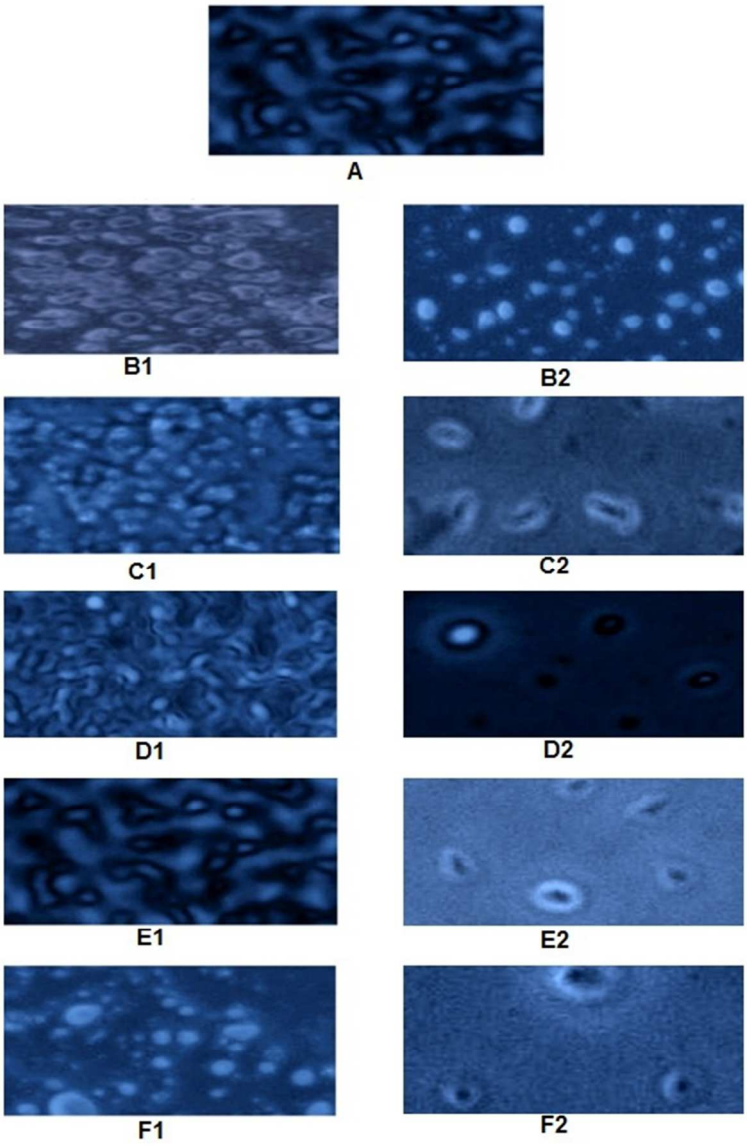

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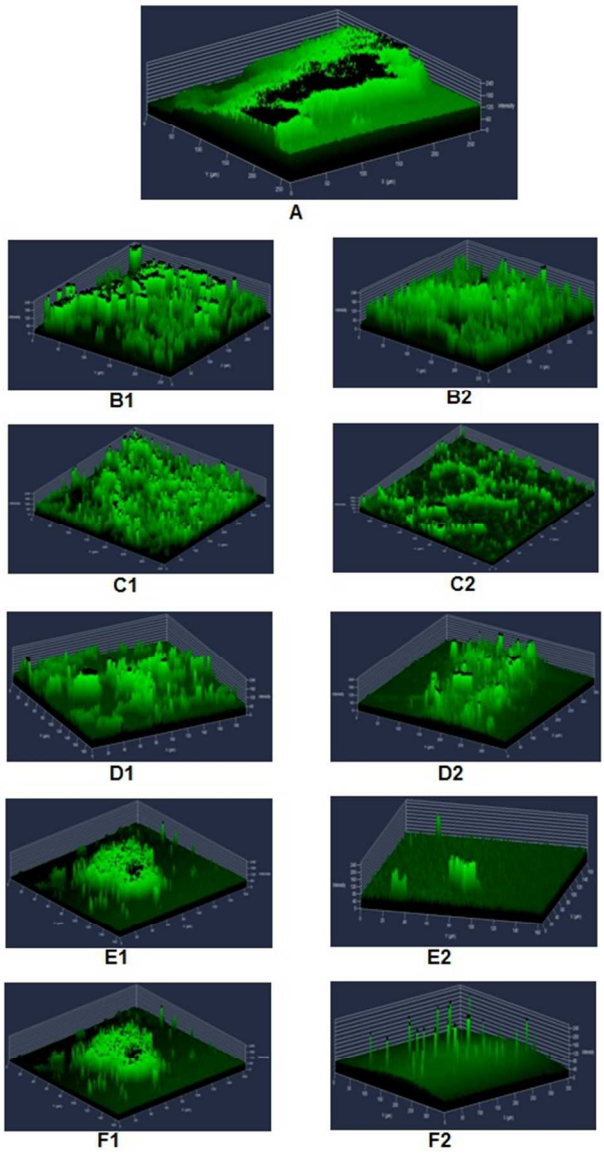

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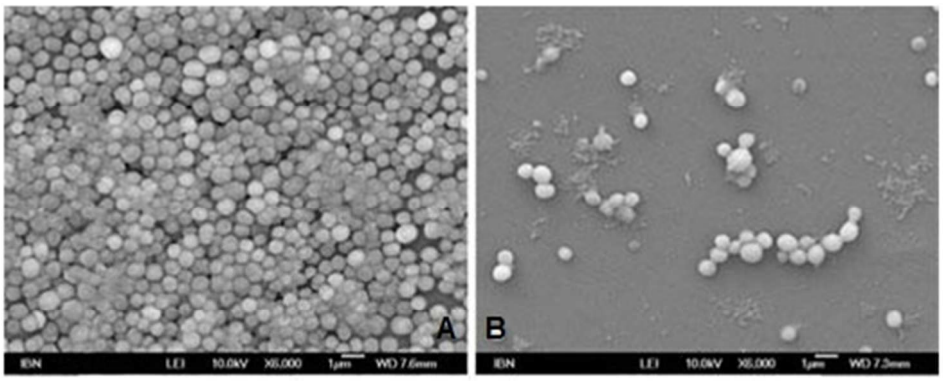

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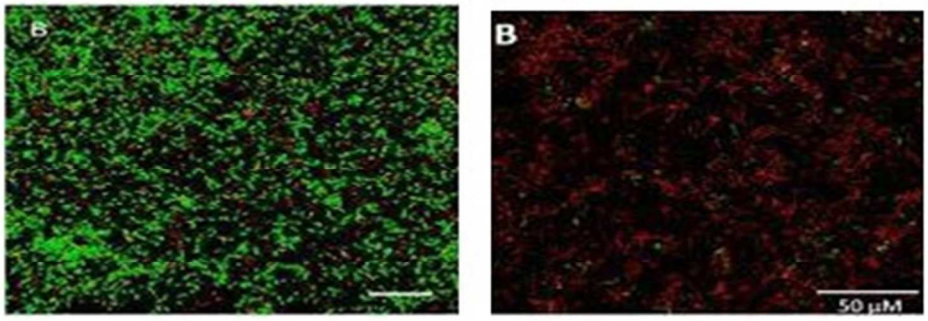

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