

ENHANCED ANTIMICROBIALS AND ESTERASE PRODUCTION ASSOCIATED TO BIOFILM FORMATION BY TWO ESTUARINE ISOLATES IN A NOVEL POLYMETHYLMETHACRYLATE CONICO- CYLINDRICAL FLASK

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[Received-01/04/2014, Accepted-17/04/2014]

ABSTRACT

A newly designed polymethylmethacrylate (PMMA) conico-cylindrical flask (CCF) with an inner arrangement consisting of eight equidistantly spaced rectangular strips mounted radially on a circular disk to provide additional surface area for microbial attachment was employed for antimicrobial compounds and esterase production by two biofilm-forming marine isolates, *Streptomyces sundarbansensis* sp nov. and BSE01, respectively. The design allowed comparison of production between (1) CCF with hydrophobic surface (PMMA-CCF), (2) CCF with hydrophilic glass surface (GS-CCF), and (3) standard unbaffled Erlenmeyer flask (EF). Compared to the EF, antimicrobial activity increased from 31 mm to 41 mm and esterase activity from 1070 ± 10 U/mL to 3120 ± 10 U/mL, respectively, in the PMMA-CCF but decreased from 31 mm to 17 mm for antimicrobial activity and from 3345 ± 10 U/mL to 765 ± 5 U/mL for esterase activity, respectively, in the GS-CCF. Growth of *Streptomyces sundarbansensis* sp nov. was also higher in the PMMA-CCF by 1.12 fold and 3.12 fold, respectively, compared to the EF and GS-CCF. Similarly, growth of BSE01 was also higher in the PMMA-CCF by 391 fold and 1181 fold, respectively, compared to the EF and GS-CCF. Temporal pattern of biofilm progression based on two-channel fluorescence detection of extracellular polymeric substances and whole cells in a confocal laser scanning microscope followed by application of PHLIP and ImageJ volume viewer software demonstrated formation of matured, stable, well-developed, thick biofilm on PMMA in contrast to glass surface for both the isolates. Thus, antimicrobial compounds and esterase production were positively associated with vessel surface area and hydrophobicity, suspended cell growth, and biofilm formation.

Keywords: *Streptomyces*, antimicrobial, bacteria, esterase, biofilm, confocal laser scanning microscopy

1. INTRODUCTION

The intertidal zone, which occupies the upper edge of the world's coasts extending over 1,600,000 km, is probably the most important coastal habitat given its biological productivity and economic value. It comprises rocky platforms, sandy beaches, mudflats, estuaries, salt marshes, mangrove forests, certain coral reefs and human-made infrastructures. Intertidal microbial communities often occur as biofilms which are high-density attached communities embedded in extracellular biopolymer matrices [13]. The microbial biofilm is a common adaptation of natural bacteria and other microorganisms. In the fluctuating environments of intertidal systems, biofilms form protective microenvironments and may structure a range of microbial processes [6].

Surface attachment and biofilm formation are known to influence metabolite production by microorganisms. Marine surface associated microorganisms may require conditions that resemble their native environment in order to produce the maximum amount of bioactive metabolites. For example, several studies have shown an increased production of antimicrobial compounds when the surface associated bacteria were grown, *in vitro*, to form surface attached biofilms [14].

Presently, there are no small-scale vessels that provide significantly enhanced surface area as well as property for surface attachment of biofilm-forming microorganisms. It would therefore be beneficial to have an appropriate small-scale vessel that enable researchers to verify if surface attachment and biofilm formation would increase production of antimicrobial compounds by intertidal actinobacteria. A newly designed shaking flask, the conico-cylindrical flask (CCF) containing an inner arrangement that helps biofilm formation was made. Compared to Erlenmeyer flask having similar volume, the CCF provided more than 80% additional surface for biofilm formation and growth. The conico-cylindrical

flask (CCF) has a diameter close to that of a 500-ml Erlenmeyer flask, thus can be easily fit into a rotary shaker for routine small-scale studies. The surface properties of the inner arrangement of the CCF's can be varied by using different surfaces (hydrophobic/hydrophilic) [22] and, at the same time, also provided a facility for varying the modes of aeration (shaking, stationary, external aeration through a pump) [22].

The CCF was successfully used for the production of different metabolites e.g. protease, [22], melanin [11], antibiotic substance [11], cellulase [10], xylanase [10] and riboflavin [9] obtained from the marine sources. In the present investigation a comparative studies were made for an antimicrobials production by *Streptomyces sundarbansensis* sp. nov. [2, 17, 3] and an esterase production by the marine inhabitant bacteria (BSE01) [19] cultivated both in the CCF and in the traditional Erlenmeyer flask. Thus, the aims of the present investigation were, first, to study the application of the novel small-scale vessel both for an actinobacterial as well as bacterial cultivations, second, to study the effect mode of aeration and third, to examine the effect of the surface attachment of intertidal estuarine actinobacterial strain *Streptomyces sundarbansensis* sp nov. on the production of antimicrobial compounds as well as an esterase production by the intertidal inhabitant bacteria (BSE01).

In this study a salt-tolerant estuarine actinobacterial strain *Streptomyces sundarbansensis* sp nov. [2], designated as MS1/7^T (=MTCC 5272^T) isolated from the *Sundarbans* was used. The strain produces a cytotoxic antimicrobial of molecular weight 300.2 and predicted molecular formula C₂₀H₂₈O₂ [17] and also 2-allyloxyphenol [2, 3].

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The second marine isolate, a *Bacillus* sp. isolated from the Sundarbans region of the Bay of Bengal produces an extremely dimethylsulfoxide tolerant esterase [19].

2. MATERIALS AND METHODS

2.1. Microorganisms and media composition

The first marine isolate, a salt-tolerant estuarine actinobacterial strain *Streptomyces sundarbansensis* sp nov., designated as MS1/7^T (=MTCC 5272^T) [2] was isolated from the intertidal regions of the Sundarbans delta off the Bay of Bengal. The novel actinomycete produces a cytotoxic antimicrobial of molecular weight 300.2 and predicted molecular formula $C_{20}H_{28}O_2$ [17] and also 2-allyloxyphenol [2, 3] at shake flask level. The compound inhibits three Gram-positive and three Gram-negative multiple drug resistant (MDR) bacteria (defined as that disease-causing bacterium which can survive in the presence of structurally unrelated antimicrobial compounds targeted at distinct bacterial physiological mechanisms to eradicate the disease causing bacterium), seven non-clinical Gram-positive, four Gram-negative bacteria and five fungi [17].

The first strain was maintained on an enrichment medium (EM) (all units in g/L, K_2HPO_4 0.5, casein 3.0, starch 10.0, peptone 1.0, yeast extract 1.0, malt extract 10.0 and agar 15.0, distilled water 500 ml and natural seawater 500 ml, pH 7.4) [19] slant, stored at 4 °C, and subcultured every month. Sucrose based medium (SBM) (all units in g/L, starch 2.0, soybean meal 2.0, yeast extract 0.5, $CaCO_3$ 0.32, $CuSO_4$ 0.005, $MnCl_2$ 0.005, $ZnSO_4$ 0.005, sucrose 170 g/L, seawater 500 ml, distilled water 500 mL, pH 10 was used as the production medium and $5.0 \text{ mL of } 8 \times 10^9 \text{ CFU/ml}$ spore and substrate mycelium suspension was used as inoculum.

The second marine inhabitant bacteria, a *Bacillus* sp. [BSE01] isolated from the sediments of the Lothian Island of the Bay of Bengal produces an extremely dimethylsulfoxide tolerant esterase [19] and it was maintained on Marine Agar (MA) 2216 media [18] containing (g/L) peptone, 5; yeast extract, 1; $FeCl_3$, 0.1; $MgCl_2$, 8.8; NaCl, 19.45; Na_2SO_4 , 3.24; $CaCl_2 \cdot 6H_2O$, 1.8; KCl, 0.55; $NaHCO_3$, 0.16; KBr, 0.08; $SrCl_2$, 0.034; H_3BO_3 , 0.022; Na_2SiO_3 , 0.004; NaF, 0.0024; NH_4NO_3 , 0.0016; Na_2HPO_4 , 0.008; agar, 15; pH 7.0–7.2. Marine Broth (MB) 2216 medium supplemented with 90 g/L sucrose (pH adjusted to 7.0 before autoclaving) was used as optimized medium for esterase production by BSE01. Sucrose was autoclaved separately at 121 °C for 15 min and added aseptically to MB 2216 medium [22]. One percent (v/v) of a 24-h culture was used as inoculum.

2.2. Flask configurations and operating conditions

The CCF made of polymethylmethacrylate (PMMA-CCF) was designed along with engineers from M/s Plastic Abhiyanta, Kolkata, India [22]. The three flask configurations studied in the initial experiments were polymethylmethacrylate conico-cylindrical flask (PMMA-CCF), a cylindrical flask (CF) and a 500-mL Erlenmeyer flask (EF). The cylindrical flask (CF) was a CCF without the inner arrangement. The production of antimicrobials by *Streptomyces sundarbansensis* sp nov., in these two novel configurations were compared with that in a standard 500-ml Erlenmeyer flask made of borosilicate glass (EF), the third flask configuration. The PMMA-CCF contained three parts, namely a lower cylindrical portion, an upper funnel portion and an inner arrangement. The dimensions were (a) lower cylindrical portion: height 150 mm, outer diameter 80 mm, thickness 5 mm; (b) upper funnel portion: diameter of the funnel base 90 mm, diameter of funnel top 40 mm. The two parts were joined by screwing the upper part into the lower part. The inner arrangement

consisted of eight equally spaced rectangular strips mounted radially on a circular disk (height of the strip 150 mm, thickness 5 mm, diameter of the disk 70 mm, thickness 5 mm). For the surface attachment study, the fourth configuration (GS-CCF) where 16 autoclaved borosilicate microscope glass slides (75 mm X 25 mm X 1.35 mm) were aseptically attached to both sides of the eight-strip inner arrangement of the PMMA-CCF with a strong non-toxic adhesive (Fevi Kwik, Pidilite Industries Ltd., Mumbai, India). This provided a hydrophilic surface to the inner arrangement of the GS-CCF, while the PMMA-CCF had hydrophobic surface characteristics. One hundred milliliters of medium was added to each vessel. The surface area of the inner arrangement was 706.4 cm². The surfaces of the inner arrangement, of both PMMA-CCF and GS-CCF were roughened with Grade 50 sandpaper [22]. Quantitative measurement of roughness was obtained for roughened PMMA surfaces of 15 rectangular strips of the inner arrangement and the same number of roughened microscope glass slides. The roughness average value (Ra) was determined on a stylus-type profilometer (Surtronic 3+, Taylor Hobson Ltd., England). The traverse length was 4.0 and the cut-off length was 0.8 mm. A traverse speed of 1 mm/s and a Gaussian filter were applied. Talyprofile (Taylor Hobson, England) software was employed for data analysis. For sterilization, the PMMA-CCF and GS-CCF were disassembled, the components washed thoroughly in tap water and immersed in 3 % (v/v) sodium hypochlorite for 5 h. The parts were then surface-sterilized under UV light (TUV15W/G15T8, Philips, The Netherlands) in a laminar airflow bench for 30 min. Ports on the top lid of the PMMA-CCF were available for air inlet and exhaust. Three modes of aeration were examined in each of the three reactor configurations i.e. PMMA-CCF, CF and EF: shaking at 140 rpm in an orbital shaker (with cotton plugs), orbital shaking with external

aeration of 0.75 L/min of air (through ports on top of the PMMA-CCF) and external aeration without shaking. Cultivation temperatures for *Streptomyces sundarbansensis* sp. nov and BSE01 were 30 and 37 °C, respectively [19, 21]. Samples were aseptically withdrawn at 24, 48, 72, and 96 h. All cultivations were done thrice in duplicate sets for each vessel configuration.

2.3. Analytical methods

2.3.1. Assay of antimicrobial compound

Determination of antimicrobial activity was carried out following the method described in [21] by centrifuging the liquid medium containing the suspended cells and plating 150–200 µL of the supernatant against *Staphylococcus aureus* MTCC 96 as the test microorganism. Maximum antimicrobial activity at a defined time interval is described as peak antimicrobial activity (PAMA) corresponding to the diameter of zone of inhibition.

2.3.2. Assay of enzymatic activity

Esterase production will be determined by the methods reported earlier [19]. For standard assays, the esterase activity was measured by the spectrophotometric method using p-nitrophenyl acetate as substrate. 50 µL of 2mM p-nitrophenyl acetate was mixed with 250 µL of diluted enzymes and 200 µL of Mc Ilvain buffer pH (7.2) and incubated at 40°C for 30 minutes. The reaction was stopped immediately by addition of 500 µL chilled buffer and suspended particles were removed by centrifuging at 12,000 rpm for 2 minutes. The amount of p-nitrophenol released by esterase-catalysed hydrolysis was measured spectrophotometrically at 410 nm against the enzyme free blank. One unit of esterase was defined as the amount of enzyme needed to liberate 1 µmole p-nitrophenol per minute under this condition.

2.3.3. Determination of soluble protein

Soluble protein was measured following the Bradford method as described earlier [8]. The color reagent was prepared by dissolving 100 mg

of Coomassie Brilliant Blue G-250 (SRL, Mumbai, India) in 50 mL of 95 % ethanol. To this solution, 100 mL of 85 % ortho-phosphoric acid (SRL, Mumbai, India) was added. The volume was made up to 1000 mL and filtered through Whatman No. 1 filter paper before use. To 100 μ L of appropriately diluted culture supernatant sample, 1000 μ L of the prepared reagent was added, mixed thoroughly, and the absorbance was recorded at 595 nm against distilled water blank. Soluble protein concentration was determined against a calibration curve prepared with bovine serum albumin standard in the range 0–100 μ g/mL.

2.3.4. Measurement of planktonic cell growth
Planktonic cell growth, defined as growth measured from samples withdrawn from suspension and not from the biofilm was determined by recording cell dry weight and viable cell count. Culture samples (1.0 mL) were centrifuged at 9300 g (Eppendorf Model 5415D, rotor: F45–24-11) for 5 min. The supernatant was discarded and the cell pellet resuspended in an equal volume of distilled water and recentrifuged. The biomass was dried at 105 °C overnight and weighed. Viable cell growth expressed as colony forming units (CFUs) per mL was determined by plating 100 μ L of a suitably diluted culture on enrichment medium (EM) and incubation at 30 °C for 24 – 48 h.

2.3.5. Measurement of biofilm formation

In the experiments related to the effect of flask configuration on antimicrobial activity, biofilm formation was quantified by scraping of an area of approximately 45 cm² area of the total biofilm surface with a sharp scalpel at the end of the experiment and measuring the dry weight on an electronic digital balance (AFCOSET, Model ER-180A, Mumbai, India) as described [22]. For the experiments studying the effect of surface properties on antimicrobial activity, biofilm formation in the inner arrangement of the ES-CCF was assessed by an attachment assay [5]. Non- and

poorly attached bacteria were removed by placing the inner arrangement on a sterile absorbent paper. The inner arrangement was incubated at 60 °C for 30 min, submersed in 0.1 % crystal violet for 15 min at room temperature and unbound dye was removed by rinsing with phosphate-buffered saline. The inner arrangement was immersed in 2 mL of 33 % acetic acid to solubilize the dye bound to the biofilm, and the absorbance was measured using a spectrophotometer at 590 nm.

2.3.6. Estimation of extracellular polymeric substance (EPS)

The EPS in suspension was estimated as described earlier [15, 20]. To 1 mL of cell-free supernatant, 2 mL of distilled alcohol (90 %) was added and incubated for 12 h at -20 °C. The EPS was obtained by centrifugation at 8100g (Eppendorf Model 5415D, rotor: F45–24-11) for 15 min. The precipitate was air dried for 4 h and dissolved in 2 mL of distilled water. The polysaccharide content was measured using the anthrone method [20]. Glucose was used as standard, and the absorbance was measured at 625 nm to estimate the concentration of hexose sugars. Biofilm EPS was determined following the method described earlier [1]. For polysaccharide quantification, the samples were first dried at 40 °C for 2 h, resuspended in 1 N NaOH and heated at 80 °C for 30 min in a water bath. The samples were centrifuged at 8100 g (Eppendorf Model 5415D, rotor: F45–24-11) and 4 °C for 15 min and the polysaccharide concentration was determined in the supernatant.

2.3.7. Estimation of lactone type quorum sensing compounds

Following the method described earlier [23], 5 mL of culture broth was centrifuged at 5,000 rpm for 10 min, the supernatant extracted twice with equal volumes of ethyl acetate, the combined extracts dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The quantification of lactone type quorum sensing compounds was done described earlier [27]. The dried residues were dissolved in 100 μ L of distilled water, following

which 40 μL was taken in a 96-well plate and 50 μL of a 1:1 mixture of hydroxyl amine (2 M)/NaOH (3.5 M) was aliquoted and mixed with the samples. Subsequently, 50 μL of 1:1 mixture of ferric chloride (10 % in 4 M HCl)/ 95% ethanol was added. A dark brown color developed in all the samples containing lactone type compounds, the absorbance of which was determined spectrophotometrically at 520 nm in a multiscanner (Bio-Rad 680 XR). The concentration of lactone type compounds in the culture broth was determined using γ -valerolactone (Sigma-Aldrich) as the standard. All experiments were performed thrice in duplicate sets and the values reported are the averages of six determinations.

2.4. Confocal laser scanning microscopy of *Streptomyces sundarbansensis* sp. nov and BSE01

Biofilm architecture was determined by confocal laser scanning microscopy (CLSM) described earlier [10]. For this study, polymethylmethacrylate slides and borosilicate microscope glass slides (75 mm X 25 mm X 1.35 mm³,) were aseptically attached to both sides of the eight-strip inner arrangement of the PMMA ES-CCF with Fevi Kwik[®] (Pidilite Industries Ltd., Mumbai, India). Slides were aseptically taken out (two each) after 24 h, 48 h and 72 h of cultivation of both the isolates on PMMA surface, and glass surface by applying a minimal amount of sterile warm water to debond the adhesive (following the manufacturer's instruction). The slides were washed twice with 1 mL of 1 X PBS buffer and excess fluid was removed by using Whatman filter paper. For EPS staining, the slides were incubated in the dark for 20 min with 200 μL of 100 $\mu\text{g}/\text{mL}$ (in PBS) fluorescein isothiocyanate-concanavalin A (FITC-conA, Sigma). Excess FITC-Con A was removed by two rinses of 1 mL PBS and one rinse with 1 mL TRIS (all units in g/L ; EDTA 18.6; TRIS 0.158; NaCl 20; KC1 0.4). Thereafter, slides were incubated for 2 min using

200 μL of 10 μM SYTO 64 (Molecular Probes) dissolved in TRIS-buffer to stain the cells. Excess SYTO 64 was removed by 2 rinses of 1 mL TRIS and one rinse of 1 mL PBS. Biofilm development was visualized by using a confocal laser scanning microscope (Leica TCS SP2, Germany) equipped with an argon laser and helium-neon laser. HCX PL APO CS 40 X 1.25 oil-immersion lens was used as the objective. The parameters for the CLSM were set once and applied evenly as much as possible for the rest of the experiments in order to ensure that there could be quantitative comparison of the results. For visualization of FITC-con A, excitation was set at 488 nm, emission at 520 nm whereas for visualization of SYTO 64, excitation was fixed at 543 nm and emission at 620 nm.

2.5. Image analysis

PHLIP software (<http://phlip.sourceforge.net>) is open-source software which was specifically designed for three-dimensional (3D) biofilm analysis [12]. PHLIP automatically sets the threshold value using the Otsu algorithm and calculates the architectural parameters. PHLIP runs on the Matlab platform and requires an additional definitions file, which was automatically produced by Auto-PHLIP-ML software ([http://sourceforge.net/projects /auto-phlip-ml](http://sourceforge.net/projects/auto-phlip-ml)). Auto-PHLIP-ML also removes bias from biologically insignificant pixels by removing extraneous images. In this work PHLIP calculations were used to determine the biovolume, mean thickness, substratum coverage, total spreading, roughness coefficient and area-to-volume ratio. PHLIP was run in the "no connected volume filtration" (CVF) mode. Data were exported in an HTML format and were statistically analyzed using Origin[®]. ImageJ (<http://rsb.info.nih.gov/ij/>) was used for imaging using the "Volume Viewer 1.31" plugin (<http://rsb.info.nih.gov/ij/plugins/volume-viewer>) [12, 7].

3. RESULTS AND DISCUSSION

3.1. Effect of mode of aeration on antimicrobials production and cell growth by *Streptomyces sundarbansensis* sp. nov.

From the data on cell growth and antimicrobial activity (Table 1), it is apparent that of the three modes of aeration considered, orbital shaking at 140 rpm is most favorable for antimicrobials production and cell growth for all flask configurations. Antimicrobial compounds production by MS1/7^T in the ES-CCF was much higher i.e. 38 mm than the antimicrobial compound production at shake flask level i.e. 33 mm reported earlier [21]. Therefore, in further experiments, the top lids in the CCF and CF were replaced by sterile cotton plugs similar to those commonly used standard Erlenmeyer flasks.

Table 1

I		II		III	
PAMA	DCW	PAMA	DCW	PAMA	DCW
Conico-cylindrical flask 38±1	0.3789±0.0028	24±1	0.1739±0.0024	No activity	0.0649±0.0028
Cylindrical flask 18±2	0.0942±0.0008	16±2	0.0641±0.0006	No activity	0.0035±0.0004
Erlenmeyer flask 30±1	0.2635±0.0022	21±1	0.1198±0.0012	No activity	0.0461±0.0006

Peak antimicrobial activity (PAMA expressed as diameter of zone of inhibition in mm) and dry cell weight (DCW, g/mL) of suspension cells for *Streptomyces sundarbansensis* sp nov., grown under three modes of aeration, I – shaking at 140 rpm in an orbital shaker (with cotton plugs), II – orbital shaking with external aeration of 0.75 L/min of air and III – external aeration without shaking in three flask configurations, conico-cylindrical flask, cylindrical flask and Erlenmeyer flask. Data shown in bold indicate highest values obtained with three modes of aeration for *Streptomyces sundarbansensis* sp nov. Zone of inhibition data included 7 mm hole diameter.

3.2. Effect of flask configuration on antimicrobial production, cell growth and biofilm formation by *Streptomyces sundarbansensis* sp. nov.

Fig.1 A represents the effect of flask configuration on antimicrobials production by *Streptomyces sundarbansensis* sp nov. Although antimicrobial activity was same for both the CCF and the EF for the first 48 h, the CCF showed higher antimicrobial activity

after longer incubation period. Maximum antimicrobial activity was obtained in the CCF (39 ± 1 mm at 72 h) compared to the EF (31 ± 1 at 48 h) and CF (19 ± 2 mm at 48 h). Planktonic cell growth was higher in the CCF compared to the CF and EF (Fig.1 B). Cell suspension EPS was significantly higher in the CCF (Fig.1 C). Utilization of soluble protein (Fig.1 D) was faster in the CCF than in the EF or CF. Biofilm formation (as recorded by average weights) was higher in the CCF (0.7448 ± 0.0512 g) compared to the EF (0.2584 ± 0.0151 g) and CF (0.0354 ± 0.0021 g). Maximum amount of biofilm EPS values of 0.6234 ± 0.041 g/mL culture and 0.0837 ± 0.002 mg of EPS/g of biofilm were obtained in the CCF compared to the amounts of biofilm EPS formed in EF and CF cultivations.

3.3. Effect of surface property of the inner arrangement on antimicrobials production, cell growth and biofilm formation by *Streptomyces sundarbansensis* sp. nov.

The roughness average Ra (PMMA) was estimated as 2.51 ± 0.02 µm while Ra (glass) was determined to be 2.39 ± 0.02 µm. Thus, the two materials were of nearly equal roughness. Antimicrobials production by the isolate *Streptomyces sundarbansensis* sp nov. was higher in the PMMA-CCF where the inner arrangement was without glass slides than the GS-CCF, when lined with glass slides (Fig.2 A). Biofilm formation, as determined by attachment assay, was reduced drastically when the glass slides were used (OD590 = 0.421 ± 0.002 for PMMA-CCF and OD590 = 0.081 ± 0.003 for GS-CCF). Also, the concentration of cell suspension EPS was higher in the PMMA-CCF compared to the GS-CCF (Fig.2 B). Suspended cell growth was 60-fold higher when the isolate was cultivated in the vessel whose inner arrangement was without glass slides (26 × 10⁷ CFU/mL with glass and 16 × 10⁹ CFU/mL without glass). The cultivation with the

hydrophobic surface allowed faster consumption of soluble protein (Fig.2 C). The concentration of the lactone type quorum sensing compounds was higher in the PMMA-CCF as well as maintained a

Thus, in following experiments, sterile cotton plugs similar to those regularly used standard Erlenmeyer flasks were used.

Table 2

I		II		III	
PEA	DCW	PEA	DCW	PEA	DCW
Conico-cylindrical flask					
3120±10	0.3442±0.0024	1130±12	0.0281±0.0012	No activity	0.0123±0.0021
Cylindrical flask					
735±12	0.3251±0.0009	621±8	0.0174±0.0004	No activity	0.0119±0.0005
Erlenmeyer flask					
1070±10	0.3312±0.0013	955 ±9	0.0262±0.0010	No activity	0.0127±0.0007

steady level till the end of the cultivation in contrast to the concentration attained in the GS-CCF (Fig. 2 D). The quorum sensing compounds, most probably, promoted positive cooperation among the cells that could lead to biofilm formation. In the PMMA-CCF, the marine isolate grew as distinct thick biofilm (Fig.3 A), while in the GS-CCF, it formed a thin biofilm structure (Fig.3 B). Thus, the growth of *Streptomyces sundarbansensis* sp nov. was correlated with the hydrophobic reactor surface, biofilm formation, EPS synthesis, and concentration of quorum sensing compounds, results indicate the association between antimicrobial compounds production by the actinobacterium with the other aforesaid five parameters.

3.4. Effect of mode of aeration on esterase production and cell growth by BSE01

From the data on cell growth and esterase activity (Table 2), considering three modes of aeration, orbital shaking at 140 rpm is most favorable for esterase production and cell growth for all flask configurations. Esterase production by BSE01 was maximum in the ES-CCF i.e. 3120±10 U/mL than the esterase production at shake flask level i.e. 1070±10 U/mL. Table 2 showed that during cultivation with shaking at 140 rpm, the highest esterase production (3120±10 U/mL) as well as suspended growth (0.3442±0.0024 g/mL dry wt.) occurred in the hydrophobic surface vessel (PMMA-CCF), followed by CF (735±12 U/mL; 0.3251±0.0009 g/mL dry wt.), and finally the EF (1070±10 U/mL; 0.3312±0.0013 g/mL dry wt.).

Peak esterase activity (U/mL) and dry cell weight (DCW, g/mL) of suspension cells for BSE01, grown under three modes of aeration, I – shaking at 140 rpm in an orbital shaker (with cotton plugs), II – orbital shaking with external aeration of 0.75 L/min of air and III – external aeration without shaking in three flask configurations, conico-cylindrical flask, cylindrical flask and Erlenmeyer flask. Data shown in bold indicate highest values obtained with three modes of aeration for BSE01.

3.5. Effect of flask configuration on esterase production, cell growth and biofilm formation by BSE01

Maximum esterase activity as well as planktonic growth was recorded in the CCF in comparison with the EF and the CF (Fig.4 A and Fig.4 B). Biofilm formation (as recorded by average weights) was higher in the CCF (0.0128 ± 0.0008 g) compared to the EF (0.0046 ± 0.0009 g) and CF (0.0038 ± 0.0002 g). The polysaccharide content of the biofilms formed in the CCF (5.424 ± 0.0012 mg/ml) was higher in comparison with the polysaccharide content measured in the EF (3.854 ± 0.0018 mg/ml) and the CF (2.582 ± 0.00216 mg/ml). Cell suspension EPS was found to be maximum in the CCF in contrast to the EF and the CF (Suppl. Fig. 1A). Utilization of soluble protein was also faster in the PMMA-CCF than in the EF or CF. (Suppl. Fig. 1B).

3.6. Effect of surface property of the inner arrangement on esterase production, cell growth and biofilm formation by BSE01

Esterase production by the isolate BSE01 was also higher in the PMMA-CCF where the inner

arrangement was without glass slides than the GS-CCF, when lined with glass slides (Fig.5 A). Biofilm formation, as determined by attachment assay, was reduced drastically when the glass slides were used ($OD_{590} = 0.311 \pm 0.002$ for PMMA-CCF and $OD_{590} = 0.0012 \pm 0.0003$ for GS-CCF). Also, the concentration of cell suspension EPS was higher in the PMMA-CCF compared to the GS-CCF (Fig.5 B). Suspended cell growth was 1181-fold higher when the isolate was cultivated in the vessel whose inner arrangement was without glass slides (22×10^7 CFU/mL with glass and 26×10^{10} CFU/mL without glass). The cultivation with the hydrophobic surface allowed faster consumption of soluble protein (Fig.5 C). The concentration of the lactone type quorum sensing compounds was higher in the PMMA-CCF as well as maintained a steady level till the end of the cultivation in contrast to the concentration attained in the GS-CCF (Fig.5 D). In the PMMA-CCF, the isolate grew as thick biofilm (Suppl. Fig. 2A) compared to the GS-CCF where very negligible amount of biofilm formed (Suppl. Fig. 2B). Thus, the growth of BSE01 was correlated with the hydrophobic reactor surface, biofilm formation, EPS synthesis, and concentration of quorum sensing compounds, that indicated the positive effect of the abovementioned five parameters on esterase production by BSE01.

3.7. Confocal laser scanning microscopy of *Streptomyces sundarbansensis* sp nov. and BSE01 biofilm

Biofilm architectural parameters were generated from FITC-conA fluorescence (green channel) and SYTO 64 fluorescence values (red channel). As shown in Fig.6 A, biovolume represented the overall volume of the biofilm, and it also provided an estimate of its biomass. For *Streptomyces sundarbansensis* sp nov. it is evident that the biovolume of the biofilm on PMMA surface attained 81.54 % higher biovolume as compared to the biofilm on glass surface. Biofilm thickness

provided a measurement of the spatial size of the biofilm. Average thickness provided information about the upper extent of the biofilm. Mean thickness on PMMA surface showed an increasing trend compared to decreasing trend recorded for the film on glass surface (Fig.6 B). Substratum coverage (Fig.6 C) was also 41% higher for the PMMA surface compared to the glass surface. Total spreading (Fig.6 D) increased by 5.37 % for the PMMA surface compared to the glass surface. The biofilm roughness coefficient provided a measure of how much the thickness of the biofilm varied and was an indicator of its heterogeneity. Decrease of roughness coefficient of the biofilm grown on both the PMMA and glass surface indicated that the film assumed an ordered structure as shown in Fig.6 E. The surface area/biovolume ratio reflected the fraction of the biofilm that was exposed to the nutrient flow and thus may indicate how the biofilm adapted to the environment. The surface area/biovolume ratio (Fig.6 F) was also 125.64 % higher for the PMMA compared to the glass surface.

Fig.7 A–B, at an early stage of development of the biofilm on the PMMA surface (when the biofilm was 1 day old), appearance of green and red fluorescence indicated initiation of recruitment of cells embedded in the EPS matrix. Thereafter, emergence of predominant red fluorescence from the 2nd to the 3rd day (Fig.7 B–C) signified formation of a mature biofilm. As evident from Fig.7 D–F, at an early stage of development of the biofilm on the glass surface (when the biofilm was 1 day old), appearance of strong green fluorescence indicated intense deposition of EPS. Absence of red fluorescence indicated insignificant mobilization of cells in the biofilm and more remained in the planktonic state. Thereafter, emergence of red fluorescence at the 2nd and 3rd days (Fig.7 E–F) indicated formation of a biofilm, albeit less developed than the biofilm on the PMMA surface.

As shown in Fig. 8 (A), it is evident that the biovolume of the biofilm on PMMA surface attained higher biovolume as compared to the film on glass surface. Mean thickness on PMMA surface showed an increasing trend compared to decreasing trend recorded for the film on glass surface (Fig. 8(B)). Substratum coverage (Fig. 8(C)), and total spreading (Fig. 8(D)) were also higher for the PMMA surface compared to the glass surface. Decrease of roughness coefficient of the biofilm grown on both the PMMA and glass surface indicated that the film assumed an ordered structure as shown in Fig. 8 (E). The surface area/biovolume ratio reflected the fraction of the biofilm that was exposed to the nutrient flow and thus may indicate how the biofilm adapted to the environment. The surface area/biovolume ratio (Fig. 8 (F)) was much much higher for the PMMA surface compared to the glass surface.

Fig. 9 A–B, at an early stage of development of the biofilm on the PMMA surface (when the biofilm was 1 day old), appearance of green and red fluorescence indicated initiation of recruitment of cells embedded in the EPS matrix. Thereafter, emergence of predominant red fluorescence from the 2nd to the 3rd day (Fig. 9 B–C) signified formation of a mature biofilm. As evident from Fig. 9 D–E, at an early stage of development of the biofilm on the glass surface (when the biofilm was 1 day and 2 days old, respectively), low extent of green fluorescence indicated feeble production of EPS. Absence of red fluorescence indicated insignificant mobilization of cells in the biofilm and more remained in the planktonic state. Thereafter, emergence of very less amount of red fluorescence at the 3rd days (Fig. 9 F) signified formation of a very thin biofilm, albeit less developed than the biofilm developed on the PMMA surface.

The design of the novel PMMA-CCF was beneficial to higher cell attachment and consequently biofilm formation that favored more antimicrobial compounds as well as esterase

production. This was reflected in the biomass of the biofilm being formed in the novel CCF was higher compared to the EF or CF and the same being greater on the hydrophobic surface than the hydrophilic surface of the inner arrangement of the CCF. The PMMA-CCF allowed extended surface area for the attachment of cells to the PMMA surface and thus biofilm formation was enhanced. The increased production could originate from the biofilm cells (as shown by higher biofilm formation in the PMMA-CCF, Fig.3 A and Suppl. Fig. 3A) or from the positive cooperation among suspended cells (as shown by higher production of quorum sensing molecules, Fig.2 D and Fig.5 D).

The combined effect of higher surface area and hydrophobicity of the PMMA was responsible for stable biofilm formation that ultimately enhanced both the antimicrobial activity and the esterase production in the PMMA -CCF.

The conclusions of these studies are similar to Yan et al. [25] where a modified roller bottle elicited the production of antimicrobial compounds from two epibiotic marine bacterial strains isolated from the surface of a marine alga. These isolates were grown as a stable biofilm on the inside of a rolling bottle. The culture supernatant exhibited a different antibiotic spectrum when the strains were grown using the agar roller bottle method compared with shake flask cultures. The glass surface did not appear to be able to mimic the ecological niche (seaweed surface) of these strains. An air-membrane surface (AMS) bioreactor was designed earlier [26] that allowed bacteria to grow attached to a surface as a biofilm in contact with air. When the strain isolated from the surface of a marine alga [26] was grown in this reactor, cells produced antimicrobial compounds, which they did not produce when they were grown in shake flask cultures. The physical environment of the bacteria, when they were grown in the AMS bioreactor was similar to the natural environment on the surface of seaweed. The roller bottle as well as the AMS cultivation allowed increased

production of these compounds. These results implied that surface attachment was a significant factor affecting metabolism and that biofilm formation was an important factor in the production of antimicrobial compounds by the marine epibiotic bacteria. The PMMA-CCF that promoted surface attached growth of the marine isolate, the intrinsic natural mode of growth of *Streptomyces sundarbansensis* sp nov., was more conducive for cell proliferation and antimicrobials production.

Cultivations in the novel PMMA-CCF allowed convenient interchange of growth surfaces and, in combination with the standard EF, established that vessel surface characteristics has a remarkable influence on antimicrobial compounds as well as esterase production. The present study is the first report on the effect of the surface characteristic (hydrophobic or hydrophilic) of the surface to which biofilm growth occurred has pronounced effect on the suspended growth, biofilm formation, antimicrobial compounds and esterase production by *Streptomyces sundarbansensis* sp nov. and BSE01, respectively. Surface roughness plays an important role in the wettability of a surface and reported values of the contact angle have traditionally shown a wide range of variability when they have not been corrected for roughness effects. Differences in the contact angle hysteresis of surfaces with an Ra greater than 0.1 μm was related to roughness of a surface, whereas for surfaces with an Ra less than 0.1 μm , any difference in hysteresis could not be related to surface roughness but might be due to chemical heterogeneity of the surface reported earlier [24]. The roughened surfaces in our study had a Ra value higher than 0.1 μm and therefore any change in contact angle may be related to surface texture. When a water droplet completely wets a rough surface on which it sits, the impact of surface roughness on contact angle is given by the Wenzel equation [16]. Wenzel's relation shows that surface roughness increases the hydrophobicity of

a hydrophobic surface and hydrophilicity of a hydrophilic surface. This could be the reason for higher biofilm formation on the rough, hydrophobic PMMA-CCF surface in comparison to the hydrophilic GS-CCF surface for both the estuarine isolates.

CLSM has been applied in the research on biofilm formation by the *Streptomyces sundarbansensis* sp nov. and the BSE01 for the first time. The design of the novel PMMA-CCF allowed easy attachment and removal of standard size slides and subsequent successful CLSM analyses. The present study is the first report on the temporal pattern of biofilm development by monitoring volumetric parameters of *Streptomyces sundarbansensis* sp nov. and BSE01 biofilm based on two-channel fluorescence detection through the application of the PHLIP image analysis software. Results demonstrated that the progression of biofilm formation varied depending on the surface characteristic of the surfaces on which the isolates were grown.

4. CONCLUSIONS

The marine isolate, *Streptomyces sundarbansensis* sp nov. and BSE01 showed diverse levels of antimicrobial activities and esterase production, variable extents of cell adhesion and biofilm formation when both the microorganisms were cultivated in the novel conico-cylindrical flask in conjunction with the standard un baffled Erlenmeyer flask and differently constructed shaking vessels. Antimicrobial compounds production by *Streptomyces sundarbansensis* sp nov. and the esterase production by BSE01 were strongly dependent on biofilm formation. The fact that vessel surface characteristics crucially influenced productivity of intertidally derived *Streptomyces sundarbansensis* sp nov. and BSE01 have been highlighted for the first time. Thus, the conico-cylindrical flask that has the advantage of possessing a high level of clarity (92 % light transmission), good surface hardness, excellent mechanical stability, corrosion resistance to high

salt concentrations, resilience to low-dose UV exposure as well as sodium hypochlorite, more conducive to biofilm formation, can immensely facilitate the determination of the factors that would play a critical role in the bioprocesses investigated. As biofilm formation favoured enhanced antimicrobial compounds and esterase production by these isolates, future studies may be focused on the application of molecular tools that will enhance biofilm formation, and ultimately further enhancement in antimicrobial compounds and esterase production.

ACKNOWLEDGEMENTS

CSIR Senior Research Associateship (8584-A/2012-Pool) to S. Sarkar is thankfully acknowledged. The author wishes to thank Dr. Arun Bandyopadhyay and Mrs. Banasri Das for their help during confocal microscopy.

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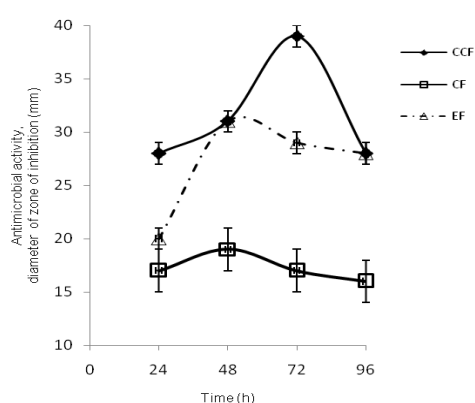


Fig.1 A

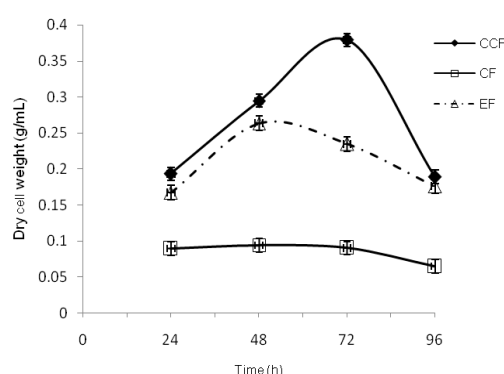


Fig.1 B

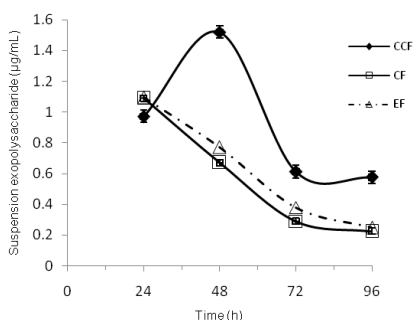


Fig.1 C

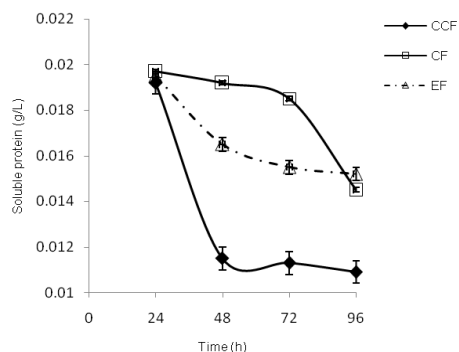


Fig.1 D

Fig.1 Effect of flask configuration on (A) antimicrobial activity (B) dry cell weight (C) suspension exopolysaccharide and (D) medium soluble protein during cultivation of *Streptomyces sundarbansensis* sp nov. Zone of inhibition data included 7 mm hole diameter. Error bars represent standard deviation of six determinations. cultivation of *Streptomyces sundarbansensis* sp nov. for 4 days.

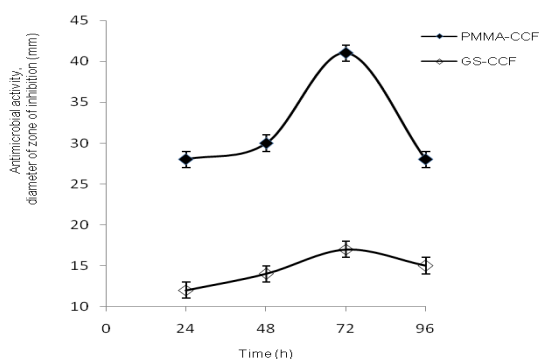


Fig.2 A

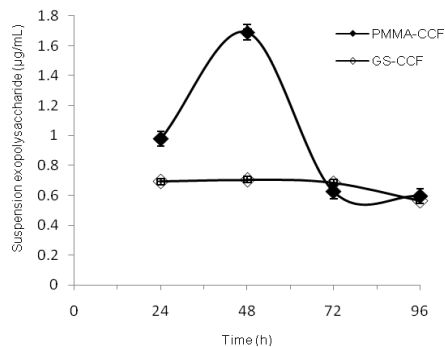


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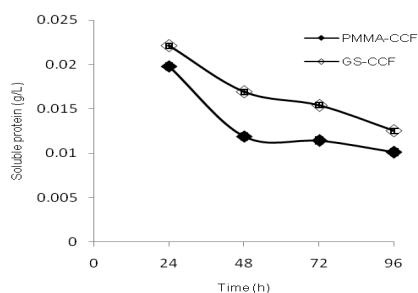


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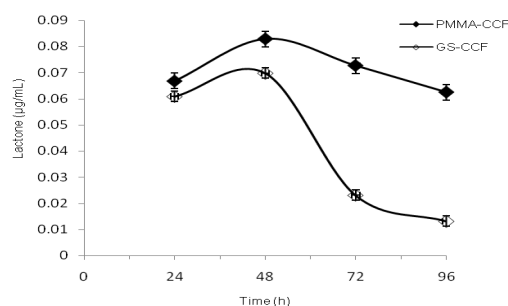


Fig.2 D

Fig.2 Effect of surface property of the conico-cylindrical flask on (A) antimicrobial activity (B) suspension exopolysaccharide (C) medium soluble protein and (D) quorum sensing compounds during cultivation of *Streptomyces sundarbansensis* sp nov. Zone of inhibition data included 7 mm hole diameter. Error bars represent standard deviation of six determinations.



Fig.3 A

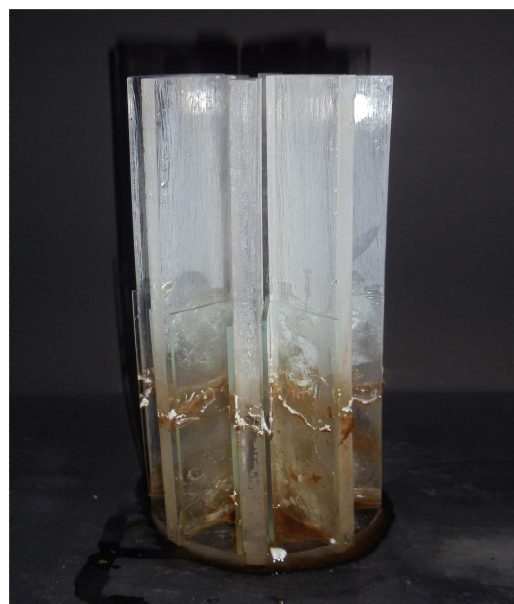


Fig.3 B

Fig.3 Appearance of (A) the inner arrangement of PMMA-CCF and (B) the inner arrangement of GS-CCF after cultivation of *Streptomyces sundarbansensis* sp nov. for 4 days.

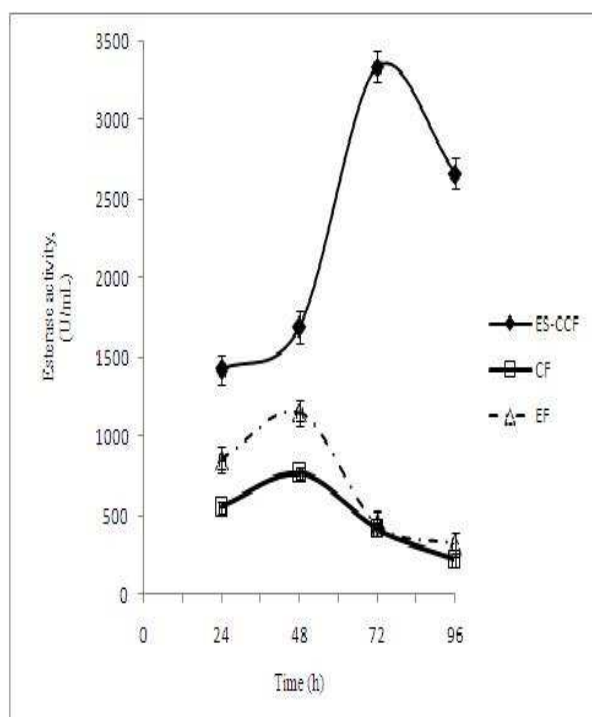


Fig.4 A

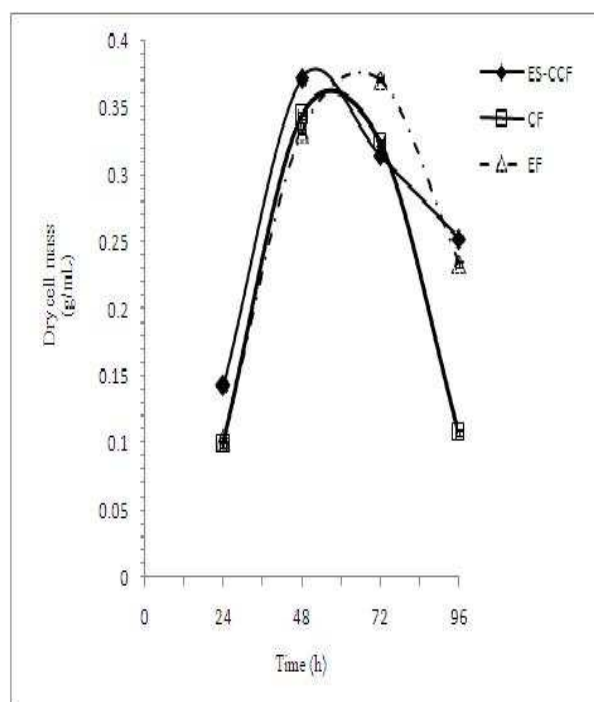


Fig.4 B

Fig.4 Effect of flask configuration on (A) esterase activity (B) dry cell weight during cultivation of BSE01. Error bars represent standard deviation of six determinations.

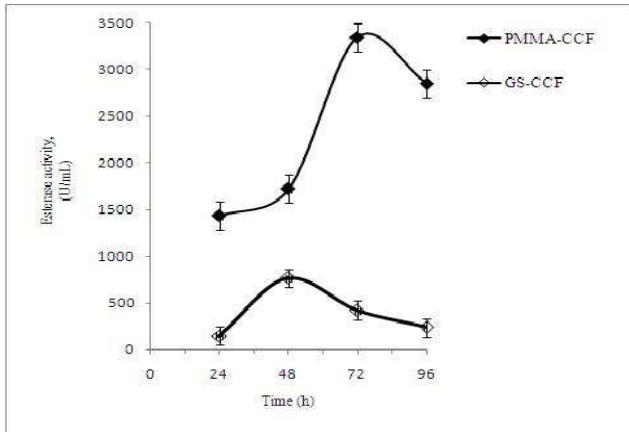


Fig.5 A

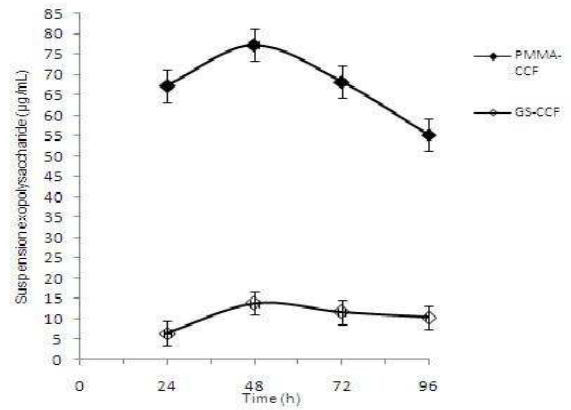


Fig.5 B

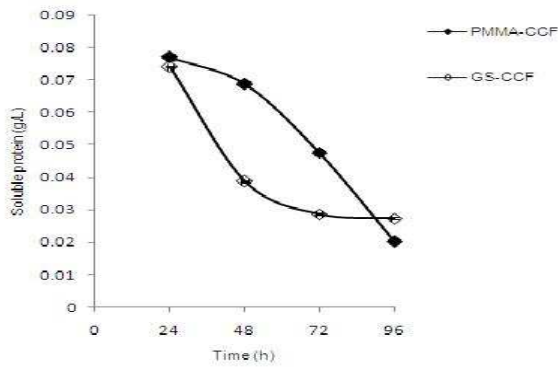


Fig.5 C

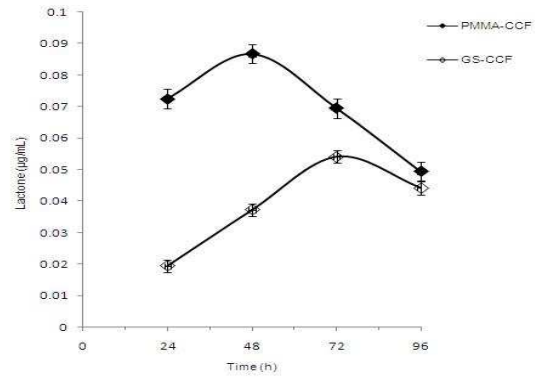


Fig.5 D

Fig.5 Effect of surface property of the inner arrangement of the conico-cylindrical flask on (A) esterase activity (B) suspension exopolysaccharide (C) medium soluble protein and (D) quorum sensing compounds during cultivation of BSE01. Error bars represent standard deviation of six determinations.

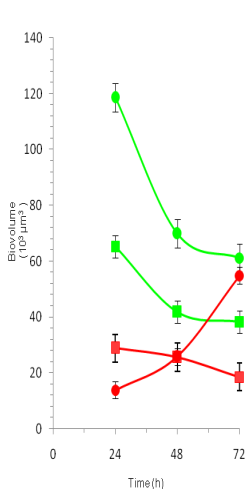


Fig.6 A

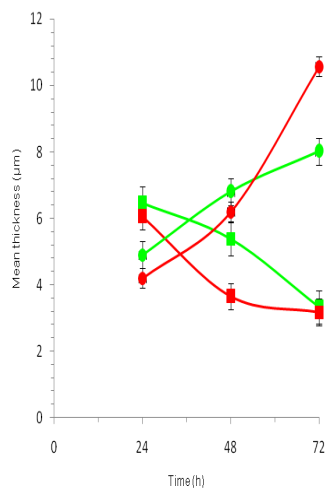


Fig.6 B

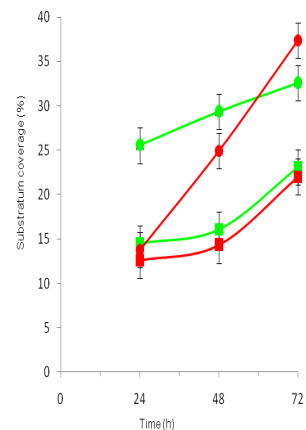


Fig.6 C

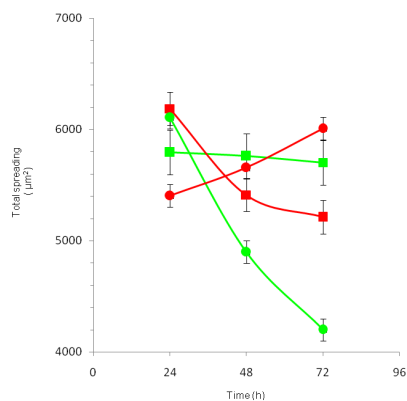


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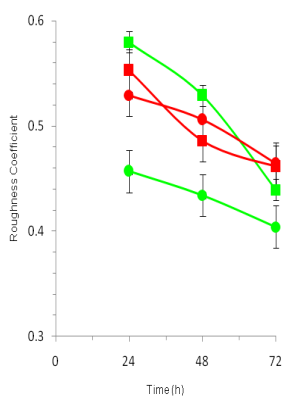


Fig.6 E

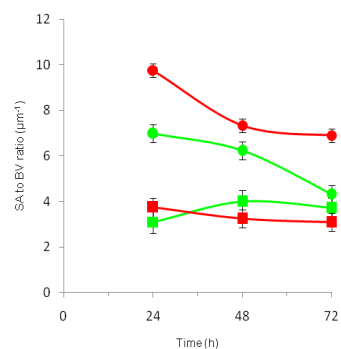


Fig.6 F

Fig.6 Biofilm architectural parameters during *Streptomyces sundarbansensis* sp nov. biofilm development: (A) biovolume, (B) mean thickness, (C) substratum coverage, (D) total spreading, (E) roughness coefficient, and (F) surface area (SA) to biovolume (BV) ratio. Symbol representation: red closed square red channel on glass surface, green closed square green channel on glass surface, red closed circle red channel on PMMA surface, green closed circle green channel on PMMA surface. Error bars represent standard deviation of six determinations. Color only in online version

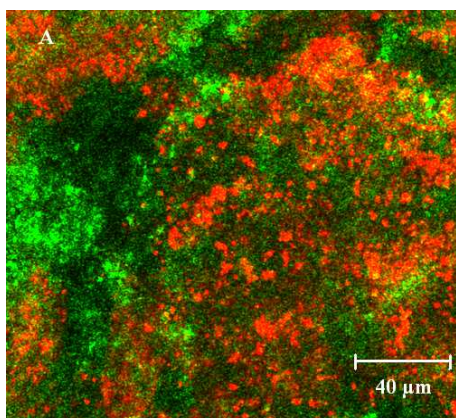


Fig.7 A

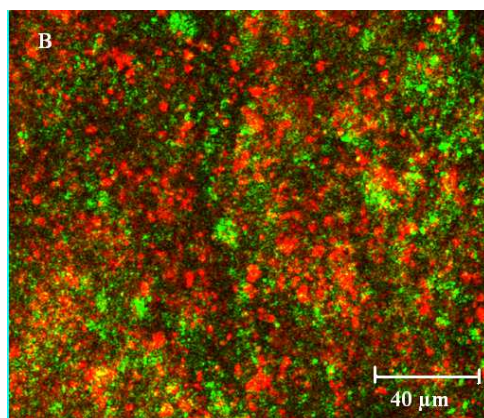


Fig.7 B

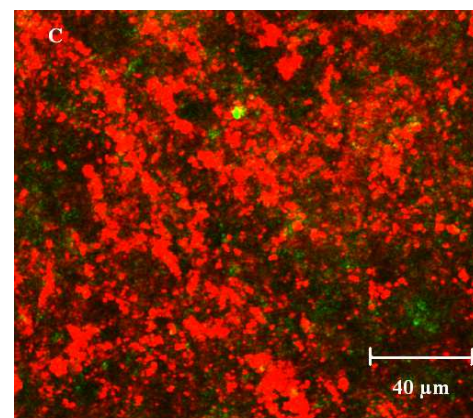


Fig.7 C

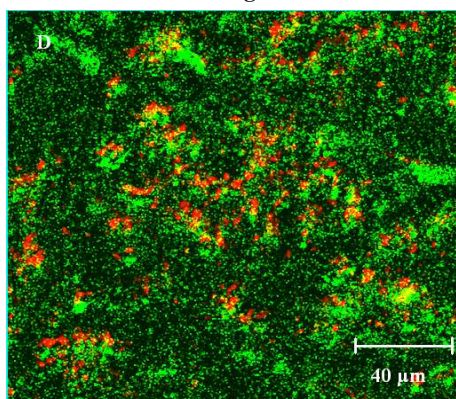


Fig.7 D

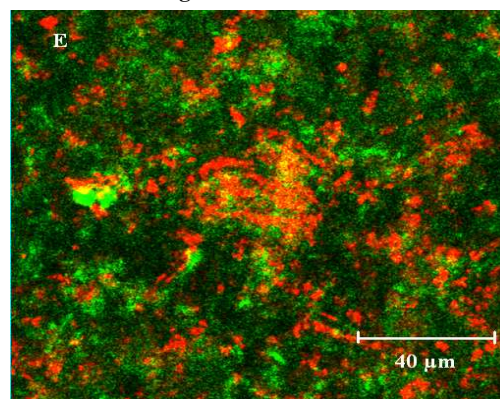


Fig.7 E

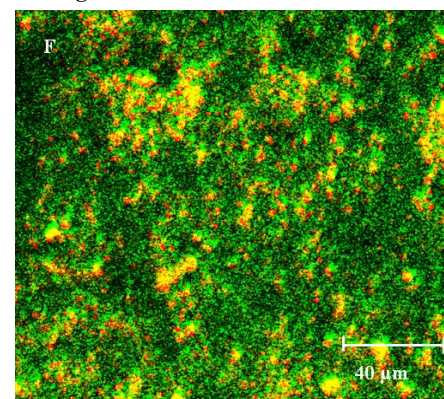


Fig.7 F

Fig.7 Typical CLSM micrograph of *Streptomyces sundarbansensis* sp nov. biofilm following analysis by ImageJ using the volume viewer 1.31 plugin and observed in the x–y plane after A 1, B 2, C 3 days on PMMA surface and after E 1, F 2, and G 3 days on glass surface. Color only in online version

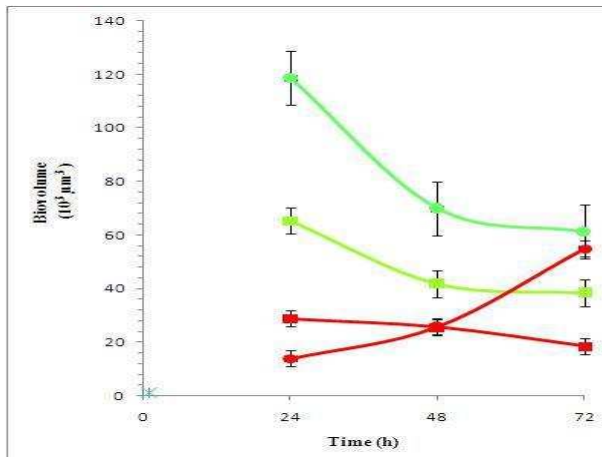


Fig.8 A

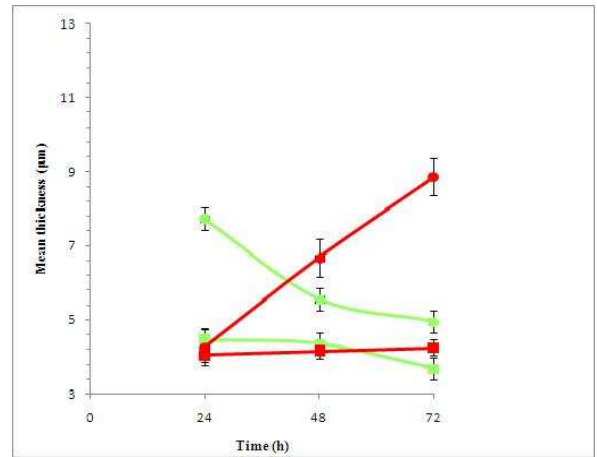


Fig.8 B

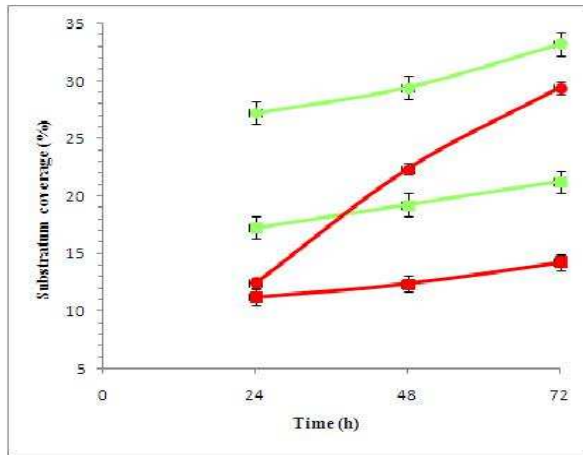


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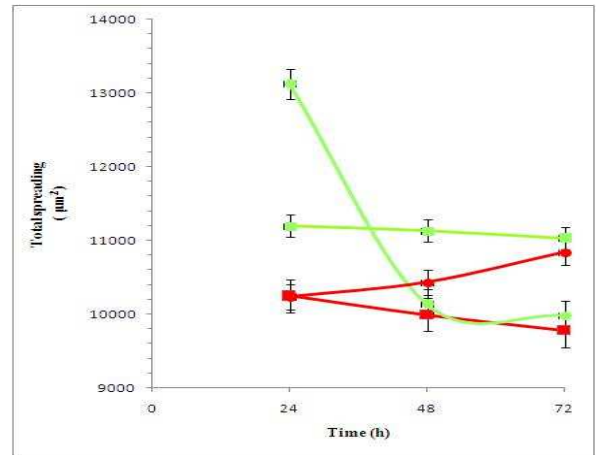


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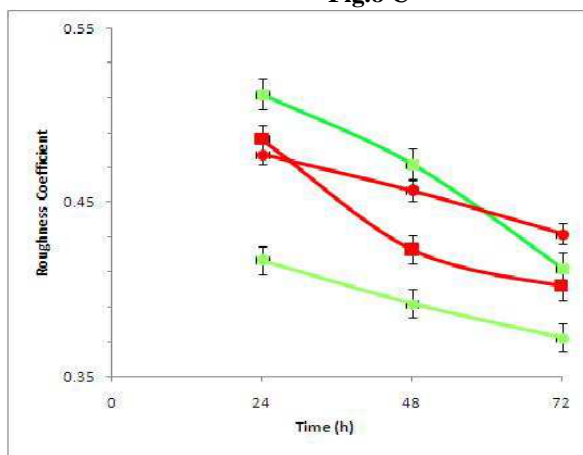


Fig.8 E

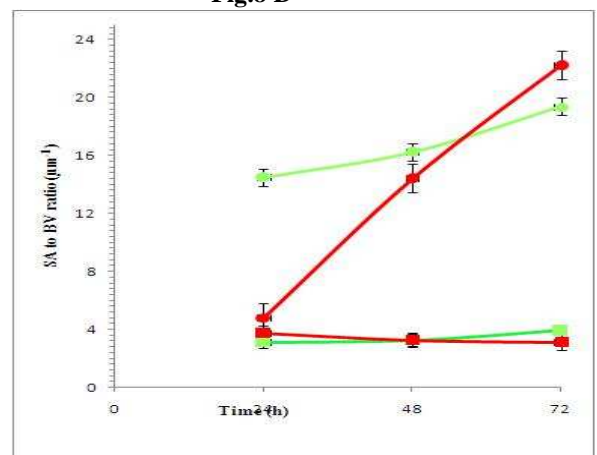


Fig.8 F

Fig.8 Biofilm architectural parameters during BSE01 biofilm development: (A) biovolume, (B) mean thickness, (C) substratum coverage, (D) total spreading, (E) roughness coefficient, and (F) surface area (SA) to biovolume (BV) ratio. Symbol representation: red closed square red channel on glass surface, green closed square green channel on

glass surface, red closed circle red channel on PMMA surface, green closed circle green channel on PMMA surface. Error bars represent standard deviation of six determinations. Color only in online version.

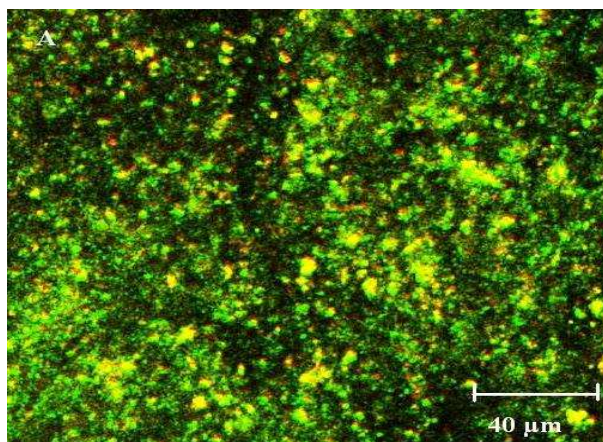


Fig.9 A

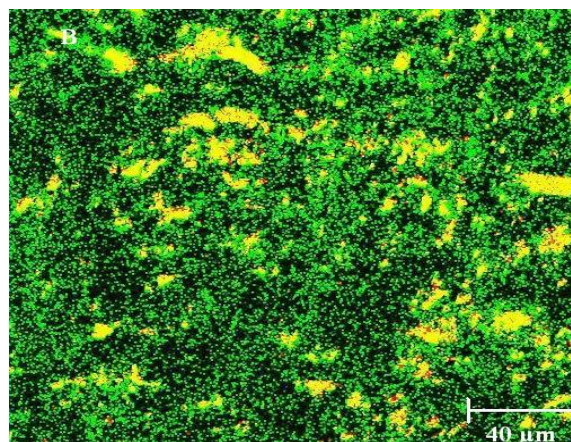


Fig.9 B

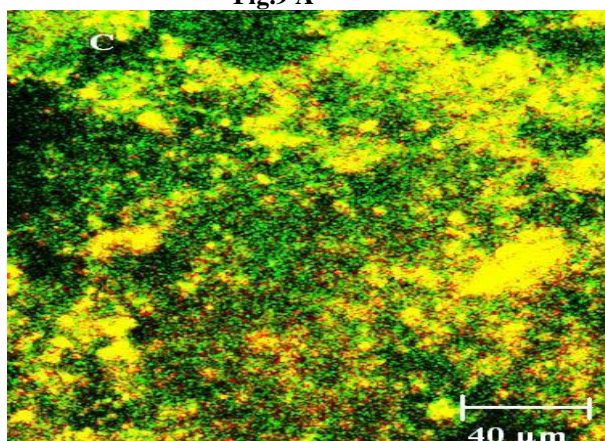


Fig.9 C

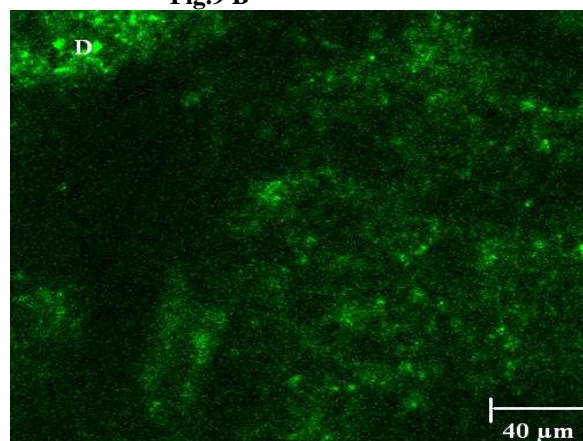


Fig.9 D

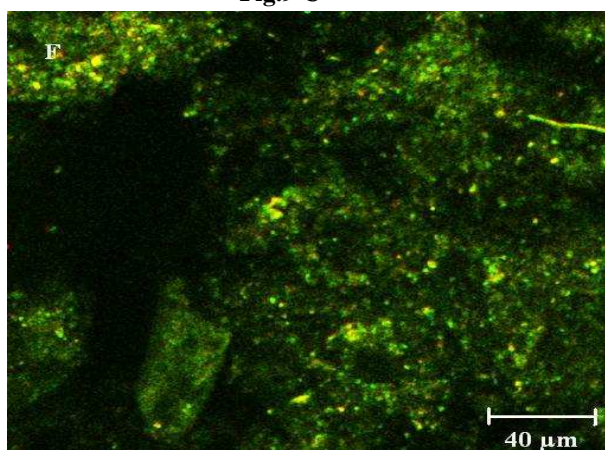


Fig.9 E

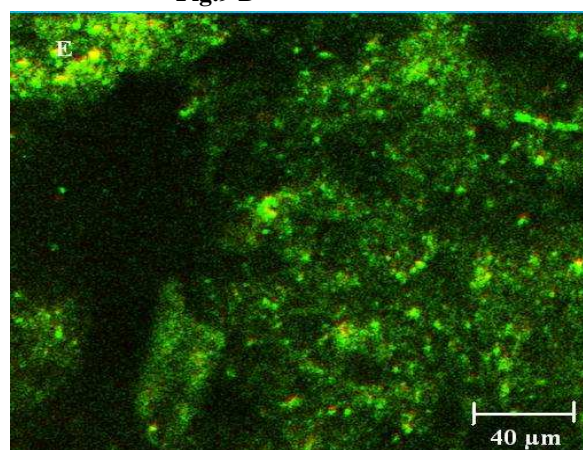
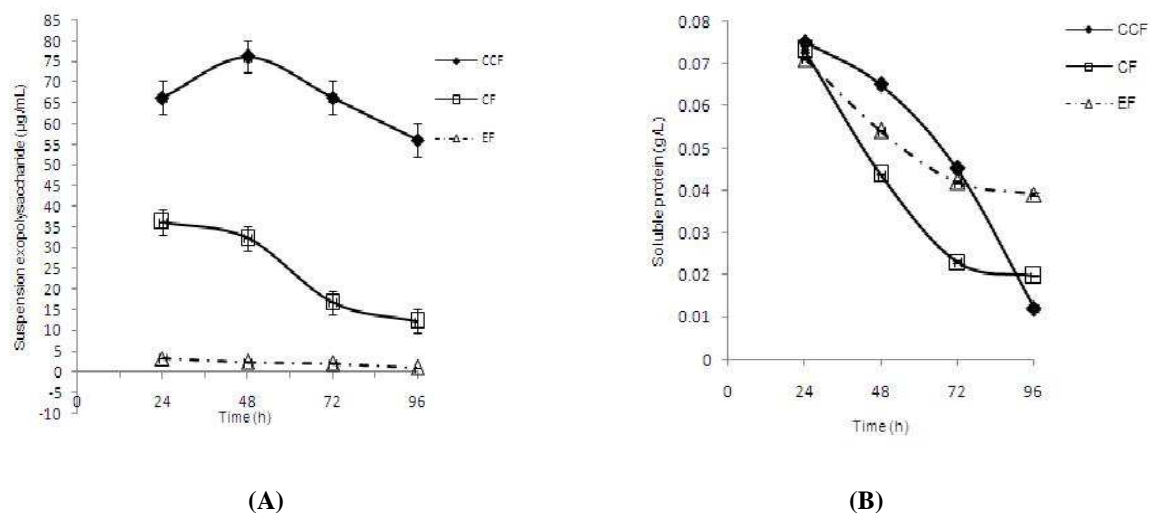
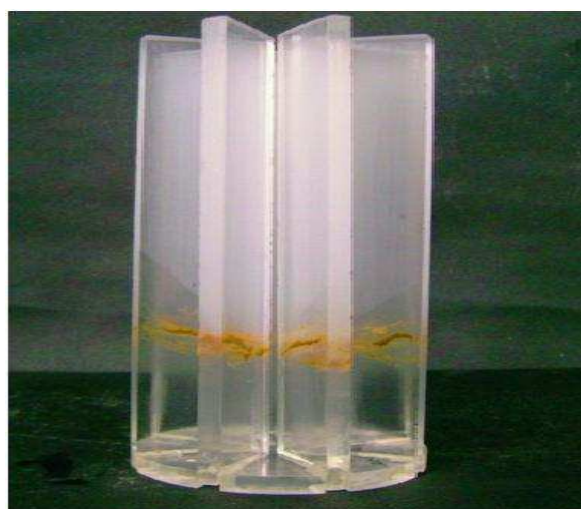


Fig.9 F

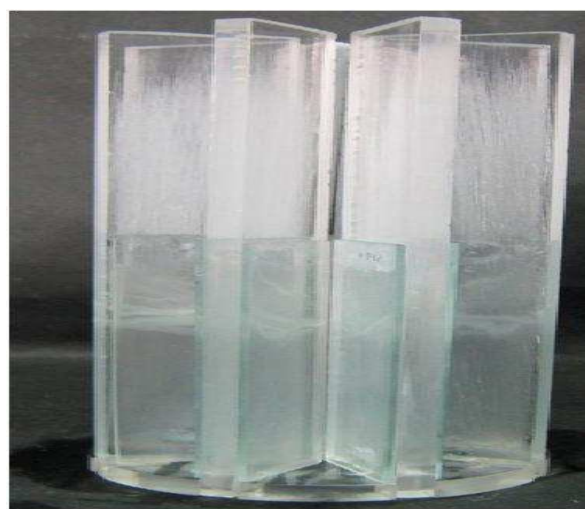
Fig.9 Typical CLSM micrograph of BSE01 biofilm following analysis by ImageJ using the volume viewer 1.31 plugin and observed in the x-y plane after A 1, B 2, C 3 days on PMMA surface and after E 1, F 2, and G 3 days on glass surface. Color only in online version



Suppl. Fig. 1 Effect of flask configuration on (A) suspension exopolysaccharide and (B) medium soluble protein during cultivation of BSE01. Error bars represent standard deviation of six determinations.



(A)



(B)

Suppl. Fig. 2 Appearance of (A) the inner arrangement of PMMA-CCF and (B) the inner arrangement of GS-CCF after cultivation of BSE01 for 3 days.