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Research Article
Biofilm Formation and its Susceptibility Testing of *Myroides odoratimimus* SKS05-GRD Using MBEC™ High-throughput Assay

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Abstract

Background and Objective: Bacterial biofilms are highly regarded for their increasing potential to resist antibiotics and cause infections especially on food and beverage based substrates. These bacteria with a potential to develop biofilms are now researched with the hindsight to control the contamination they can cause. In this study the biofilm forming ability of *Myroides odoratimimus* (*M. odoratimimus*) SKS05-GRD has been evaluated using different substrates and different time intervals. **Materials and Methods:** Determination of minimal inhibitory concentration (MIC) and minimal biofilm eradication concentration (MBEC) of each antimicrobial agents was compared to test efficacy of the antibiotics and nanoparticles. Bioanalytical instrumentation such as scanning electron microscopy (SEM) and Fourier Transform Infra Red (FT-IR) spectroscopic analysis and statistical validation (ANOVA) on the pre-formed biofilm was carried out. **Results:** Growth curve analysis revealed an exponential phase at 47 min while optimum biofilm forming potential was observed at 72 h. Parametric evaluations of media and substrate revealed food flavobacterium medium and plastic was favoured by the bacterium for optimum biofilm formation. Although, silver nanoparticles by themselves render antibiofilm activity, results of MIC, MBEC, SEM and FT-IR analysis suggest enhanced reduction in biofilm formation when the nanoparticles combined with tetracycline. **Conclusion:** This study confirms the potential of *Myroides odoratimimus* SKS05-GRD for biofilm formation and the eradication of the same using tetracycline complexed with silver nanoparticles.

Key words: Antibiotic, biofilm, efficacy, *Myroides odoratimimus*, silver nanoparticle

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Bacterial biofilms usually adhere to many abiotic materials such as soil particles, medical implants, plastics and metals by building a slimy substance[1]. The first bacteria provide an adhesion site for other bacterial colonies act as pioneers and hold the matrix together. Bacteria that fail to attach to any surface on their own tend to use earlier colonists as an anchor and grow in the matrix.

Comparing to planktonic forms, biofilms are highly resistant to antibiotic and detergents, which is mainly due to the extracellular polymeric substances. Stable biofilm formations have always been found to be facilitated by lateral gene transfer[2]. However, certain microorganisms such as *Pseudomonas aeruginosa* have shown no difference in resistance to antimicrobials when present as biofilm or stationary-phase planktonic cells[3]. Several studies have been conducted to determine the ability of bacterial communities to adhere and form biofilms on food contact surfaces and determined that the bacteria attach to surfaces such as plastics, glass, stainless steel and rubber[4]. The formation of biofilms creates a major problem in the food industry as it may represent an important source of contamination for material or food stuffs coming into contact with them leading to food spoilage or transmission of disease[5].

Myroides was isolated by Stutzer[6] in 1923 for the first time, members of the genus *Myroides* have historically been classified as *Bacterium faecale aromaticum*, the first strain isolated from the human intestine. Since then this organism has been isolated from human urine, faeces, wound discharges, sputum and blood. In the food environment, *Myroides* strains have been isolated from dairy sources and shown to produce thermostable proteases and lipases which may play a role in the spoilage of dairy products[6].

In the present study, the ability of *Myroides odoratimimus* SKS05-GRD (JQ178355) for biofilm formation is evaluated using different substrates and different medium and its inhibition using nanoparticles and antibiotics is evaluated. This study targets the efforts to understand the underlying mechanism using a combination of both nanoparticles and antibiotics which may enhance the antibacterial effect of the nanoparticles and arrest bacterial growth resulting in the inhibition of biofilm.

MATERIALS AND METHODS

Geography of the study: The present study was conducted using soil, chicken and meat samples collected from in and around Coimbatore, Tamil Nadu, India during the period of 2014-2017. The chicken and meat samples collected from vendors and the soil samples were brought to lab in sterile containers until further processing.

Isolation and maintenance of *Myroides* species: The entire chemical used in this study was of commercial grade from Himedia Laboratories Private Limited, India. The soil sample and chicken, meat extract were serially diluted from 10^{-2} to 10^{-8}. Each of the dilutions was spread plated on to Shieh medium (polymyxin-10 μg mL^{-1}, neomycin-5 μg mL^{-1}, peptone-5 g, yeast extract-0.5 g, sodium acetate-0.01 g, barium chloride-0.01 g, potassium dihydrogen phosphate-0.1 g, magnesium sulphate-0.3 g, ferrous sulphate-0.001 g, sodium bicarbonate-0.05 g, calcium chloride-0.0067 g, pH-7.2, agar-10 g, distilled water-1000 mL) and incubated at 37°C for 3 days. The cultures showing yellow pigmentation were maintained on food flavobacterium medium (beef extract-10 g, peptone-10 g, sodium chloride-5 g, agar-12 g, distilled water-1000 mL, pH-7.5).

Biochemical characterization and 16s rRNA sequencing: Methods for the identification of members of the *Flavobacteriaceae* are described earlier[7]. The yellow pigment producing isolates were selected and subjected for morphological and biochemical characterization. Cellular morphology was determined by Grams stain and observed under microscope. Flexirubin pigment production was determined by staining the bacterial colonies with 20% KOH. The strain SKS05-GRD was further submitted to the Chromous Biotech Pvt. Ltd, Bangalore for 16s rRNA sequencing. Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out.

Growth curve analysis: One percent of the overnight culture of *Myroides odoratimimus* SKS05-GRD was inoculated into FFB and incubated at 37°C in a shaking incubator. The growth was analyzed for every half an hour in UV-Vis spectrophotometer (HACH DR6000, USA) for five and half hours. A graph was plotted with optical density against time and the doubling time was analyzed. The doubling time is the period of time required for a quantity to double in size or value.

Screening of different substrates for optimum biofilm formation: Different substrates such as Teflon (10×0.5 mm), glass (22×22 mm), plastic (20×30 mm), steel and polyvinyl (20×30 mm) were selected, washed with detergent, rinsed with distilled water and air-dried. The dried substrates were

aseptically inoculated into the overnight culture and incubated for 96 h at 37°C. After regular 24 h incubation period, the substrates were aseptically removed from the broth culture for biofilm quantification using crystal violet binding assay. The absorption of re-solubilized glacial acetic acid was measured at a wavelength of 437nm using Hach's UV spectrophotometer at 490 nm[8]. Control wells without culture were maintained.

Microtiter plate method for optimization of growth media to form biofilm: The overnight culture was taken at a concentration of 9.83×10^{-3} CFU mL^{-1}, diluted to 10 and 100-fold and incubated into FFB, tryptic soy broth (TSB), brain heart infusion (BHI) broth, FFB+2.5% glucose, TSB+2.5% glucose and BHI+2.5% glucose in the 96 well microtiter plate for 24, 48, 72 and 96 h. The planktonic cells that remained floating in the broth after each incubation time were removed by washing with distilled water and the biofilm formed in the wells of the microtitre plate was stained with 1% crystal violet for 15 min. Destaining was carried out by adding ethanol followed by re-solubilization of the dye bound to adherent cells with 2.5 mL of 33% glacial acetic acid. The biofilm was quantified using an ELISA plate reader (Biorad, India) at 650 nm.

Selection of antibiotics for screening the susceptibility of *Myroides odoratimimus* SKS05-GRD: The overnight culture was swabbed on the solidified sterilized FFB agar. Selected antibiotic discs (HiMedia, India) such as tetracycline, chloramphenicol, tobramycin, gentamycin, streptomycin, ciprofloxacin, ampicillin, rifampicin, ofloxacin, imipenem, enrofloxacin, penicillin, kanamycin, polymyxin, piperacillin, vancomycin, methicillin, trimethoprim, amikacin and gatifloxacin were placed accordingly and incubated for 24 h. The diameter of the zone of inhibition was measured and the sensitivity and resistivity of each antibiotic has been assessed according to the observed diameter.

Preparation of silver nanoparticles (AgNP): Two methods of preparation of silver nanoparticles were conducted in this study to screen for the antimicrobial sensitivity of nanoparticles. Silver nitrate ($AgNO_3$) was reduced with polyvinyl pyrrolidone (PVP) as a stabilizer.

- **Ethylene glycol based silver nanoparticles:** Silver nitrate weighed to 157 mg was dissolved in 100 mL of ethylene glycol along with 5 g PVP
- **Glucose-based silver nanoparticles:** The $AgNO_3$ (157 mg) and 5 g of PVP was dissolved in 100 mL of 40% (w/v) of glucose syrup

As a confirmation for the conversion of all silver nitrate as nanoparticles, 5 mL of NaCl was added to the samples. The turbidity of solution indicates the presence of ionic silver while clear solution confirms the completion of the reaction.

High-throughput antimicrobial susceptibility testing of microbial biofilms calgary biofilm device method (CBD): The overall protocol is mentioned in Fig. 1. Overnight culture was taken from FFB and transferred to 100 mL of freshly sterilized FFB broth. Two hundred microliters of the diluted culture (9.83×10^{-5} CFU mL^{-1}) was added to all the wells of the 96-well microtitre MBEC™ Assay Plate, closed with the lid containing attached polystyrene pegs and incubated on a rocking table for 72 h at room temperature. The 3 day old bacteria that adhered on the pegs of the modified calgary device or challenge plate were washed with sterile phosphate-buffered saline (PBS) (NaCl-8 g, KCl-0.2 g, Na_2HPO_4-1.44 g, KH_2PO_4-0.24 g, distilled water-100 mL, pH 7.4) and inserted into the media containing various antibiotics/nanoparticles. The rinsed biofilms were immersed in the antimicrobials of the challenge plate and incubated for 24 h at 37°C on a rocking table. A neutralizing agent (L-histidine-0.05 g, L-cysteine-0.05 g, reduced glutathione-0.1 g) was mixed with 0.1 mL of tween 20 and 10 mL of FFB medium. Hundred microlitre of the prepared neutralizing medium was added to each well in the challenge plate. The lid containing the pegs was transferred into a recovery plate containing freshly sterilized FFB medium. The remaining biofilm was removed from pegs by intense vortexing for 30 min and incubated for 24 h. To determine the minimum inhibitory concentration (MIC) values, turbidity (visually) in the wells of the challenge plate was checked.

In order to determine the minimum biofilm eradication concentration (MBEC™) values, the turbidity (visually) in the wells of the recovery plate was checked. Hundred microlitre of the each recovery plate media was serially diluted and spread plated on FFB agar for viable cell counts of the biofilm after the treatment with appropriate antimicrobials and the percentage and log percentage survival was calculated by the below-mentioned formula described by Jass *et al.*[9]:

$$\text{Survival (\%)} = \frac{\text{Remaining CFU of recovery plate}}{\text{CFU of initial biofilm}} \times 100$$

$$\text{Log \% survival} = \log10\ (\text{\% survival})$$

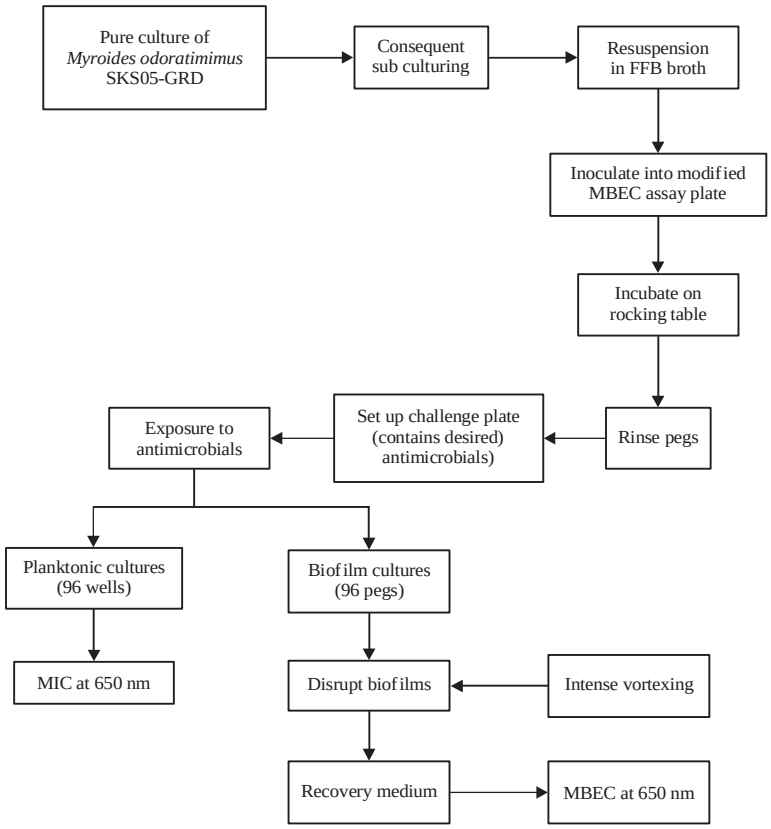

Fig. 1: High throughput antimicrobial susceptibility testing protocol using modified MBEC™ assay plate

Effect of AgNPs on the growth of *Myroides odoratimimus* SKS05-GRD: Among the concentrations of 5-50 µg mL^{-1} of AgNP suspension (ethylene glycol based and glucose based) used above, the concentration that showed the minimum inhibitory concentration was further studied along with the antibiotic. To assess if the nanoparticles aid in the inhibition of growth at lower concentrations, 20 µg mL^{-1} of tetracycline and 10 µg of AgNPs was added to FFB and incubated along with the culture for 24 h at 37°C. After incubation, the optical density of the broth was observed at 490 nm.

Screening for inhibitory potential on biofilm formed by *Myroides odoratimimus* SKS05-GRD: The AgNP suspension (in different concentration ranging from 5-50 µg mL^{-1}, ethylene glycol based and glucose based) was added to FFB broth at a gradient increase of 5 µg along with the 100 µL overnight culture of *Myroides odoratimimus* SKS05-GRD and incubated for 72 h. After incubation, the plate was stained with 1% crystal violet. Ethanol was finally added to each stained well and after the dye has solubilized, the optical density of each plate had been taken using ELISA reader at 650 nm. The MBEC™ assay using CBD was carried out with 20 µg mL^{-1} of tetracycline +10 µg of AgNPs and incubated for 24 h at 37°C.

Scanning electron microscope (SEM) and FT-IR spectroscopic analysis to evaluate the antibiotic and nanoparticles susceptibility of *M. odoratimimus* SKS05-GRD: To discover the effect of antibiotics, its combination, nanoparticles with and without antibiotics on biofilm formed by *Myroides odoratimimus* SKS05-GRD, the lyophilized samples of the master plate (72 h culture grown in FFB in a modified calgary biofilm device without any antibiotics) and the challenge plate (biofilm treated with different antibiotics and their combination in FFB medium for 24 h) were processed for SEM analysis. The cells were harvested by centrifugation at 6500 rpm for 15 min and fixed using 2.5% (v/v) aqueous glutaraldehyde for 2 h. The cells were dehydrated using a gradient of ethyl alcohol (10-100%) and a final wash done with absolute ethyl alcohol and dried the cells using lyophilization. The dried cells were subjected to FT-IR analysis (SHIMADZU 8400S) and scanning electron microscopy (HITACHI S-4500) after gold plating.

Statistical analysis: The influences of different media at various dilutions were analyzed by one-way ANOVA test. The differences were considered significant at p = 0.05 significance level.

RESULTS

Biochemical screening of *Myroides odoratimimus* SKS05-GRD AND 16S rRNA sequencing: The results of the entire IMViC test were found to be negative. Strain showed positive result for catalase test, urease test and KOH assay confirming the production of flexirubin pigments. Strain revealed a positive reaction for oxidase test, DNAse test and reduced nitrite. Positive reaction was observed for lipase test without any carbohydrate metabolism confirming the isolated strains as genus *Myroides.* Additional biochemical testing revealed positive reactions for deaminase test, gelatin hydrolysis, casein hydrolysis and starch hydrolysis whereas chitin hydrolysis, esterase test, congo red test, CMC hydrolysis and esculin hydrolysis revealed negative results (Table 1). The sequenced strain was found to be *Myroides odoratimimus* that belongs to the genus *Myroides* and formerly called as *Flavobacterium odoratum*. The FASTA sequence obtained was submitted to GenBank and accession number JQ178355 was obtained.

Growth curve and assessment of biofilm formation: The optical density values of the strain grown at 30 min interval was observed and plotted on a graph. The doubling and the generation time have been deduced by the exponential phase values as 47 min and 1.6 h, respectively. The biofilm formation is found to be ideal after 72 h although faint biofilm formation was seen when observed at 48 h.

Biofilm formation using different parameters (substrates and media): Based on the graphical representation of optical density values obtained by incorporating various substrates in food flavobacterium medium (FFB) and tryptic soy broth (TSB), teflon was found to support high biofilm formation of *Myroides odoratimimus* SKS05-GRD (JQ178355). Plastic showed high adherence of the bacterium in FFB, least growth was shown using plastic+TSB.

Analysis of the undiluted overnight culture for biofilm formation on staining with crystal violet could not confirm if the biofilm formation has been due to fresh growth of the culture by attaching itself to the substrate. At 9.83×10^{-3} CFU mL^{-1}, the culture showed effective biofilm formation by crystal violet staining. This characteristic feature might be due to effective aeration of the culture leading to fresh growth in media. The influences of different media at various dilutions were analyzed by statistical method of analysis of variance (ANOVA) and 100-fold dilution was observed to give optimum result.

The results obtained indicate that the commercial media (FFB) shows optimum biofilm formation and suggests that this may be due to efficient uptake of the components of the media for growth. Comparison of biofilm formation using FFB and FFB+glucose shows similar results, therefore, FFB was used as the media for further biofilm analysis. On comparison with the various time intervals using $9.83^{-}10^{-5}$ CFU mL^{-1} concentration of cells, optimum biofilm formation using FFB was seen at 72 h of incubation (Fig. 2).

Antibiotic susceptibility of *Myroides odoratimimus* SKS05-GRD: The organism was found to be sensitive to chloramphenicol, tetracycline, imipenem and gatifloxacin at the different concentration of the ready to use discs (Himedia). The organism showed resistance to other antibiotics such as rifampicin, erythromycin, piperacillin, vancomycin, sulphamethizole and nalidixic acid, etc. at the given concentration (Table 2). Tetracycline, chloramphenicol, amoxicillin, ciprofloxacin, cephatoxime, erythromycin, rifampicin and ampicillin were taken for further analysis to confirm the concentration at which the biofilm formed by *M. odoratimimus* SKS05-GRD is rendered sensitive.

Table 1: Biochemical characterization of *Myroides odoratimimus* SKS05-GRD

Biochemical test	Result	Biochemical test	Results
Morphology	Yellow, Mucoid with rhizoidal growth, fruity odour	Catalase test	+
Gram's staining	Gram negative rods	Urease test	+
Motility	+	KOH assay	+
Indole	-	Oxidase test	+
Methyl red	-	DNase test	+
VP test	-	Nitrite reduction	+
Citrate	-	Nitrate reduction	-
Lipase test	+	TSI and H_2S production	+ Without H_2S production
Deaminase test	+	Chitin hydrolysis	-
Esterase test	-	Gelatin hydrolysis	+
Casein hydrolysis	+	Starch hydrolysis	-

+: Positive result, -: Negative result

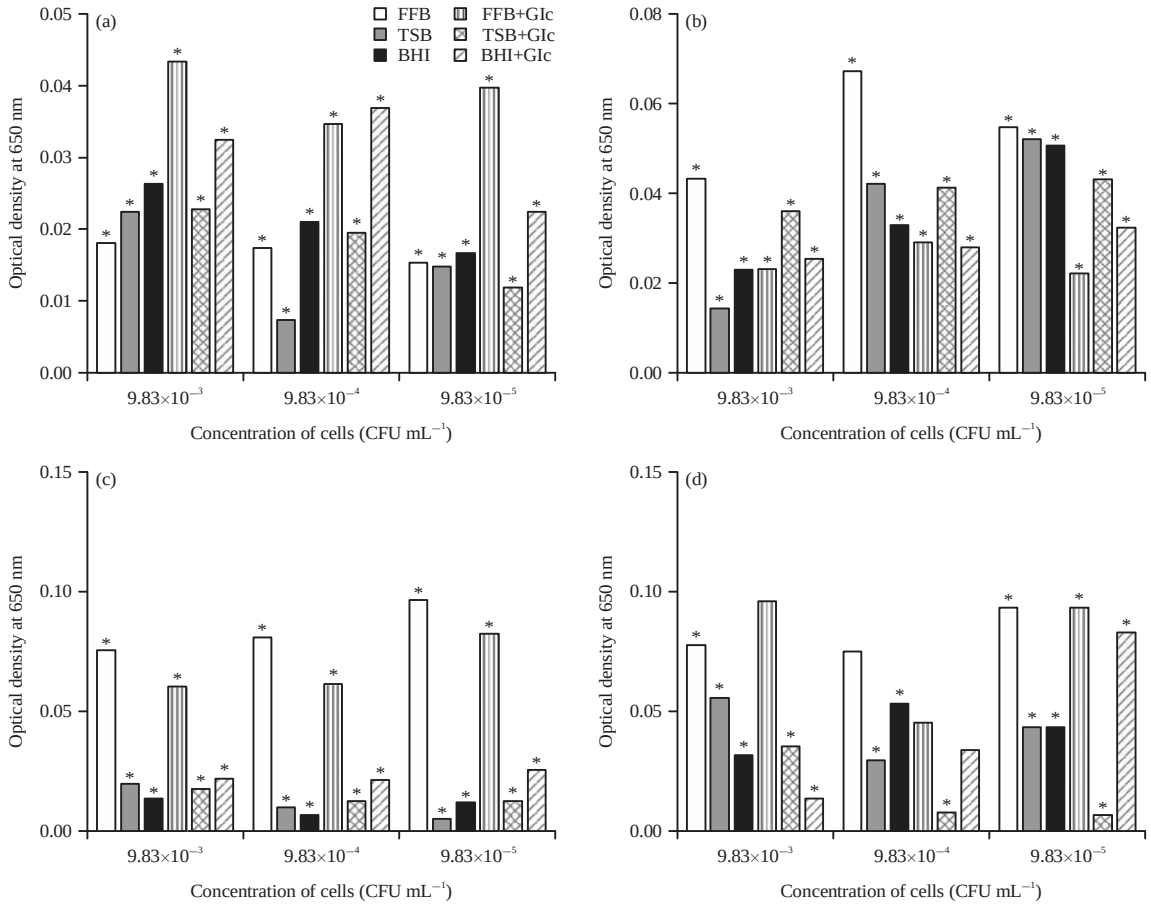

Fig. 2(a-d): Biofilm formation of *M. odoratimimus* SKS05-GRD of different cell density in static microtiter plate in different media at different time interval. Biofilm formation was measured by crystal violet staining at 650nm (A_{650}) on 96 well plate reader and the results are expressed (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h as mean of at least 3 independent experiments. *Significant relationship between biofilm formation of *M. odoratimimus* SKS05-GRD in FFB media in the cell concentration of 9.83×10^{-5} CFU mL^{-1} at 72 h (showing higher biofilm formation) and other medium, time intervals and cell density in the study

Table 2: Susceptibility of *Myroides odoratimimus* SKS05 GRD for tetracycline and antimicrobial agents assayed with CBD

Concentration (μg mL^{-1})	Tetracycline	Glucose based silver nanoparticles	Ethylene glycol based silver nanoparticles	Tetracycline+glucose based AgNP	Tetracycline+ethylene glycol based AgNP
Planktonic population (MIC)					
60	0.851±0.027	0.043±0.002[a]	0.024±.295	0.040±0.025[a]	0.042±0.002[a]
50	0.98±0.017	0.069±0.050[a]	0.061±0.115[a]	0.043±0.125[a,b]	0.053±0.042[a,b]
40	0.4±0.016	0.070±0.085	0.071±0.065[a]	0.061±0.130[a]	0.073±0.017[a,d]
30	0.401±0.018	0.086±0.268	0.078±0.031[a]	0.073±0.107[a]	0.082±0.013[a,d]
20	0.098±0.297	0.089±0.229	0.291±0.057	0.088±0.082[a]	0.084±0.004[a]
Biofilm population (MBEC)					
50	0.024±0.013	0.041±0.001[a]	0.045±0.004[a]	0.041±0.002[a]	0.044±0.001[a]
40	0.198±0.008	0.041±0.000[a]	0.041±0.000[a]	0.044±0.000[a,b,c]	0.042±0.001[a]
30	0.166±.010	0.040±0.000[a]	0.041±0.000[a]	0.042±0.001[a]	0.042±0.000[a]
20	0.135±0.114	0.041±0.000	0.043±0.000	0.04±0.004	0.041±0.000

The values given are obtained by measuring the turbidity of 650 nm (A_{650}) on a 96 well plate reader and expressed as Mean±SD at significance $p<0.05$. [a]Significant relationship between treatment of biofilm with tetracycline and other antimicrobial agents in the study, [b]Significant relationship between treatment of biofilm with glucose based silver nanoparticles and other antimicrobial agents in the study, [c]Significant relationship between treatment of biofilm with ethylene glycol based silver nanoparticles and other antimicrobial agents in the study, [d]Significant relationship between treatment of biofilm with tetracycline+glucose based silver nanoparticles and tetracycline+ethylene glycol based silver nanoparticles in the study

Table 3: MIC and MBEC values of antibiotics, nanoparticles and antibiotics coupled with nanoparticles when treated on preformed biofilm of *Myroides odoratimimus* SKS05-GRD

Antibiotics	Minimal Inhibitory concentration (MIC) (μg mL^{-1})	Minimal biofilm eradication concentration (MBEC) (μg mL^{-1})
Tetracycline	<20	50
Chloramphenicol	<20	100
Amoxicillin	<20	90
Ciprofloxacin	30	90
Cephatoxime	30	>100
Erythromycin	<20	80
Rifampicin	60	>100
Ampicillin	80	>100
Nanoparticles		
G-AgNP	20	5
E-AgNP	20	<5
G-AgNP with tetracycline (20 μg mL^{-1})	20	20
E- AgNP with tetracycline (60-10 μg mL^{-1})	20	20

[a]G-AgNP: Glucose based silver nanoparticles, E-AgNP: Ethylene glycol based silver nanoparticles

The master plate containing 9.83×10^{-5} CFU mL^{-1} concentration of *Myroides odoratimimus* SKS05-GRD was dispensed into modified calgary Biofilm device and incubated for 72 h. This biofilm which was grown in the pegs was then treated with antibiotics for 24 h using challenge plate (MIC) followed by a recovery of remaining biofilm attached to the peg by intense vortexing (MBEC™). The difference in the absorbance at 650 nm of planktonic cultures obtained through this procedure to understand the effect of antibiotics on biofilm showed tetracycline having maximum eradication potential followed by chloramphenicol and amoxicillin. The reduced levels of biofilm formation when treated with different antibiotics in triplicates extended to reveal the statistical significance among all treatment groups. All concentrations of study among all the antibiotics were checked at significance level of 95% ($p<0.05$).

The colony forming units of the contents from the recovery plate was serially diluted and spread plated on FFB agar plate resulted in reduced CFU mL^{-1} of *Myroides odoratimimus* SKS05-GRD (JQ178355) as the concentration of antibiotic added increased in the challenge plate.

Assessment of potential inhibition and eradication by silver nanoparticles on *Myroides odoratimimus* SKS05-GRD (JQ178355) biofilm: In this study, tetracycline showed effective anti-biofilm potential at 50 μg mL^{-1}, this study was carried out by narrowing down the concentrations of tetracycline and nanoparticles based antimicrobial agents from 20-60 μg mL^{-1}. The MIC of the antimicrobials was observed at 20 μg mL^{-1} except in ethylene glycol based silver nanoparticles showed the minimum inhibitory concentration at 30 μg mL^{-1}. The MBEC against *M. odoratimimus* SKS05-GRD when compared among the silver nanoparticles and tetracycline complexed silver nanoparticles showed that the eradication was at 20 μg mL^{-1} whereas, tetracycline alone showed the same effect at 50 μg mL^{-1} (Table 3).

Analysis of biofilm eradication using scanning electron microscopy: The morphological changes of 24 h bacterial cells, biofilm formed by *Myroides odoratimimus* SKS05-GRD at 72 h and planktonic population treated with glucose based silver AgNPs and antibiotic (TET) complexed glucose based AgNPs were observed by SEM (Fig. 3). The SEM analysis conducted at a magnification-5,000X and -20,000X revealed intense layering of biofilm. In the 24 h cells (Fig. 3a) treated as control, rod shaped intact cell surface with no damages were observed. However, in the biofilm population at 72 h (Fig. 3b), there are many irregular fragments on the cell surface indicating increase in cellular growth of the population. The treatment of biofilm with antibiotics as effect on its eradication was further confirmed using scanning electron microscope. The details obtained on the visual examination of cells or layer in the biofilm formation proves the effect of the antimicrobials with nanoparticles used in this study. Biofilm population treated with glucose based AgNPs (Fig. 3c) and antibiotic (TET) complexed glucose based AgNPs at 72 h (Fig. 3d) showed no changes in morphology when compared to the 24 h planktonic cells indicating that the cells were inhibited from biofilm formation.

Variations in molecular spectrum in untreated and treated *M. odoratimimus* SKS05-GRD biofilms by FT-IR Spectroscopy analysis: Representative FTIR spectrum of the 24 h bacterial cell (*M. odoratimimus*), planktonic population (antibiotic treated Biofilm-MIC) and biofilm population (planktonic population subjected to fresh media) were subjected to FTIR analysis for identifying changes in the bond formations present in the cellular contents of the cells (Fig. 4).

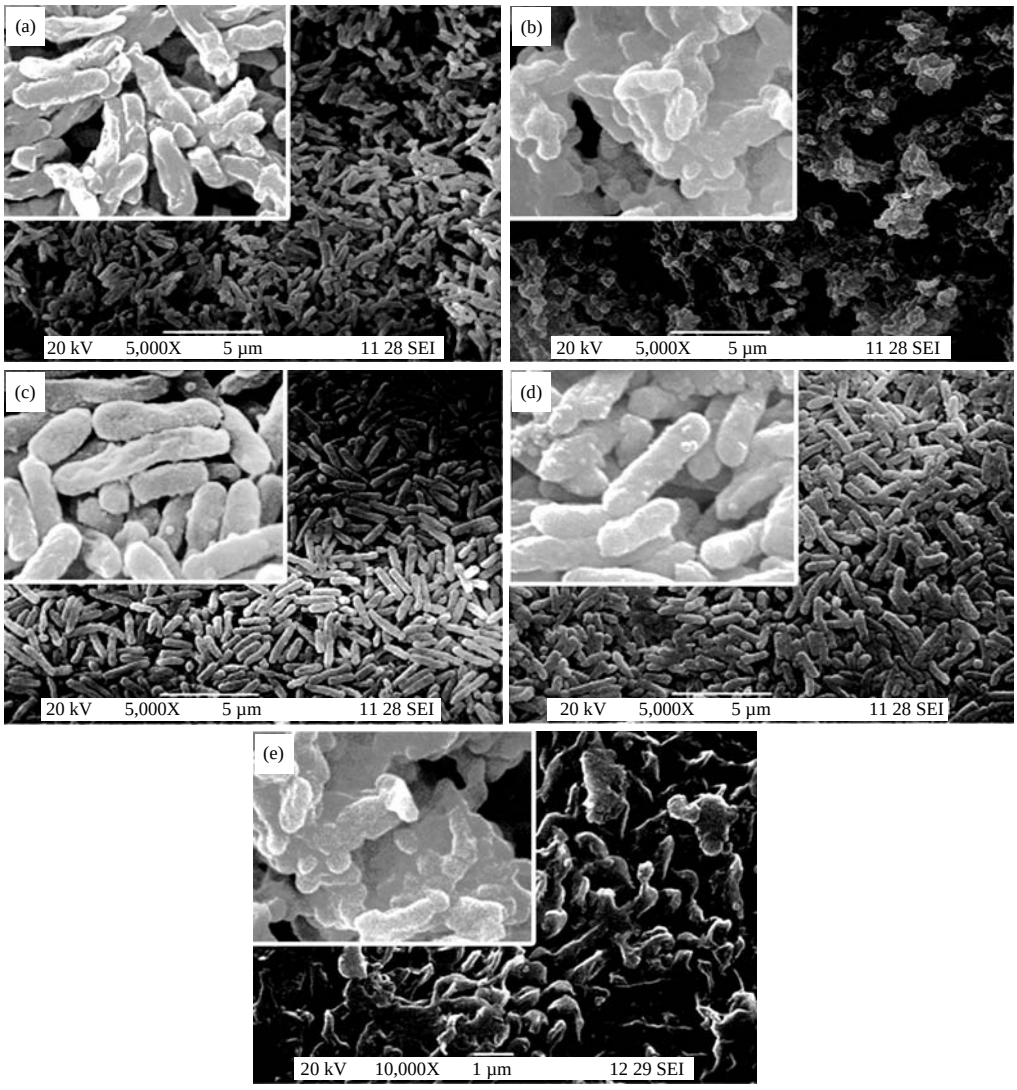

Fig. 3(a-e): Visual examination of eradication of biofilm using scanning electron microscopy, (a) *Myroides odoratimimus* SKS05-GRD at 24 h (Inset bar 1 µm, magnification-20,000X), (b) *Myroides odoratimimus* SKS05-GRD biofilm before antibiotic exposure at 72 h. Bar-5 µm (Inset bar 1 µm, magnification-20,000X), (c) Micrograph of *Myroides odoratimimus* SKS05-GRD biofilm after exposure with glucose based nanoparticles for 24 h. Bar-5 µm (Inset bar 1 µm, magnification-20,000X), (d) *Myroides odoratimimus* SKS05-GRD biofilm after exposure with tetracycline conjugated glucose based nanoparticles for 24 h. Bar-5 µm (Inset bar 1 µm, magnification-20,000X) and (e) SEM Micrograph of *Myroides odoratimimus* SKS05-GRD biofilm after exposure with tetracycline for 24 h. Bar-5 µm (Inset bar 1 µm, magnification-20,000X)

Alkynyl stretch between two carbon atoms was found to be in a highly stretched condition in the control plate (Fig. 4a). In planktonic population (Fig. 4c) a characteristic bent was found in the same bond with slight stretching as in the biofilm population (Fig. 4b). Each spectra exhibited characteristic absorption of wave number where the position of peaks may vary between 4000-600 cm^{-1}. Planktonic and biofilm population of single antibiotic were compared with that of the 24 h culture. Wave number 4000-3500 cm^{-1} corresponding to amide N-H bond shows a stretch in the control but was bent in the planktonic population. The same bond formation was found to stretch in the presence of fresh media in the biofilm population. The prominent absorption bending around 2900 cm^{-1} are mainly due to a C-H asymmetric stretching of $>CH_2$ in fatty acids from the bacterial cell wall. Slight difference between the spectra of 24 h, planktonic and

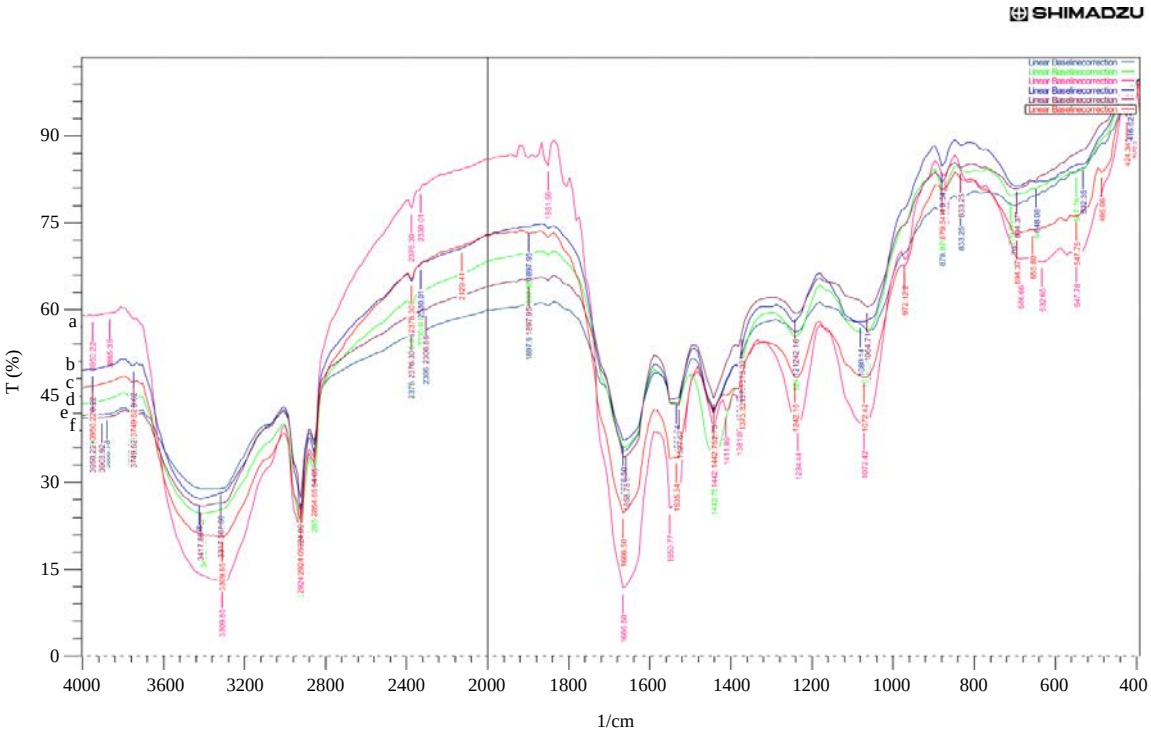

Fig. 4: Fourier Transform Infra Red (FT-IR) spectroscopy spectra of *Myroides odoratimimus* SKS05-GRD on treatment with nanoparticles. a: *Myroides odoratimimus* SKS05 GRD biofilm, b: Population of *Myroides odoratimimus* SKS05 GRD treated with glucose based nanoparticles, c: 24 h old *Myroides odoratimimus* SKS05 GRD d: Population of *Myroides odoratimimus* SKS05 GRD treated with tetracycline conjugated glucose based nanoparticles, e: Planktonic population of *Myroides odoratimimus* SKS05 GRD treated with tetracycline conjugated glucose based nanoparticles and f: Planktonic population of *Myroides odoratimimus* SKS05 GRD when treated with glucose based nanoparticle

biofilm population was observed in the peak intensity of 2400-2000 cm^{-1}. The wave number position of absorption peaks 1700-1500 cm^{-1} containing amide I and II band of protein and peptides shows significant changes. Absorption at wave numbers between 1200 and 900 cm^{-1} corresponds to absorption bands of carbohydrate in microbial cell walls shows differences. The bending to aromatic C-H bond indicated by a frequency decrease was found to be in wave number 900-700 cm^{-1} seen in the control plate was found stretched in bacterial cells in the planktonic and biofilm population. About 700-500 cm^{-1} shows a fingerprint region which can be confirmed specific to *M. odoratimimus* SKS05-GRD. These atomic changes in chemical bonding indicate similar patterns in control and biofilm population with distinct variation in planktonic population treated with antibiotics (Fig. 4).

The stretching and bending of bonds was more distinct in biofilm population treated with antibiotic (TET) complexed glucose based silver AgNPs when compared to biofilm population treated with glucose based silver AgNPs. This indicates antibiotic (TET) complexed glucose based silver AgNPs have more efficient anti-biofilm potential. The highest bending of this bond was seen in the biofilm treated with TET+AgNP in the challenge plate indicating that the nanoparticles might have an effect on the alkenes present either in the glycocalyx or within the cells. Stretching of the C=N bond was seen in the wave numbers corresponding from 1080-1065 cm^{-1} indicating changes in the DNA, RNA and Phospholipids. Similar stretching was seen in C-N (1245-1235 cm^{-1}) and C=O (1667-1657 cm^{-1}) indicating changes in amide I and II bond of proteins along with C-H rocking of $>CH_2$ in fatty acids and proteins at 720 cm^{-1} (Fig. 4).

DISCUSSION

The assessment of biofilm activity in different substrates by *Myroides odoratimimus* SKS05-GRD (JQ178355) is studied. The generation time of the bacterium has to be analyzed so that the proper incubation period needed by the organism to

form complete biofilm maturation period can be found out. The growth curve analysis is an empirical model of the evolution of a quantity over time. The time period required for the division of bacteria into daughter cell by a binary fission suggests the generation time needed by the organism. The culture is initiated in the appropriate medium and the turbidity is analysed every half an hour using spectrophotometer[10].

As a contact surface for biofilm formation wood was used in developed countries. Recent studies on assessment of biofilm formation by *Listeria monocytogenes* and *Listeria* spp., on wood, steel and glass surfaces suggest that there has been a reduction in the use of wood due to its porosity and absorbance as organic matter leads to entrapment and cross contamination of bacteria[8]. Glass and stainless steel were then researched as biofilm formation surfaces among which stainless steel shown to resist impact damage better than glass but accumulates bacteria on developed surface cracks and is vulnerable to corrosion[11]. Based on these studies contact surfaces such as wood, glass, stainless steel, plastic, teflon, polyvinyl were selected in this study.

Reports also suggest that glass and stainless steel are hydrophilic material while wooden, plastic and teflon are hydrophobic material. Hydrophobic materials are reported as surface that provides a greater bacterial adherence. In accordance with some earlier studies[8], the results of wood based contact surface could not be confirmed as the observation of the uptake of dye could not be concluded specific to the bacterial based biofilm due to the hydrophobic nature of absorbance of dye by wood. A number of studies have shown contradictory results with no relationship found between the bacterial strain surface hydrophobicity and the extent of initial binding to either a hydrophilic or hydrophobic substrate[12]. The presence of gliding bacteria in aquatic environment and their ability to adhere to various substrates has suggested that gliding bacteria are likely to be members of microbial biofilm.

Microtiter plates have been widely used because they are simple, reproducible and quantitative method. However, the staining measurements reflect the total amount of biofilm but do not give any information about the viability[13]. Many flavobacterium species, pathogens and opportunistic pathogens included, have been identified in biofilm material collected from environments[14]. Some studies have also reported the inability of *F. columnare* and *F. johnsoniae* cultures to form biofilm in undiluted nutrient broth[15].

In the present study, the ability of *Myroides odoratimimus* SKS05-GRD (JQ178355) to form biofilms using undiluted concentration (9.83×10^{-3} CFU mL^{-1}), 10-fold dilutions (9.83×10^{-4} CFU mL^{-1}) and 100-fold dilution (9.83×10^{-5} CFU mL^{-1}) in culture media with and without glucose as a carbon source for 96 h at every 24 h interval was reported. Studies regarding biofilm formation have been conducted within a period of 24-48 h. The biofilms were metabolically highly active in the first 8 h, but as the biofilm matured and the complexity increased (24-48 h) the metabolic activity reached a plateau, but remained high probably reflecting the increased number of cells that constituted the mature biofilm. This study took into account 96 h of growth due to the longer generation time of the bacterium.

The antibiotic resistance of *Myroides odoratimimus* SKS05-GRD (JQ178355) to multi-antibiotics in this study was also supported[16]. Their study concluded that resistance of *Myroides* to a wide range of antimicrobial agents commonly used against Gram negative non-fermentative bacterial infections, including β-lactam and aminoglycosides. The CBD and the MBEC™ assay have proved to be reliable, rapid and reproducible methodologies for effective selection of clinically used antibiotics or agents that pose to yield clinical benefits[17]. In case of the preformed biofilm, tetracycline, chloramphenicol, amoxicillin and erythromycin showed the best results in biofilm removal even at the concentrations of 20 μg mL^{-1} and caused removal of 50% of an already formed biofilm. On the other hand, ampicillin, ciprofloxacin, cephatoxime and rifampicin showed the least results in biofilm removal. However, tetracycline showed efficient biofilm eradication potential at concentration of 50 μg mL^{-1} when compared to other antibiotics which showed eradication at the concentration more than 90 μg mL^{-1}.

The efficacy of nanoparticles and nanoparticles complexed with tetracycline was measured in terms of zone of inhibition (mm) against various concentrations of nanoparticles. Specific antibiotic activities showed no variation against any of the concentrations although efficient antimicrobial activity was found as the zone of inhibition averaged ~20 mm against the entire tested nanoparticles. These nanoparticles alone and in conjugation with tetracycline proved to be efficient anti-biofilm agent. The concentration for effective inhibition and eradication of the biofilm was determined using MIC and MBEC assays. Well diffusion assay showed zone of inhibition from 10 μg mL^{-1} concentration. The zone of inhibition increased linearly with increase in concentrations of AgNPs. Hence, confirmed bacterial growth inhibition is dose dependent. Similar descriptive studies in other Gram negative bacteria shows indulgences of proton motive force in membranes of *E. coli* when AgNPs are exposed in various concentration[18].

Several other studies demonstrated that the silver nanoparticles show the efficient antibacterial activity against *E. coli* and *S. aureus*[19,20]. The results resolve that the treatment of AgNPs can obstruct the growth as well as the synthesis of exopolysaccharide or slime formation. These nanoparticles diffuse directly into the exopolysaccharide layer through the pores that meant for nutrient transportation and acts as an anti-microbial agent[21]. Despite, the regulation of biofilm suppression, there were fluctuations in the inhibition rate at each nanomolar concentration for the stains used. Similar observations with AgNPs against Gram negative biofilm producers in contact lens[22].

The use of scanning electron microscopy in biofilms has previously used to reveal its fine structure to understand the physiology, ecology and thickness providing a visual effect of antibacterial and antifungal agents[23]. The results obtained on SEM analysis were in accordance with previous studies where morphological changes were observed on the bacterial cell. In the present study, treatment by glucose based AgNPs and antibiotic (TET) complexed glucose based AgNPs indicate eradication of biofilm[24,25]. Nanoparticles and antibiotic conjugated nanoparticles revealed complete disintegration of the biofilm and resulted in individual cells at 1 µm magnification.

The FTIR analysis of whole microbial cells has been utilized as a reliable technique for microbiological analysis, including identification of microorganisms, study of microbial metabolism and antibiotic susceptibility of other cell-drug interactions. The results of the present study at wave number 3600-3000 cm^{-1} show significant peaks that are mainly due to N-H stretching of amide A in the proteins. Not much of a variation was seen among the wave numbers ranging from 2925-2855 cm^{-1} indicating no changes in molecular bonding of fatty acids. This may be due to FT-IR spectra of intact bacterial cells cannot provide complete information of specific cellular components due to overlapping absorbance bands[26].

Wave widths ranging from 2800-1700 cm^{-1} showed a major difference in the peaks with wide range of stretching and bending corresponding to C (triple bond) C among all tests. As the spectral measurements may provide insight regarding the condition or injured microbes and how certain types of injury could occur, the major difference may be due to the use of sonication in this procedure. Wave width ranging from 1700-1000 cm^{-1} shows distinct variation in amide I and II bands of proteins and peptides, fatty acids bending vibration proteins and phosphate carrying compounds and bands of carbohydrates in bacterial cell wall dominated by ring vibration in various polysaccharides[27].

CONCLUSION

This study has, therefore, confirmed the potential of *M. odoratimimus* SKS05-GRD (JQ178355) to form biofilm. Optimum medium and dilution of the culture was also determined that yield effective biofilm formation. As the property of biofilm formation has been studied to restrict the activity of antibiotics by a resistive layer of exopolysaccharides, the study was extended to analyze the effect of antibiotics for eradication of biofilm. The visual description obtained after analysis using SEM revealed significant changes in the morphology of cells between treated and untreated biofilm. FT-IR analysis revealed that there were some bond stretching and bond bending when the biofilm was treated with antibiotics and AgNPs when compared to untreated biofilm. On recovery plate, the bond stretching and bond bending were slowly reverting back to the original untreated biofilm structure of 24 h. The potency of anti-biofilm activity by MBEC against *Myroides odoratimimus* SKS05-GRD was higher when tetracycline conjugated AgNPs were used.

SIGNIFICANCE STATEMENTS

This study provides an insight into the synergistic action of antibiotics and nanoparticles that can be used to target the biofilm formed by bacterial samples. As the study is based on the quantification and visual analysis using bio-analytical methods, many researchers will be able to confirm the biofilm formation of other microbes and validate their findings.

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