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**SPECTROSCOPIC PROSPECTS OF EXPLORING EXOPOLYMERIC SUBSTANCES
PRODUCED BY *STAPHYLOCOCCUS* SP. MB377 FOR SIMULTANEOUS
ACCUMULATION OF TRIPHENYLMETHANE DYE+METALS**

TAHIR U^{*1} AND KHAN UH²

1: Microbiology and Biotechnology Laboratory, Department of Environmental Sciences, Fatima
Jinnah Women University, Rawalpindi, Pakistan

2: School of Agriculture and Environment, The University of Western Australia, Crawley, 6009,
Perth

***Corresponding Author: E Mail: urujtahirjavaid@gmail.com**

Postal Address: Microbiology and Biotechnology Laboratory, Department of Environmental
Sciences, Fatima Jinnah Women University, Rawalpindi, Pakistan, 46000

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ABSTRACT

Present research focuses on exploring roles of exopolysaccharides from *Staphylococcus* sp. in uptake of co-contaminants. Highest uptake of cadmium was recorded by EPS matrix (95.67%) and *Staphylococcus* sp. MB377 (96.45%) without malachite green. Copper removal efficiency of *Staphylococcus* sp. (92.64%) and EPS (90.68%) enhanced on addition of dye. EPS displayed highest cadmium uptake (95.49%) with 1.08 mg g⁻¹ adsorption rate under the dye stress. Quantification of dye degradation/decolorization represented decline in color removing efficiency of exopolymeric matrix upon addition of copper, cadmium and chromium. Approximately 85.10%, 84.76% & 83.90% dye removal was recorded under cadmium, chromium and copper stress. *Staphylococcus* sp. displayed effective decolorization of dye combined with chromium (81.63%) following cadmium (79.65%) and copper (79.35%). Dye binding affinity of EPS was found to be highest in cadmium stress (0.86 mg g⁻¹). Ultraviolet-Visible kinetics represented variable peak patterns indicating possible transformation of contaminants in to different metabolites and

accumulation by EPS. Disappearance of peaks and formation of new ones in FTIR spectra revealed adsorption of pollutants on to functional groups existing on surface of exopolymeric matrix, further observed via SEM. Results suggested that *Staphylococcus* sp. MB377 and exopolymeric substances from *Staphylococcus* sp. can effectively be applied for bioremoval of co-contaminants.

Keywords: Co-contaminants; Extracellular polymeric substances; Fourier Transform Infrared spectroscopy; Sorption; *Staphylococcus* sp., Scanning electron microscopy

INTRODUCTION

Environmental contamination by discharge of co-contaminants originating from industrial activities has led to irreparable damages to the soil and aquatic systems. Since many industries apply different chemicals, metals and coloring agents in a bulk for manufacturing, processing and finishing their final products. Consequently, they generate large volumes of effluents loaded with different types of contaminants which are directly released into the water bodies or dumped on to open land spaces without any preliminary treatment [1]. Metals and dyes either individually and combined are utilized in diverse areas as textiles, leather processing, medicines, food processing industries and pigment manufacturing. Though may not be harmful in lower concentrations but their continuous usage and accumulation in to environmental compartments has led to considerable environmental deterioration/problems which has raised serious health concerns and needs to be addressed [2-5]. Co-existence of these pollutants in to various environmental

compartments adversely impact and increase pressure on flora and fauna, aquatic ecosystems and ultimately the humans because of their toxicity, persistence and non-degradable nature [6]. These circumstances have arose the need to seek new approaches for developing more efficient and cost-effective treatments in order to minimize and even exterminate the co-contaminants existing in various environmental compartments. Numerous physicochemical technologies have been applied so far, which to some extent have been proven suitable for controlling the pollution but could not remediate the pollutants permanently. Application of microbes and their natural mechanisms, and products (Bioremediation) has proven to be more useful and effective in reducing the toxic nature of pollutants persisting in the effluents or even elimination of these contaminants. Microbial communities respond to pollutants variably depending upon their concentration, nature and availability, type of microbial species as well [1, 7-9]. Moreover, microbial

treatments are actively growing as more cost effective, efficient and ecofriendly alternatives. They boost the remediation and/or restoration of polluted environments with their distinctive catabolic features (intracellular accumulations or enzymatic transformations) and biomolecules along with minimum pollution buildup in the surroundings [10, 11].

Members of genus *Staphylococcus* are the most pathogenic bacteria. They are commonly known as skin colonizing bacteria and cause many community-acquired and nosocomial infections [12]. They are also known to cause food-borne illnesses and food poisoning [13, 14]. However, *Staphylococci* are also playing crucial roles in influencing the ecosystems and industries. Diverse life styles and high metabolic adaptations and/or versatility promote their existence and prevalence in wide range of environmental conditions [15]. A number of different *Staphylococcus* sp. have been applied for remediation of contaminants, for instance, biosurfactant from *Staphylococcus* sp. strain 1E has been applied for remediation of hydrocarbons [16]. Likewise, different species of *Staphylococcus* have also been assessed and applied for their potential to remediate heavy metals [8, 17, 18] and different dye stuffs like Navy N5RL1 [19], Methyl red [20] and Remazol Brilliant

Blue R [21]. *Staphylococcus aureus* has been applied for the degradation of dimethoate as well [22]. To best of knowledge none of the *Staphylococcus* sp. was evaluated for simultaneous uptake of dye molecules combined with metals. Therefore, *Staphylococcus* sp. MB377 and extracellular polymeric substances extracted from the bacterium were analyzed and explored for their potential to accumulate and uptake malachite green dye combined with different metals (Cd, Cu & Cr).

MATERIALS AND METHODS

STRAIN USED FOR EXTRACTION OF EXOPOLYMERIC (EPS) MATRIX

Staphylococcus sp. MB377 (GenBank accession number MBPE01000000) identified as biofilm former was used. Extracted and purified extracellular polymers [23] from *Staphylococcus* sp. MB377 and bacterial cells with intact EPS were assessed for estimating simultaneous accumulation and/or uptake efficiencies for malachite green and metals (Cr, Cd & Cu). Scheme of experiments along with composition of medium presented in **Table 1** was utilized for estimating the sorption efficiency of exopolymer (EPS) and the bacterium.

EXPERIMENTS WITH EXOPOLYMERIC (EPS) MATRIX

For analyzing the uptake of metals by exopolymeric substances (EPS) with and without addition of malachite green via atomic absorption spectrophotometry, treated medium after filtration was divided into 2 portions i.e. C1 and C2 without any dye addition and 3 portions i.e. C1, C2 & C3 for medium containing the dye after filtration using the experimental procedure described in detail by [24], and metal uptake efficiencies with and without malachite green were calculated using eq 1, 2 and 3 [24]. Dye decolorization/degradation with and without metals was quantified using Ultraviolet-Visible (UV-VIS) eq 4 and absorption spectroscopy, while the dye uptake/bioaccumulation yield was quantified via HPLC following methodology of [24] using eqs 5, 6 and 7.

EXPERIMENTS WITH STAPHYLOCOCCUS SP. MB377

Concentration of metals and bioaccumulation yield for metals without and with dye was estimated using similar methodology and formulae given in eqs 8, 9 and 10 mentioned by [24], dye decolorization/degradation with and without metal stress (using UV-VIS and absorption spectroscopy) was determined using eq 4 and bioaccumulation yield of dye with and without metals addition by *Staphylococcus* sp. MB377 was also analyzed

using HPLC procedure and calculated using eq 11 [24].

The composition of M9 medium, all the experimental conditions and procedures with EPS and *Staphylococcus* sp. MB377 were same as performed and described by [24].

FTIR SPECTROSCOPIC ANALYSIS OF BACTERIAL BIOMASS AND EPS FOR DETERMINATION OF FATE OF DYE MOLECULES WITHOUT AND WITH METAL STRESS

FTIR (Fourier-transform infrared) analysis of bacterial biomass and EPS matrix was performed using IR spectroscopy in 4000-400 cm^{-1} region (15 scan speed). For determining the changes in peak positions control was compared with the obtained spectra.

SEM ANALYSIS OF EXOPOLYMERIC (EPS) MATRIX

EPS matrix before and after treatment of dye combined with metals was observed through SEM (MIRA 3 TESCAN). Samples of biopolymeric matrix were dried in desiccator and mounted on stubs (stainless steel) containing adhesive tape (double coated). Carbon coating of stubs was performed by placing in sputter coater chamber containing 8-10 psi of argon. Coated samples were observed in scanning electron microscope.

RESULTS AND DISCUSSION

ASSESSMENT OF METAL UPTAKE BY *STAPHYLOCOCCUS* SP. MB377 AND EPS

Cadmium removal efficiency of EPS (95.67%) and *Staphylococcus* sp. MB377 (96.45%) was observed to be highest in contrast to that of copper (90.47%, 92.39%) and chromium (87.97%, 90.95%), which might be attributed to resistance potential of the strain and binding affinities of charged functional moieties present on the surface of extracellular polymeric substances [25]. Copper sorption by *Staphylococcus* sp. MB377 (92.64 %) and EPS (90.68%) enhanced slightly with the addition of malachite green contrary to copper alone (92.39 and 90.47%) **Table 2** provided in supplementary data. While, [24] reported enhancement in chromium sorption (91.12%) in presence of dye by extracellular polymeric matrix obtained from *Bacillus subtilis* MB378. Sorption trend of metals by EPS and *Staphylococcus* sp. MB377 in medium containing dye molecules is shown below:

EPS: Cd+MG (95.49%) >Cu+MG (90.68%) >Cr+MG (86.31%)

***Staphylococcus* sp. MB377:** Cd+MG (95.34%) >Cu+MG (92.64%) >Cr+MG (90.16%)

It can be noticed that *Staphylococcus* sp. MB377 sorbed the metals more efficiently in comparison with the EPS. This efficiency

might be ascribed to presence and/or availability of additional adsorption sites for bacterial cells as EPSs remained intact with the cells, which might have facilitated maximum adsorption of metals in contrast to EPS only.

QUANTIFICATION OF METAL UPTAKE BY EXOPOLYMERIC (EPS) MATRIX

Amount of metals taken up by extracellular polymeric matrix (EPS) was found to be the highest for copper (2.42 mg g⁻¹) compared to chromium (0.48 mg g⁻¹) followed by cadmium (0.86 mg g⁻¹). An increase in cadmium uptake (1.08 mg g⁻¹ of EPS) was noticed in medium containing malachite green, while it decreased for copper (2.24 mg g⁻¹) and noted to be the least for chromium (0.40 mg g⁻¹) with addition of dye molecules as presented in **Figure I (supplementary data)** provided in supplementary data. [24] reported that EPS from *Bacillus subtilis* MB378 displayed highest binding affinities for copper (1.82 mg g⁻¹) when combined with malachite green dye in contrast to copper alone (1.72 mg g⁻¹).

BIOACCUMULATION YIELD OF METALS BY *STAPHYLOCOCCUS* SP. MB377

Bioaccumulation yield of chromium by *Staphylococcus* sp. MB377 was the highest (45.65%), with second highest for cadmium

(32.15%) followed by chromium (30.80%), respectively. Almost a similar trend in bioaccumulation yield was observed for tested metals in medium containing the dye molecules (**Figure II supplementary data**). However, a reduction in bioaccumulation yield of metals (except copper) was recorded on addition of the dye as follows Cr+MG(45.07%) >Cd+MG (31.78%) >Cu+MG (30.88%).

ASSESSMENT OF DYE DECOLORIZATION/DEGRADATION BY *STAPHYLOCOCCUS* SP. MB377 AND EXOPOLYMERIC MATRIX (EPS)

Color removing efficiency of EPS increased on addition of cadmium (85.10%) and chromium (84.76%) in relation to individual dye molecules (84.49%). On the other hand, a reduction in color removing efficiency of EPS was noticed on addition of copper to malachite green dye (83.90%). Whereas, *Staphylococcus* sp. MB377 displayed effective uptake and color removal for dye molecules containing tested metals (**Table I supplementary data**). Maximum reduction in color of the dye (81.63%) was recorded on addition of chromium, followed by cadmium (79.65%) and copper (79.35%) compared to individual dye (78.92%). [26] reported effective removal of reactive black 5 (100%) and chromium (93%) simultaneously by *Serratia*

proteamaculans and *Pseudomonas putida*, and these bacterial strains reduced/removed 100% of reactive black 5 and chromium individually. [24] observed 94.91% and 82.34% of malachite green dye removal by *Bacillus subtilis* MB378 and EPS extracted from the bacterium, while a decline in dye removal efficiency was noted on addition of metals by the bacterium and EPS as well. These differences in dye uptake and removal by EPS might be due to variations in nature and composition of EPS matrices as these exopolymers have been extracted from different species. Moreover, the results indicated mediation of color removal and/or dye uptake by *Staphylococcus* sp. MB377 via biosorption or passive uptake, and/or owing to reductive enzymes present in the bacterium, which efficiently facilitated the simultaneous reduction/removal of dye molecules and heavy metals as described by [26-28].

ABSORPTION SPECTROSCOPY FOR ANALYZING DYE AND DYE+METAL UPTAKE BY *STAPHYLOCOCCUS* SP. MB377 AND EPS

Scrutiny of UV-VIS absorption spectroscopy for assessing the dye degradation kinetics with and without metal stress revealed peak shifts invariable patterns indicating transformation of dye into different intermediates or products. Spectrum for malachite green preceding any

experimentation with bacterium and EPS showed λ_{\max} at 636 nm (OD 2.471). Following treatment with *Staphylococcus* sp. MB377, significant color reduction was noticed with shifting in peak at 616 nm (OD 0.520) presented in **Figure 1A**. While the peak shifted towards 616 and 618 nm after treatment with EPS from MB377 (OD 0.381) as shown in **Figure 1A**. λ_{\max} for malachite green after addition of cadmium was obtained at 628 and 630 nm (OD 2.490). Shifts in peaks were noted at 616 nm (OD 0.507) after processing with *Staphylococcus* sp. MB377 (**Figure 1B (a)**) and at 616 and 618 nm (OD 0.378) after treatment with EPS matrix. Malachite green dye on combination with copper displayed λ_{\max} at 630 nm (OD 2.489), λ_{\max} was obtained at 616 nm (OD 0.513 & 0.400) on treatment of dye molecules containing copper by *Staphylococcus* sp. MB377 and EPS from MB377, respectively (**Figure 1B (b)**). Malachite green containing chromium displayed λ_{\max} at 628 and 630 nm (OD 2.460). Peaks for dye molecules shifted to 616 and 618 nm with OD 0.451 upon treatment by *Staphylococcus* sp. MB377 (**Figure 1B (c)**). Similar pattern of shifts in peaks (OD 0.365) were recorded by treatment with EPS matrix as presented in **Figure 1B (c)**. These shifts in peak patterns and appearance of new ones under present study

suggested degradation of the dye molecules into different intermediates and products via *Staphylococcus* sp. MB377 and sorption/accumulation on to the exopolymeric (EPS) matrix.

QUANTIFICATION OF DYE UPTAKE BY EXOPOLYMERIC MATRIX (EPS)

EPS extracted from *Staphylococcus* sp. MB377 discerned the capability of accumulating dye molecules up to 0.62-0.86 mg g⁻¹ of EPS (**Table II supplementary data**). Dye binding ability of the EPS was enhanced on addition of metals compared to dye alone by following sequence: MG+Cu (0.86 mg g⁻¹) > MG+Cu (0.66 mg g⁻¹) > MG+Cr (0.65 mg g⁻¹) > MG (0.62 mg g⁻¹).

QUANTIFICATION OF BIOACCUMULATION YIELD FOR DYE MOLECULES

Bioaccumulation of malachite green after processing with *Staphylococcus* sp. MB377 increased with addition of cadmium (0.45%), copper (0.42%) and chromium (0.26%) compared to individual dye (0.15%) as given in **Table III (supplementary data)**.

FTIR SPECTROSCOPY FOR ASSESSING THE FATE OF DYE MOLECULES WITHOUT AND WITH METAL STRESS

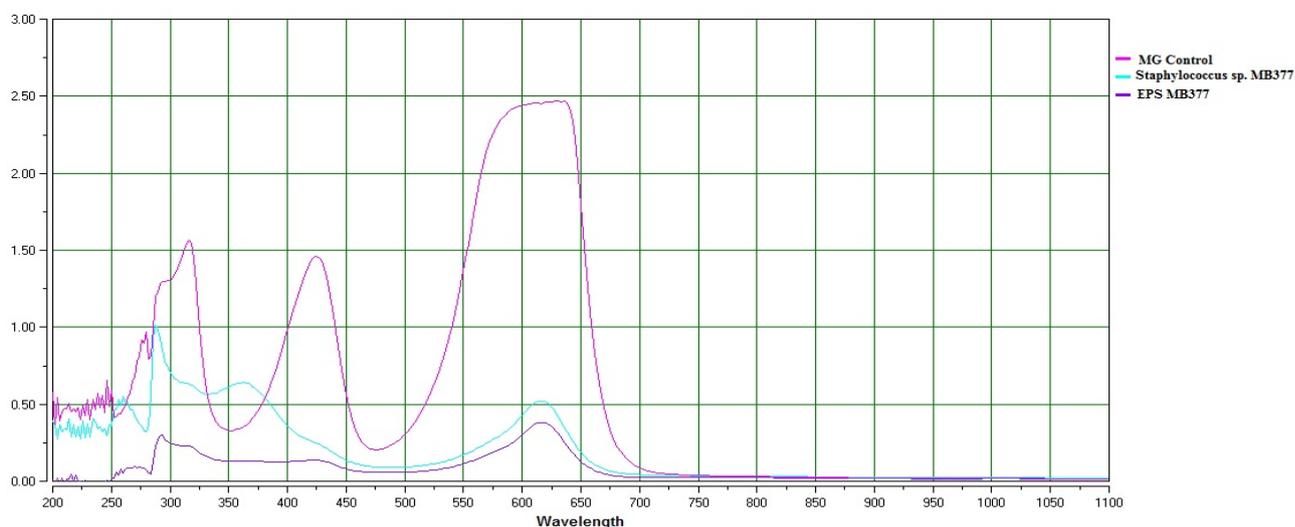
FTIR spectra of *Staphylococcus* sp. MB377 and EPS without and with malachite green, metals, and dye containing metals displayed variable peaks discerning the presence of

adequate number of adsorption spots onto exopolymeric matrix surfaces, which facilitated sorption of dye and metals. Shifting and disappearance of exiting peaks with formation of new peaks in the samples treated with dye, metals and dye combined with metals in contrast to the unprocessed ones showed involvement of the functional groups (charged and ionizable) persisting onto the surface of exopolymeric (EPS) matrix in accumulation and uptake possibly by forming interactive complexes (**Figure 2, 3 and 4**). Present work can be well explained by results of [24, 29, 30] reporting involvement of charged and ionizable functional groups (nature and composition) persisting on to the

bacterial biomass and EPS in binding, complexation and accumulation of pollutants. Major peak bands of malachite green, metals (Cu, Cd & Cr), and dye molecules containing metals together with those for exopolymeric (EPS) matrix prior to and after particular treatments have been presented in **Tables 3, 4 and 5** (supplementary data).

SEM FOR ANALYSIS OF EXOPOLYMERIC MATRIX (EPS)

Morphological appearance of exopolymeric matrix (EPS) before and after treatment of dye containing metals was examined via SEM, which was indicative of possible adsorption of the contaminants on to the surface of exopolymeric matrix as given in **Figure 5**.



(A)

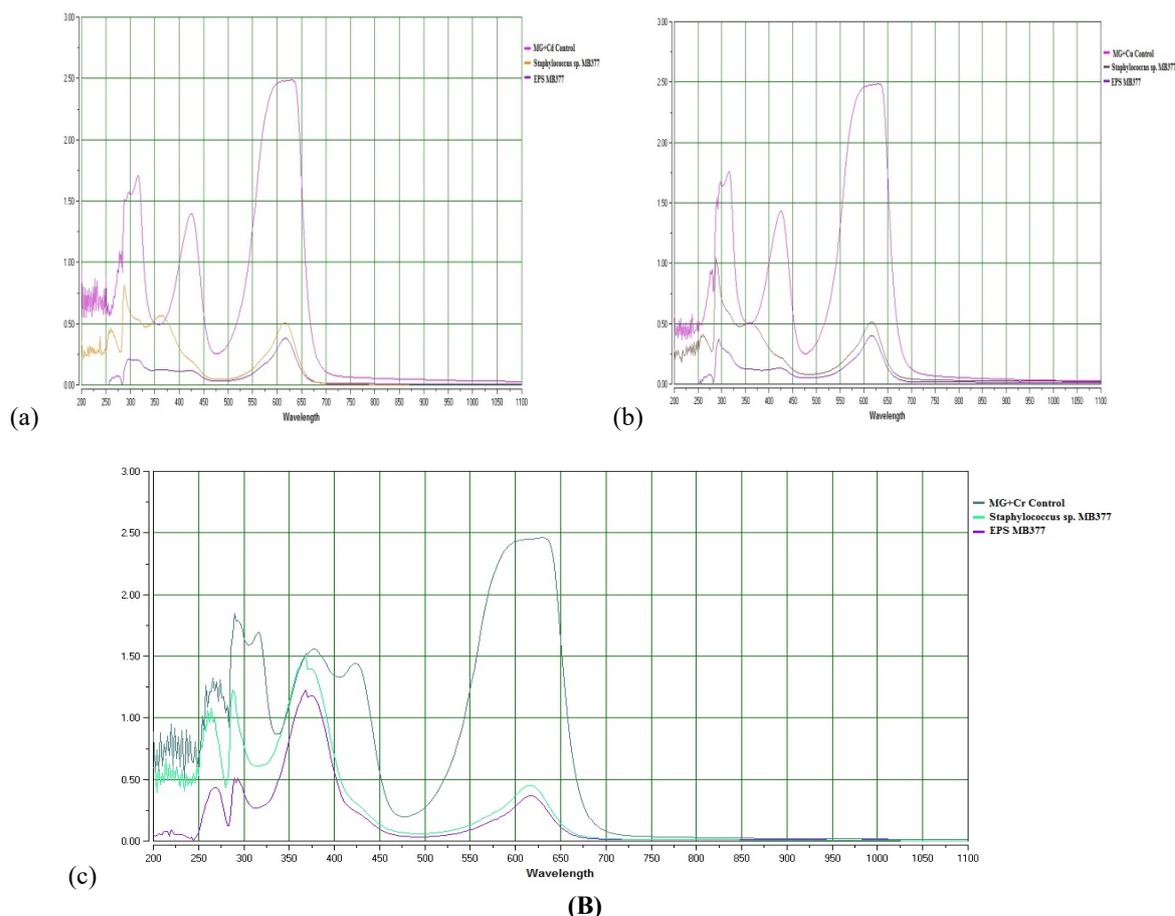


Figure 1: (A) Absorption spectrum of malachite green dye prior to and after adsorption on to the bacterial biomass and EPS. **(B)** Absorption spectra representing changes in peak patterns of dye containing (a) cadmium, (b) copper and (c) chromium before and after adsorption onto the bacterial biomass and EPS

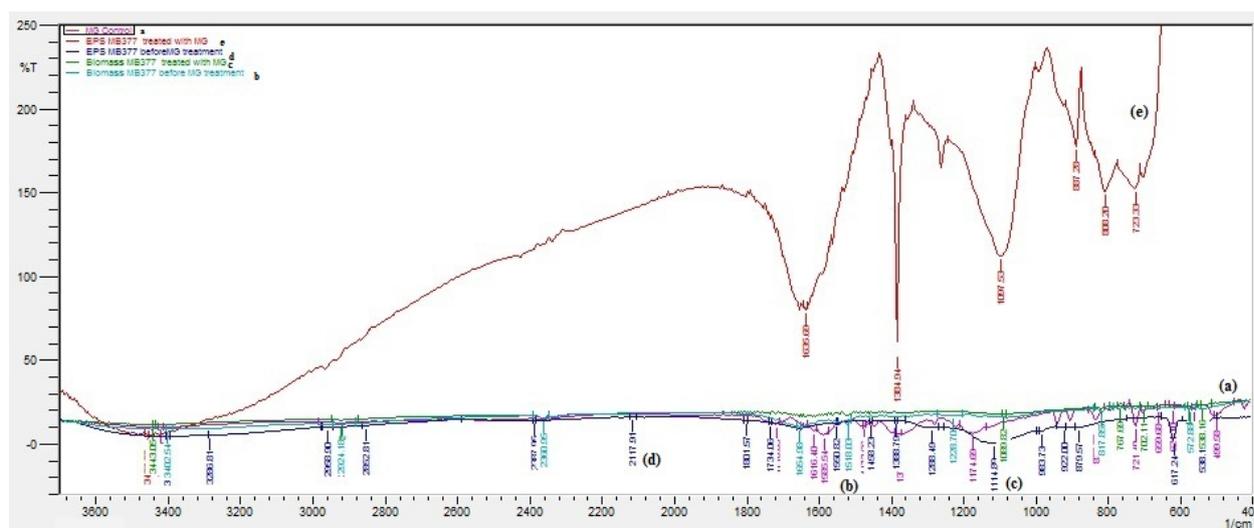
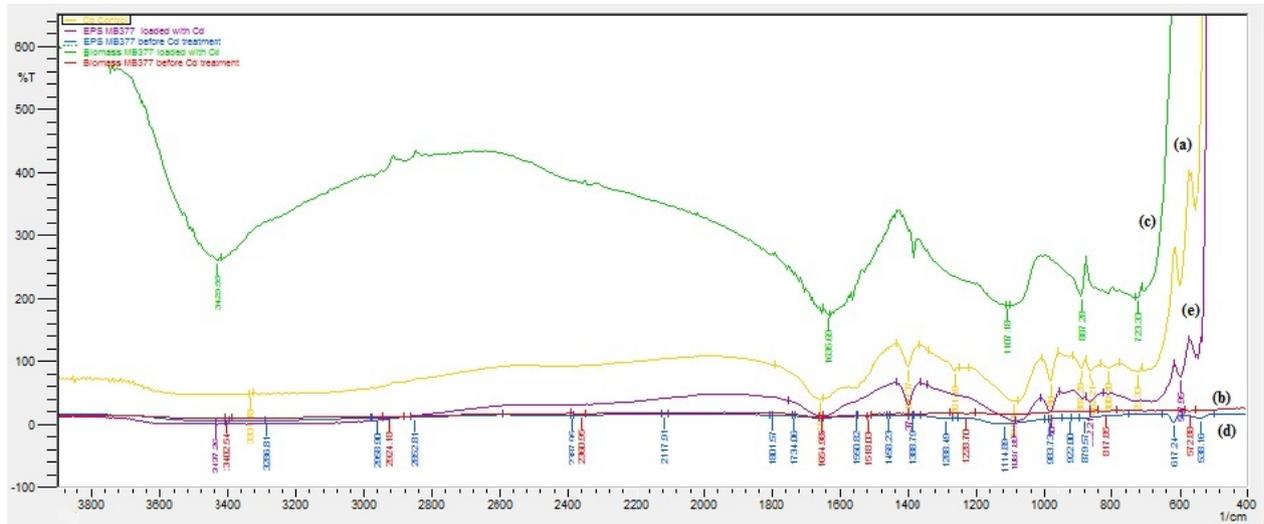
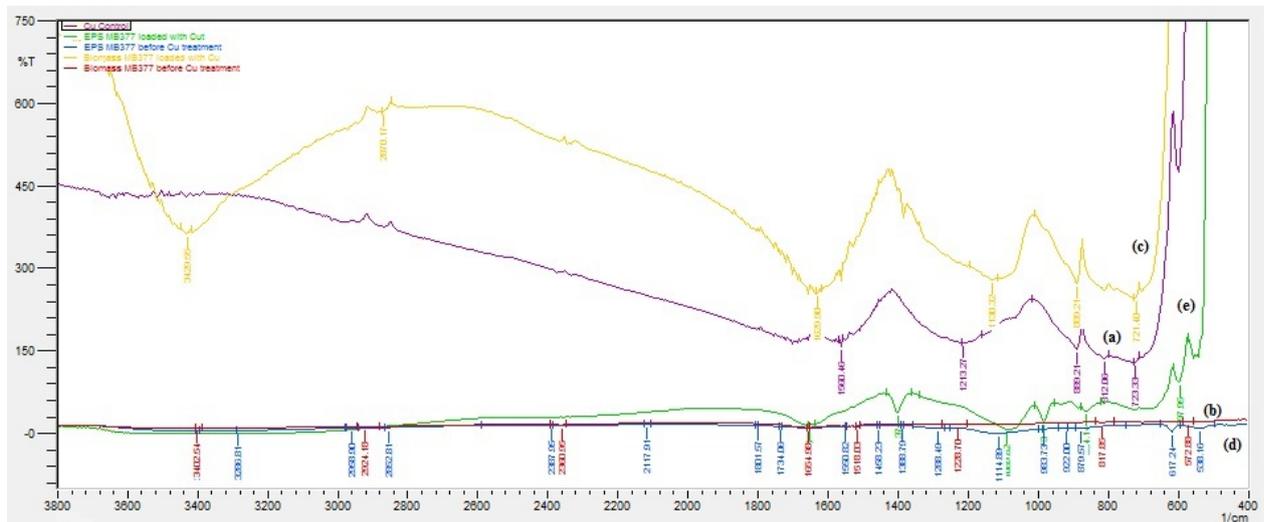


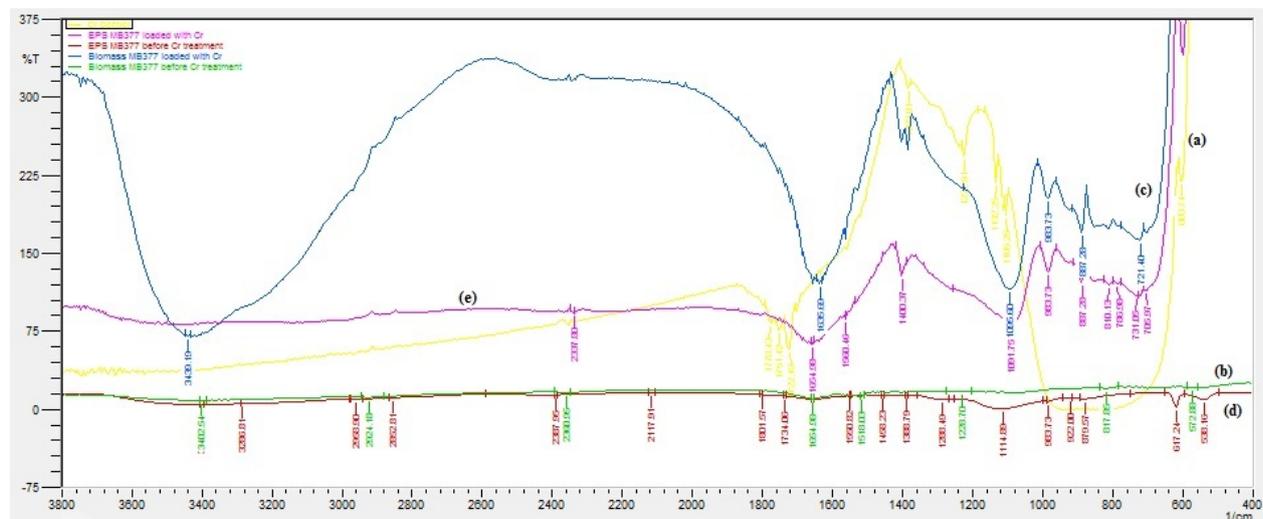
Figure 2: FTIR spectrum depicting (a) Malachite green control (b) bacterial biomass prior to dye adsorption (c) changes in the peaks of bacterial biomass after dye adsorption (d) EPS matrix before dye adsorption and (e) changes in peak patterns of EPS after dye adsorption



(A)

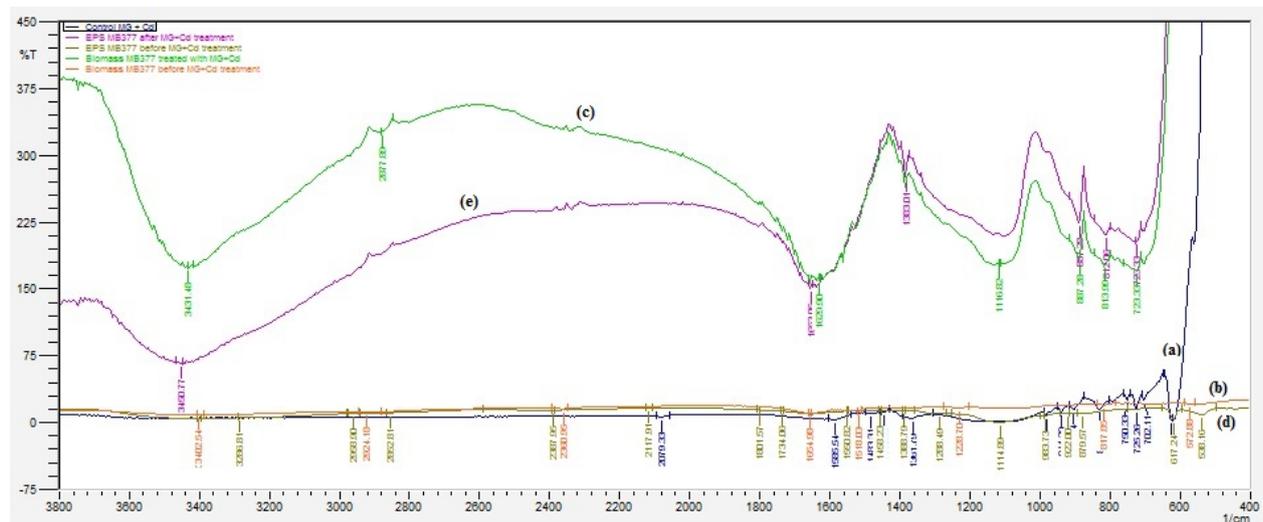


(B)

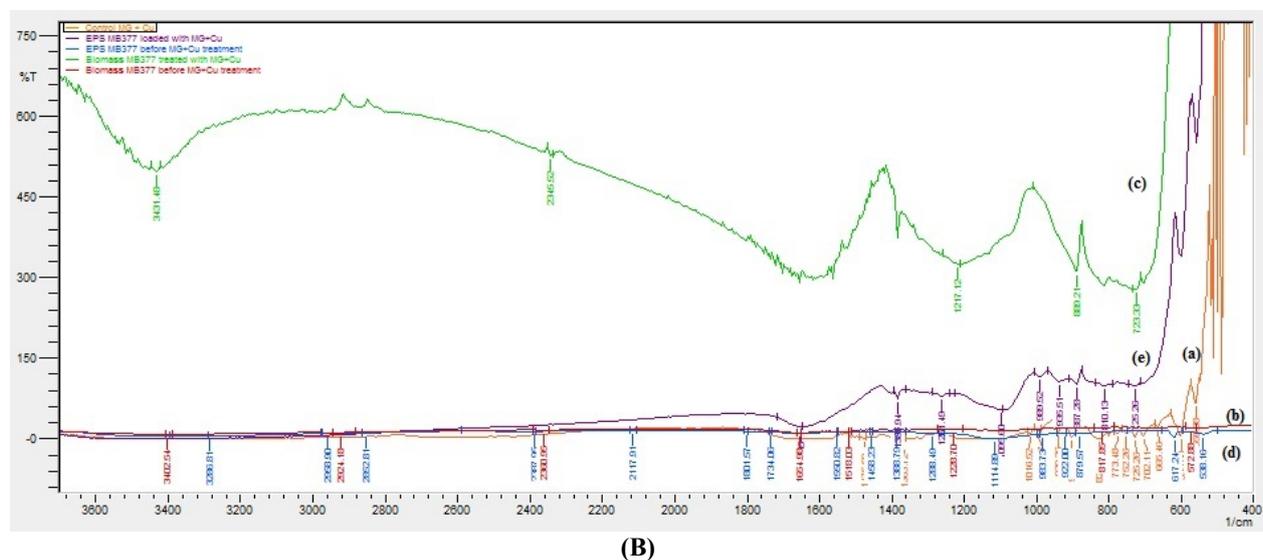


(C)

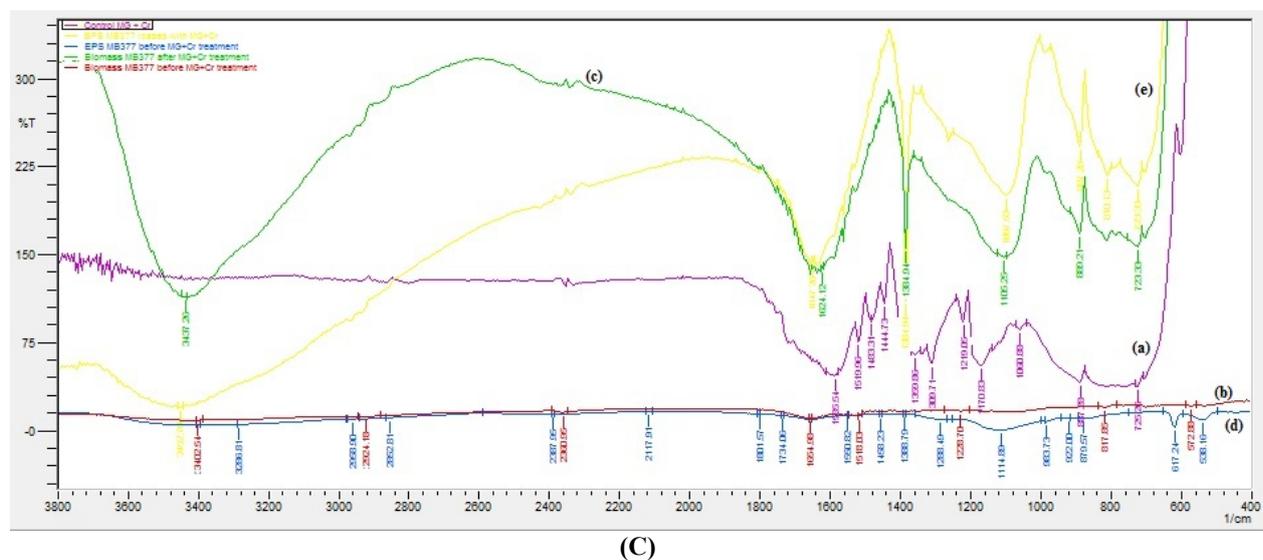
Figure 3: AFTIR spectrum representing (a) Cadmium control (b) bacterial biomass before cadmium adsorption (c) changes in peaks of bacterial biomass after cadmium adsorption (d) EPS before cadmium adsorption and (e) changes in peaks of EPS after cadmium adsorption. B FTIR spectrum representing (a) Copper control (b) bacterial biomass before copper adsorption (c) changes in peaks of bacterial biomass after copper adsorption (d) EPS before copper adsorption and (e) changes in peaks of EPS after copper adsorption. C FTIR spectrum representing (a) Chromium control (b) bacterial biomass before chromium adsorption (c) changes in peaks of bacterial biomass after chromium adsorption (d) EPS before chromium adsorption and (e) changes in peaks of EPS after chromium adsorption



(A)



(B)



(C)

Figure 4: AFTIR spectrum depicting (a) Malachite green+cadmium control (b) bacterial biomass before malachite green+cadmium adsorption (c) changes in peak patterns of bacterial biomass after malachite green+cadmium adsorption (d) EPS before malachite green+cadmium adsorption and (e) changes in peak patterns of EPS after malachite green+cadmium adsorption. B Spectrum depicting (a) Malachite green+copper control (b) bacterial biomass before malachite green+copper adsorption (c) changes in peak patterns of bacterial biomass after malachite green+copper adsorption (d) EPS before malachite green+copper adsorption and (e) changes in peak patterns of EPS after malachite green+copper adsorption. C Spectrum depicting (a) Malachite green+chromium control (b) bacterial biomass before malachite green+chromium adsorption (c) changes in peak patterns of bacterial biomass after malachite green+chromium adsorption (d) EPS before malachite green+chromium adsorption and (e) changes in peak patterns of EPS after malachite green+chromium adsorption

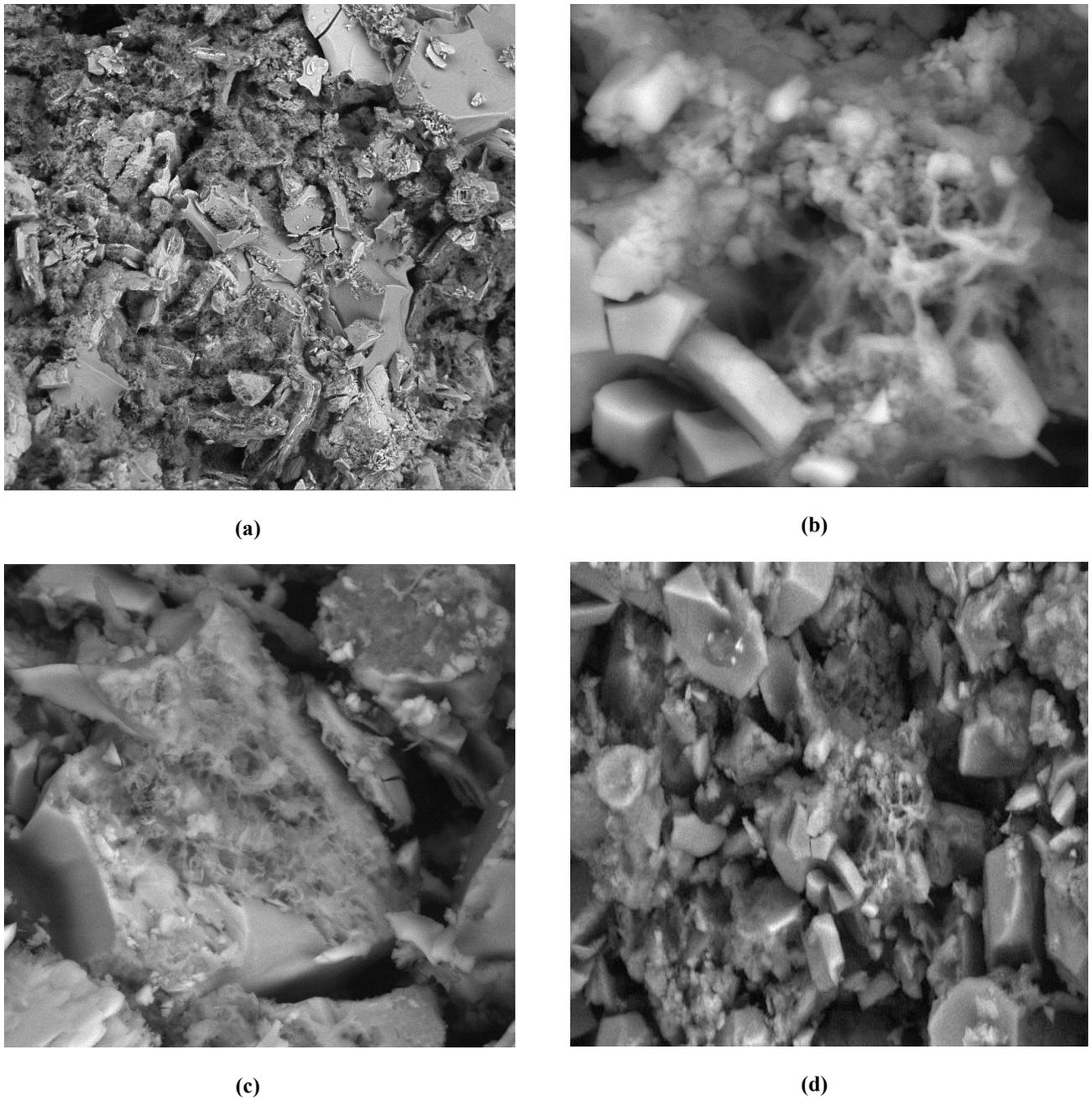


Figure 5: Scanning electron (SE) micrographs of EPS matrix (a) before any dye+metal treatment (b) after treatment with dye+cadmium (c) after treatment with dye+copper and (d) after treatment with dye+chromium

CONCLUSIONS

Existence of co-contaminants within various environmental compartments presents serious environmental concerns; hence need to be treated in an effective and economic way. Application of EPS matrix from *Staphylococcus* sp. MB377 and the bacterial cells for remediation of individual contaminants as well as systems polluted with co-contaminants under present investigation revealed effective uptake and removal efficiencies for metals, dye and dye containing the metals. Exopolymeric matrix form MB377 presented fairly good sorption of cadmium (95.49%) followed by copper (90.68%) with the addition of dye, which might be facilitated via existence of abundant/sufficient amounts of adsorption spots/sites onto the entire surface of exopolymeric (EPS) matrix, also confirmed through IR spectroscopy. Cadmium removal efficiency of EPS (95.67%) and *Staphylococcus* sp. MB377 (96.45%) was observed to be highest indicating high binding affinities of charged functional moieties/groups present on the surface of extracellular polymeric substances for the tested metals. *Staphylococcus* sp. MB377 displayed effective uptake and color removal for malachite green dye containing chromium (81.63%), cadmium (79.65%) and copper (79.35%) in comparison with the dye

molecules alone (78.92%). Results indicated that biofilm forming *Staphylococcus* sp. MB377 offer more effective and efficient removal/detoxification of co-contaminants as single system by possessing intact extracellular polymeric substances in addition to microbial biomass. An enhancement in color removing efficiency of EPS was noticed upon the addition of cadmium (85.10%) and chromium (84.76%) compared to individual dye molecules (84.49%). Scanning electron micrographs of exopolymeric (EPS) matrix prior to and after treatment with synthetic dye and metals represented possible adsorption of contaminants on to the surface of biopolymer. *Staphylococcus* sp. MB377 and the extracted biopolymeric substances (EPS) presented fairly good adsorption and transformation of co-contaminated systems, hence can be applied for construction of biologically operating bioreactors in order to treat co-contaminated wastewaters/effluents for avoiding any further serious pollution concerns.

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