Journal of Bioremediation & Biodegradation
ISSN: 2155-6199

Research Article Open Access

Characterization of Extracellular Polymeric Substances (EPS) Producing Bacteria Isolated from Biofilms Developed on Different Support Materials in Attached Growth Batch Reactors

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Received: December 16, 2019; Manuscript No. JBRBD-20-5613; **Editor assigned:** December 18, 2019; PreQC No. JBRBD-20-5613; **Reviewed:** January 02, 2020 QC No. JBRBD-20-5613; **Revised:** August 18, 2022; Manuscript No. JBRBD-20-5613; **Published:** September 19, 2022; DOI: 10.4172/2155-6199.1000516

Citation: Ali N, Sharafat I, Ikram S, (2022) Characterization of Extracellular Polymeric Substances (EPS) Producing Bacteria Isolated from Biofilms Developed on Different Support Materials in Attached Growth Batch Reactors. J Bioremediat Biodegrad 13: 516.

Abstract

Dewatering/sludge settling is the major problem that is being tackled by wastewater treatment plant. To overcome this problem, synthetic coagulants (environmental/health hazards) are used. In this contest, 50 bacteria (EPS producing capability) were selected, isolated from biofilms (different support materials). Congo red agar assay indicated 15 (slime producers), 24 (intermediate results) and 11 (non-slime) formers. Whereas tube method indicated 15 (strong), 15 (moderate), 12 (weak) slime and 8 (non-slime) producers. Microtiter plate assay showed 15 (strong) while 35 (moderate) biofilm formers. Dry weight analyses showed the greater concentration of broth (5.33-24.7 g/L) and slime EPS (1.33-13.33 g/L) in individual bacteria as compared to consortia broth (5.66-8.86 g/L) and slime EPS (3.22-6.57 g/L). EPS production was confirmed when an increase in viscosity (16,950-43,450 mPas) was measured. Over all carbohydrate content was higher in consortia (1.233-1.887 mg/ml) as compared to individual bacteria (0.504-0.610 mg/ml). Protein content varied in individual bacteria (0.227-0.52 mg/ml) as well as in consortia (0.178-0.974 mg/ml). FTIR spectra of EPS extract (bacteria and consortia) revealed the presence of functional groups of carbohydrates, proteins, lipids and nucleic acids. Great deal of EPS production was observed by consortia of six bacteria through SEM.

Keywords: Microbial biofilms; Consortia; Fourier transform infra-red spectroscopy; Scanning electronmicroscopy; Extracellular polymeric substances

Introduction

In aqueous environments, microorganisms grow in association with different surfaces and develop biofilms, which are complex assemblages of microorganisms embedded in a matrix of extracellular polymeric substances. The role of bacteria and other microbes is unique in sludge aggregation, settleability and removal by producing extracellular polymeric substances. EPS is three-dimensional, secondary metabolites, highly hydrated and gel-like, sticky or mucoid materials. It is composed of 50% to 90% of the total organic carbon *viz.* carbohydrates, proteins, lipids, nucleic acids, uronic acids and other cellular constituents. Viscoelastic properties of EPS provide mechanical stability to biomass or biofilm matrix as micro colonies divided by interstitial spaces and networks comprising a viscous and watery part called liquid phase. It helps in the formation of bioflocs in collaboration with inorganic/organic elements, filamentous microbial cells and aggregates. It facilitates bioaggregation, sludge biomass retention and solid-liquid separation due to syntrophic association by the juxtaposition of microorganisms [1].

The amount of sludge generation and its efficient removal during treatment wastewater has always been quite challenging to manage. In the environmental perspective, it must be either recycled or reused for economical disposal. However, at times, sludge treatment and its disposal alone add 50%-60% on overall wastewater treatment cost. Nevertheless, conventional activated sludge process has always been effectively used since many decades for domestic wastewater treatment. Efficient sludge settling and removal during activated sludge process is generally helped through process of flocculation and coagulation. Usually synthetic inorganic coagulants (polyaluminum chloride/aluminum sulfate) or organic polymers (polyethylene imine/ polyacrylamide derivatives) are commonly used as flocculants. The negative sludge surface charge (≈ 30 mV) is nullified by synthetic cationic polymers. They not only make the process expensive but additionally, their high molecular weight contribute to multifarious problems including;

- Large volume of sludge production
- Unstable shear stress
- Altered pH of treated water
- Decreased operational efficiency at low temperature
- Leading to environmental health and pollution issues. EPS on the other hand help to combat such issues contrary to synthetic polymers [2].

Dominant EPS producers within sludge include *Enterobacter spp*., *Yersinia spp*., *Serratia spp*., *Pseudomonas spp*., *Bacillus spp*., *Microbacterium spp*., *Aureobasidium spp*., *Sinorhizobium spp*., *Escherichia spp*., *Solibacillus silvestris*, *Achromobacter spp*., *Saccharomycete*, *Proteus mirabilis* and *Rhodococcus erythropolis*. The well-known EPS producers that belong to mesophilic group are Lactic Acid Bacteria (LAB) i.e. *Leuconostoc mesenteroides*, *Lactobacillus Bulgaricus*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactococcus lactis* and species of *Bacillus* and *Streptococcus* genera [3].

Both attached and suspended growth bioreactors work on the biocatalytic properties of bacteria, form biofilms or bioaggregates that hold and remove complex contaminants and then slowly release them in simpler forms. Previous studies on models of multistrain biofilms have demonstrated biofilm development that was highly influenced by bacterial interactions within consortia which is considered as real "heart" of the sludge system . Ecologically, rivalry and collaboration in the restricted space of the EPS matrix leads to a constant adaptation of population fitness for the cells (outermost) [4]. The interactions provide them competitive advantage of energy requiring EPS synthesis over higher cellular growth which provides them protective shield from external hostile conditions such as change in pH, temperature, against oxidizing/charged biocides and toxic materials, antibiotics, dehydration, ultraviolet radiation (distort DNA), metallic cations, host immune defenses and protozoan grazers [5]. It also plays an important role in adsorption of dissolved xenobiotics organic composites, cations, pollutants and metals (Hg, Zn, Ar, Cd, Pb and Cu) and good emulsification activity [6]. Studies have reported that bioflocculation is dependent on the growth conditions of the biofilms and is typically affected by type, amount and physicochemical characteristics of EPS. Increase in carbohydrate and protein content of EPS has direct effect on flocculating activity [7,8].

The overall aim of the present study was to identify and screen the isolated bacteria from different biofilms developed on various support materials in attached growth batch reactors under the influence of different metals (Aluminum and ferric iron (Fe^{3+})) in order to evaluate their potentials for EPS production based on various qualitative and quantitative techniques to foresee their further applications in the specific biological reactors [9,10].

Materials and Methods

Slime/biofilm formation assays

Congo red agar: Isolates were streaked and evaluated for slime production by Congo Red Agar (CRA) assay, a qualitative and simple method. The composition of EPS producing media was; brain heart infusion broth 37 g/L, Sucrose 50 g/L, Congo red dye 0.8 g/L and °Agar 10 g/L, autoclaved at 121 C for 15 minutes. Concentrated solution of Congo red dye was prepared and sterilized discretely and added to the autoclaved BHI and sucrose at °55 C. Plates were inoculated with bacteria, kept at 37° C for 24-hour and followed by overnight incubation at ambient temperature [11,12].

Tube method: Slime production of isolated bacterial strains was tested by loopful colony from fresh culture plates were inoculated in Tryptic Soy Broth (TSB) containing test tubes, kept at 37°C for overnight incubation. Bacterial broth was decanted before staining, (safranin 1% for 5 minutes). The tubes were positioned upside down and left to dry at ambient temperature. After that results were recorded and graded as strong (+++), moderate (++), weak (+) and non-slime formers [13-15].

Microtiter plate assay

The calorimetric and quantitative assay for biofilm detection has been reported as a gold- standard process. Luria-Bertani (LB) broth was used for the bacterial growth at 37°C for 24-hours. The bacterial cultures were diluted (1:100) with fresh medium and 200 µL of diluted culture was aliquoted per well of Microtiter Plate (MTP). The medium was moderately removed, after 24-hour and about 200 µL of Phosphate Buffer Saline (PBS) solution (0.1 M, pH 7.4) was used to wash three times the microtiter plate wells, then air dried for 15 minutes. After that 200 µL of crystal violet (1%) was used to stain the MTP wells for 15 minutes at room temperature. PBS buffer (200 µL) was used to wash the unbound crystal violet stain and air-dried for 15 minutes. About 200 µL of 33% glacial acetic acid was used to solubilize the unbound crystal violet. Microtiter plate count reader was used to read the plate at 630 nm [16,17].

Quantitative production of EPS

EPS producing bacterial strains were grown individually as well as in consortium on mineral salt medium. For quantitative production of EPS, the composition of mineral medium was as follows; KH_2PO_4 1 g/L, K_2HPO_4 2 g/L, NH_4Cl 1 g/L, Yeast extract 0.01 g/L, $MgSO_4.7H_2O$ 0.2 g/L, Glucose 25 g/L. The media's initial pH was set at 7.0. $MgSO_4$ and glucose were sterilized separately and then mixed aseptically with other materials before inoculation. From the PCA plates, bacterial strains were inoculated in the mineral medium, and kept at 32°C for incubation in an orbital shaker at 150 rpm for 72 hours [18,19].

After growing bacterial cultures individually and as consortium for 72 hours, the medium was centrifuged at 7500 rpm for 20 minutes at 4°C. The supernatant contained the slime EPS while pellet contained the microorganisms and capsular EPS. The bacterial broth containing both capsular and slime EPS was also used for experiments. For further analysis, the collected three types of EPS were stored at 4°C.

Measurement of dry weight of EPS

The quantitative estimation of EPS (slime/capsular) and bacterial brothwas done. After 72 hours of incubation, the consortia and bacterial broth were taken into vials and centrifuged for 15 minutes at 7500 rpm (4°C) to obtain EPS from cell mass. The one volume of absolute ethanol was added to one volume of collected supernatant (1:1) in new vials for precipitation and incubated for 24 hours at 4°C. After incubation, to collect the EPS (precipitated), the mixture was centrifuged for 15 minutes at 7500 rpm. The supernatant was decanted and the pellet (EPS) was dried at room temperature [20,21].

Analysis of EPS production

After 72 hours of incubation, the culture broth became viscous due to production of EPS. EPS production was measured in terms of viscosity of culture broth by using VP 1060 spindle R7, at 60 rpm and ambient temperature [22].

Screening of consortia for biofilm development potential

For consortia development, the six best EPS producing bacterial strains were selected for development of biofilm on small sized pebbles. A total of 4 combinations were prepared from six bacteria for biofilm development. The bacterial strains were refreshed and a loop full of bacterial cultures was inoculated in test tube containing mineral broth (composition as mentioned above) and kept at 32°C for 72-hour incubation. 1 ml aliquot of each bacterial broth was inoculated into sterilized tubes containing 9 ml mineral media, different combinations were made and pebbles of different sizes were added as a support material for biofilm development and incubated at 37°C for 10 days. After 10 days, the samples were stored for Scanning Electron Microscopy (SEM).

Characterization of slime EPS

Extracted slime EPS was characterized in terms of carbohydrate and protein content of selected six bacteria as well as four consortia. UV-sulfuric acid method was used to determine the Total Carbohydrates (TC) content of extracted EPS (glucose as a standard). Lowry method was used to determine the Total Protein (TP) content of the extracted EPS (bovine serum albumin as a standard) [23].

Scanning Electron Microscope (SEM) analysis of EPS

Initially, the dried samples (pebbles) were fixed on stub by using carbon tape, sticky on both sides and to cover the edges of sample to ensure the conduction of electron beam silver paste conduction (SPI-CHEM) was done. Secondly, high voltage and vacuum of 102 atm. was created in sputtering for gold coating by using SPI-MODULE (sputter coater). In this process, high voltage (25 mA current for 50 sec) was used to create plasma and as a result gold was deposited on target sample. Finally, the sample was loaded on the holder and placed in chamber under the column. 15 min was required to created vacuum in the chamber. Later, the EPS of biofilm was observed on the screen under 4000X, 6000X, 8,500X, 10,000X magnification power in 30 KV scanning electron microscope (JSM 5910, JEOL, Japan) [24].

Fourier transformed-infrared spectroscopy of EPS

After 72 hours of incubation or fermentation, the bacterial broth and consortia was taken into Eppendorf and centrifuged for 15 minutes at 7500 rpm (4°C) to obtain EPS. Firstly, EPS was precipitated by adding 2 volumes of 100% cold ethanol to the collected supernatant and incubated on ice overnight. The precipitates were then centrifuged at 10,000 rpm for 20 min at 4 °C and dried in an oven at 50°C overnight. FTIR (Bruker tensor 2500) was used to detect chemical composition the EPS in different bacteria and consortia [25].

Results

A total of fifty bacterial strains were isolated from biofilms developed on different support materials. Biofilm and slime EPS production abilities of the bacteria were evaluated using qualitative and quantitative assays. Selected bacterial isolates were then typically examined for EPS production and its characterization [26].

Characterization of EPS producing bacteria

Bacterial isolates (biofilm samples) were characterized on morphological (pigmentation, shape, size, margins, elevation, opacity and texture) and biochemical basis. Around 50 bacterial strains were selected on the basis of mucoid or string forming ability and subsequently screened for their ability to produce EPS. Gram staining results indicated that out of 50 isolates 8 (positive cocci), 4 (positive bacilli), 32 (negative bacilli), 5 (negative cocci) and 1 isolate was gram negative coccobacilli. On the basis of morphology, gram staining and biochemical tests it was found that majority of the bacteria were facultative anaerobes. EPS producing bacteria belong to phylum *Proteobacteria, Firmicutes, Actinobacteria*. Out of fifty bacterial strains, four were *Escherichia coli*, six belonged to *Citrobacter*, five belonged to *Serratia*, two belonged to *Enterobacter*, two belonged to *Klebsiella*, one belonged to *Salmonella*, two belonged to *Shigella*, two belonged *Erwinia* while one belonged to *Ewingella* genera (*Proteobacteria*: *Gammaproteobacteria*: *Enterobacteriales*: *Enterobacteriaceae*). Whereas, five belonged to *Neisseria* genera (*Proteobacteria*: *Betaproteobacteria*: *Neisseriales*: *Neisseriaceae*) and six belonged to *Pseudomonas* genera (*Proteobacteria*: *Gammaproteobacteria*: *Pseudomonadale*: *Pseudomonadaceae*). While, three belonged to Bacillus, five belonged to *Staphylococcus* genera (*Firmicutes*: *Bacilli*: *Bacillales*) and three belonged to Micrococcus, three belonged to *Corynebacterium* genera (*Actinobacteria*: Actinomycetales) [27]. By Maneval's capsule stain, the entire isolates showed capsule around the cells under the light microscope at 100X magnification, which indicated presence of slime EPS (Figures 1a and 1b). By Anthony's capsule stain, the selected 33 out of 50 bacterial strains were found to have capsule around the cells which showed the presence of strongly bound capsule with cell wall, a positive result. The other 17 isolates didn't show any capsule around the cells, considered as a negative result (Figure 1c and 1d) (Figure 1).

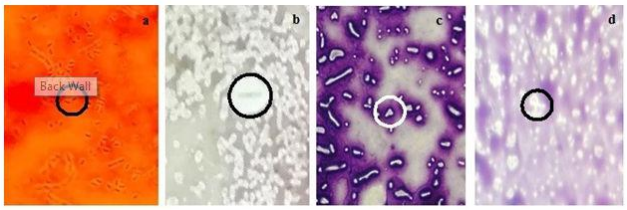

Figure 1: Micrographs of Maneval's capsule staining (a, b) and Anthony's capsule stain (c, d): Capsule layer of surrounding the bacteria (100X magnification under light microscope). Circles indicating the capsule around cells (n=50).

Biofilm formation assays

Congo red agar: In qualitative CRA assay, 30% bacterial isolates (15 of 50) with black colonies indicated strong slime/biofilm production ability. A total of 48% (24 of 50) bacterial isolates with dark red colonies and 12% (6 of 50) with light red colonies indicated the moderate and weak slime/biofilm production respectively [28]. Remaining bacterial isolates with pink colonies 10% (5 of 50) indicated non-slime producers (Figure 2).

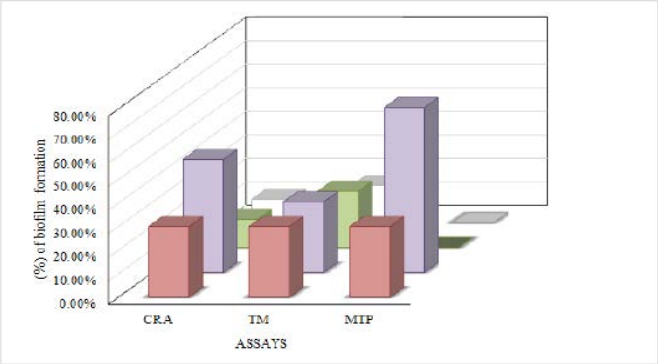

Figure 2: Comparative analysis of slime/biofilm formation by Congo red agar, tube method and microtiter plate assays.
Note: Strong, Moderate, Weak, Non adherent

Tube method

The adherence of safranin stain on the inner side of glass tubes indicated the slime production. Out of 50 bacterial strains, number of strong (+++) and moderate (++) slime producers was same i.e. 15

(30%). Whereas, 12 (24%) showed weak slime production or adherence (+) and remaining 8 (16%) bacterial strains were then non-slime producers (Figure 2).

Microtiter Plate (MTP) assay

Microtiter plate assay indicated 15 (30%) bacterial strains as strong slime/biofilm formers and 35 (70%) as moderate slime/biofilm formers. All bacterial strains were biofilm formers and none of the bacteria was detected as non-adherent (Figure 2).

Figure 2 Comparative analysis of slime/biofilm formation by Congo Red Agar, Tube method and microtiter plate assays [29].

The comparative analysis showed that 30% bacterial strains (different in each assay) were strong slime/biofilm formers. The bacterial strains as 4, 12, 13, 16, 25, 34, 39, 44, 46, 48 and 50 were strong to moderate in each assay. The other bacterial results varied in each assay.

Table 1 Microbiological characteristics of EPS producing bacterial strains (n=50) isolated from biofilms developed on different support materials in presence of Fe and Al metals.

Quantitative production of EPS

Dry weight: The slime EPS concentration varied and in the range of 1.33-13.33 g/L, produced by bacterial isolates as well as consortia. In bacterial broth, total EPS (slime, capsular and bacterial cells) was in the range of 5.33-24.7 g/L. However, the quantity of slime EPS (3.22-6.57 g/L) and total EPS (5.66-8.86 g/L) by four consortia was considerable lower than individual bacteria (Figure 3). The slime EPS produced by bacteria (1.33-13.33 g/L) varied with consortia (3.22-6.57 g/L) (Figure 3).

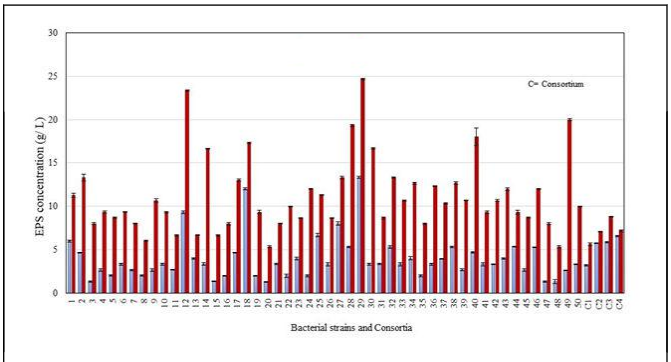

Figure 3: EPS or biopolymer concentrations produced by bacterial isolates (n=50) and 4 consortia (72 hours of incubation at initial pH 7). Note: Slime, Bacteral broth

Viscosity: After 72 hours of fermentation, viscosity of individual bacterial as well as consortia broth was measured by VP 1060 viscometer. The measured viscosity of culture broths was in the range of 16,950-43,450 mPas and of 4 consortia was 28,800-30,150 mPas. Viscosity in some specific bacteria (4, 6, 13, 16, 25, 28, 34, 39, 48, 49 and 50) was higher as compared to other bacteria and 4 consortia due to EPS production (Figure 4).

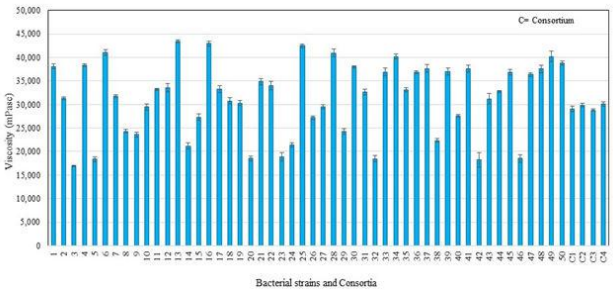

Figure 4: Viscosity of culture media by bacteria (n=50) and 4 consortia (after 3 days of fermentation at initial pH 7.0).

Selection of six best EPS producing bacterial strains and consortia development

Four combinations as consortium 1 (25, 4, 13, 39), consortium 2 (4, 25, 34, 39), consortium 3 (4, 13, 25, 48, 39) and consortium 4 (4, 13, 25, 48, 39, 48) were prepared. Further, analysis of bacterial strains and the consortia was done on the basis of biochemical characterization of the (carbohydrate and protein), FTIR spectra and SEM (Table 1).

No	Bacterial strains	Support material	Dry weight		Chemical characterization	
			Bacterial broth (g/L)	Slime EPS (g/L)	TC (%)	TP (%)
1	4	Fe 8.5, Iron	9.34	2.67	72.8	27.2
2	13	Al 6.5, PVC	6.67	4	67.11	32.9
3	25	Al 6.5, Stainless Steel	11.34	6.67	73.7	26.3
4	34	Fe 8.5, Tire rubber	12.67	4	68.3	31.7
5	39	Fe 8.5, Iron	10.67	2.67	68.78	31.22
6	48	Al 4.5, Iron	5.33	1.33	54.6	45.4
7	consortium-1	-	5.66	3.22	89.7	10.3
8	consortium-2	-	7.124	5.71	81.19	18.81
9	consortium-3	-	8.864	5.85	73.18	26.82
10	consortium-4	-	7.18	6.57	65.95	34.05

Table 1: Selection of six best EPS producing different bacteria on the basis of qualitative and quantitative assays and their Biochemical characterizations (TC and TP).

On the basis of previous assays (Maneval's and Anthony's capsule stain, CRA, TM, MTP, dry weight and viscosity) six best EPS producing bacterial strains were selected for the consortia/biofilm development. The biochemical characterization of a slime EPS (partially purified) of six selected strains and consortia.

Polysaccharide estimation of EPS

Total carbohydrate concentration in EPS produced by individual bacterial strains was in the range 0.504 mg/ml-0.91 mg/ml and of 4 consortia was in the range of 1.283-1.887 mg/ml. Maximum quantity of carbohydrate was "0.91 mg/ml" produced by bacteria '34' and 1.887 mg/ml" produced by consortium 4 (combination of 6 bacteria). Carbohydrate concentration produced by consortia was more than by individual bacteria (Table 2 and Figure 5).

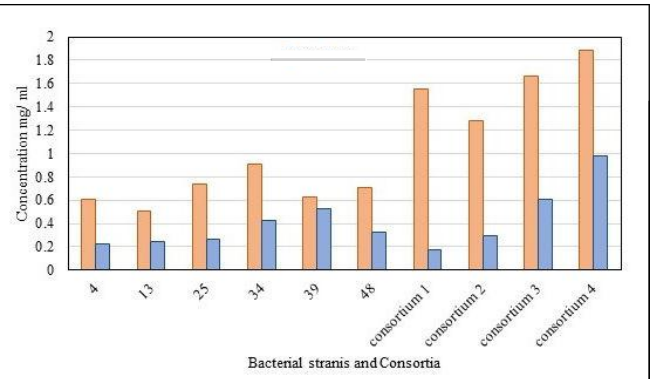

Figure 5: Total Carbohydrate (TC) and Total Protein (TP) content of partially purified EPS from six selected pure bacteria and 4 consortia. Note: TC, TP

Protein estimation of EPS. Total protein concentration in EPS produced by individual bacterial strains was in the range 0.2278-0.5214 mg/ml and by consortia (1, 2, 3, 4) was in the range of 0.1784-0.9742 mg/ml. Maximum quantity of protein was "0.5214 mg/ml" produced by bacteria '39' and "0.9742 mg/ml" was produced by consortium 4 (combination of 6 bacteria) [30]. Protein concentration was varied among individual bacteria and consortia. The total protein content was less than total polysaccharides in bacteria as well as in consortia (Figure 5).

Fourier transformed-infrared spectroscopy

FTIR spectra specified the presence of carbohydrates (800^{-1}, 200 cm^{-1}), proteins ($1,500^{-1}$, 700 cm^{-1}), lipids ($2,860^{-2}$, 930 cm^{-1}) and nucleic acids (900^{-1}, 300 cm^{-1}) of EPS. No significant difference was observed in EPS peaks of bacteria and consortia (Figure 6) which depicted that chemical composition of EPS were the same (Figure 6).

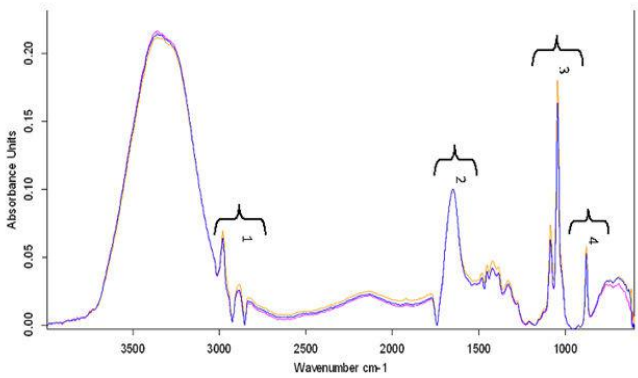

Figure 6: FTIR spectra of EPS showed by bacterial strain 4 (pink) 25 (blue) and 48 (yellow). Note: Peak 1) =C-H (alkenes), C-H (alkanes) and -CHO vibrations in lipids; Peak 2) enol and amides I/ II in proteins; Peak 3) functional group -OH, -COOH, esters, ethers and aliphatic amines vibrations in carbohydrates; Peak 4) glycosidic bond in carbohydrates.

Peaks of carbohydrates and proteins were dominant in EPS of bacteria and consortia as compared to other organic materials. The FTIR spectrum of the purified EPS samples of six bacterial strains exposed characteristic functional groups such as peak 3358.17 cm^{-1} represented the O-H bond. The absorption bands at 2970-2980 cm^{-1} represented =C-H stretch and functional group alkenes. The absorption bands at 2880-2890 cm^{-1} represented the C-H stretch and functional group alkanes. Peaks near 2830-2835 cm-1 represented -CHO stretch and functional group aldehydes. The absorption bands at 2125-2135 cm^{-1} represented -C≡C- stretch and functional group alkynes. Enol and amide groups were represented by the 1650-1540 cm^{-1} range. Peaks near 1649 cm^{-1} and 1650 cm^{-1} represented the amide and C-N bending of protein and peptide amines. Peaks 1421.72 cm^{-1} and 1483 cm^{-1} represented C-C stretch (in-ring) and functional group aromatics and 1331.26 cm^{-1} represented N-O symmetric stretch and functional group nitro compounds. Presence of monomers (galactose, glucose and mannose) was observed between 900-1200 cm-1 peaks. The absorption bands at 1086.15 cm^{-1} represented C-N stretch and functional groups alcohol, carboxylic acid, esters and ethers. Peak 1044.91 cm^{-1} represented C-N stretch and functional group aliphatic amines. The absorption bands at 878.38 cm^{-1} represented the glycosidic bond and 758.91 cm^{-1} represented C-H rock. Peak 1770 cm^{-1} represented the C=O stretch and functional group COOH. The absorption band at 841 cm^{-1} represented the asymmetric ester O-P-O stretch (nucleic acids). Carboxyl groups function as binding sites for divalent cations, showed by FTIR spectra. The EPS was more complex due to presence of different functional groups.

Extracted slime EPS was characterized in terms of carbohydrate and protein content of selected six bacteria as well as four consortia. UV-sulfuric acid method was used to determine the Total Carbohydrates (TC) content of extracted EPS (glucose as a standard). Lowry method was used to determine the Total Protein (TP) content of the extracted EPS (bovine serum albumin as standard) (Figures 7 and 8).

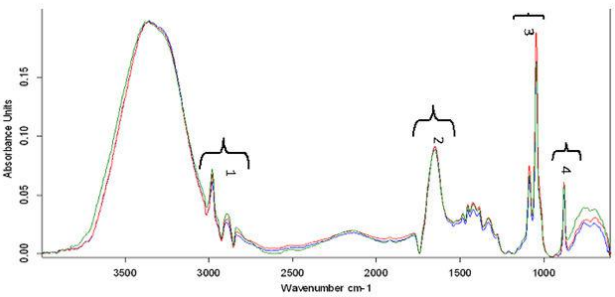

Figure 7: FTIR spectra of EPS showed by bacterial strain 13 (green), 34 (red) and 39 (blue). Note: Peak 1) =C-H (alkenes), C-H (alkanes) and -CHO vibrations in lipids; Peak 2) enol and amides I/II in proteins; Peak 3) functional group -OH, -COOH, esters, ethers and aliphatic amines vibrations in carbohydrates; Peak 4) glycosidic bond in carbohydrates.

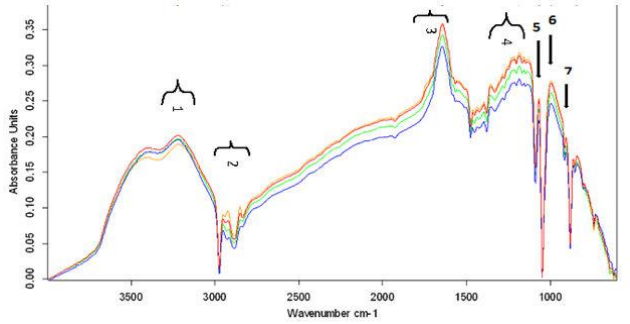

Figure 8: FTIR spectra showed by consortia 1 (yellow), 2 (green) 3 (red) and 4 (blue). Note: Peak 1) -OH group; Peak 2) CH stretch (CH2/CH3 groups); peak 3) C=O stretch (amide I), peak 4) -CH2 stretch; peak 5) C-O-C/ C-O-P stretch in carbohydrates, peak 6) asymmetric ester O-P-O stretch (nucleic acids); peak 7) -NH vibrations.

Scanning electron microscopy

Scanning electron microscopy showed the images of EPS of biofilm developed on pebbles, of 4 different consortia. Images were taken at different magnifications. Thick EPS matrix was covering the bacterial cells and transport channels were seen in the images (Figure 9).

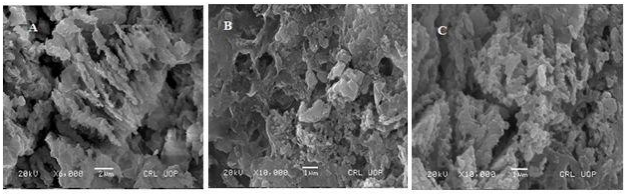

Figure 9: Scanning electron micrographs showed thick EPS matrix and channels for the transport of nutrients at different resolution power of EPS of consortia 1 (25, 4, 13, 39). (C) Exopolymeric matric covering the bacterial cells of consortia 2 (4, 25, 34, 39).

EPS surface microstructure viewed that the produced EPS exhibited compact and porous structure which indicated the potential of EPS as a thickener, viscosifying or as stabilizing agent for novel products. The microorganisms were not prominent due to viscous EPS production.

Figure 9 Scanning electron micrographs showed thick EPS matrix and channels for the transport of nutrients at different resolution power of EPS of consortia 1 (25, 4, 13, 39). Exopolymeric matric covering the bacterial cells of consortia 2 (4, 25, 34, 39) (Figure 10).

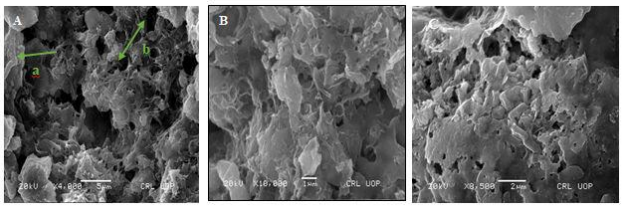

Figure 10: Scanning electron micrographs of EPS at 4,000X and 10,000X of consortia 3 (4, 13, 25, 48, 39) a) exopolymeric matrix covering the cell mass of EPS fibers for attachment of bacterial cells and transport channels; b) Cells covered by thick, mucoid EPS (consortia 3). Images were scanned at 8,500X and 10,000X resolution power of consortia 3 (4, 13, 25, 48, 39); c) Extracellular Polymeric Substances (EPS) produced by consortia 4 (4, 13, 25, 48, 39, 48) covering the bacterial cells.

Discussion

Bacterial cells on this planet have been colonized to form biofilm embedded in self producing matrix known as Extracellular Polymeric Substances (EPS), by successful means for billions of years. In our study, EPS producing bacteria were isolated from different biofilm batch reactors developed on different support materials. On the basis of morphology, gram staining and biochemical tests it was found that majority of the sludge bacteria were facultative anaerobes. The EPS (slime or capsule) production by bacterial strains not only provides shelter from unfavorable environmental circumstances such as lethal compounds, desiccation and high pressure or low temperature. It also helps in uptake of metal ions and favor the biofilm/biofloc formation by the interaction between bacterial cells and sludge solid materials.

CRA and TM showed false negative results which depicted non-biofilm producers but not by MTP method (biofilm producers). As MTP revealed 100% isolates were biofilm formers so it showed the higher sensitivity and precision. In CRA, 90% were slime producers and 10% were non slime producers but 12% isolates with red smooth colonies which were considered as the weak slime producers by some authors but others considered them non slime producers. Due to these reasons, CRA has not been reported/found not an authentic method as it gives uncertain and false positive results. In TM, 84% were slime formers and 16% were non slime formers.

In dry weight and viscosity, low EPS production reason in consortia can be attributed to the presence of competitive interactions among microorganisms. Different EPS concentrations affect other properties possess by bacteria, which might influence the chemical characteristics and bioflocculation process. EPS production depends on different factors as metabolic routes, variation in properties and chemical composition of EPS (different functional groups) of bacterial strains. The EPS organic material (carbohydrates, proteins, uronic acids) influences different properties which leads to the variation in EPS charge, acidic amino acids impart negative charge to EPS while basic amino units provide positive charge. The main factors that

govern bioflocculation process are EPS concentration, hydrophobicity and charge. For better bioflocculation either more concentration or optimal conditions of EPS are required by sludge reported by authors. Viscosity results revealed that EPS production by consortia was low than by some individual bacteria. Variation in final cell concentration had a lesser impact than diverse quantity of EPS produced (greater impact) by the bacterial strains, which was the reason of variation in viscosity ranges.

The concentration of carbohydrate content was more in EPS biochemical characterization than the protein content by individual bacterial strains as well as consortia. In comparison, the maximum carbohydrate content was 73.7%, produced by individual bacteria '25' and 89.7% was produced by consortium 1. The maximum protein quantity produced was 45.4% by individual bacteria 'B-26' and 34.04% by consortium 4. TC/TP ratio depicted that carbohydrate will have major role in bioflocculation process, sludge settling and dewatering due to interaction between divalent cations (in sludge) and anions (bacteria). Proteins help in the stabilization of EPS or biopolyme. Growth on different media and sludge, may change the composition of EPS.

Conclusion

A total of 4 combinations were made for the consortia/biofilm development. The different small sizes of pebbles were used as a support material for EPS/biofilm or consortia development. Pebbles were selected due to rough surface as bacteria adhere more strongly to the rough surfaces. FTIR spectra indicated presence of carboxylic acids, amines and amide groups, glycosidic linkages, double and triple bonds of carbon that correspond to the presence of proteins, carbohydrates, lipids and nucleic acids respectively. Higher peaks of proteins in case of EPS of consortia displayed a bit of anomalous behavior contrary to biochemical results (more carbohydrate). This could be due to the competitive inhibition between bacteria in consortia. No significant difference was observed in EPS microstructure of different consortia by Scanning electron microscope. It was compact, rough, porous due to presence of channels and grooves for transportation of nutrients and thick EPS matrix was covering the bacterial cells at different magnifications as 4,000X, 6,000X, 8,500X, 10,000X. Microorganisms were not prominent and embedded in the thick and viscous layers of EPS.

Acknowledgement

The authors sincerely thank the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan for their financial support.

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