JOURNAL OF ENVIRONMENTAL SCIENCES 115 (2022) 25–36

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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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Enhanced metronidazole removal by binary-species photoelectrogenic biofilm of microaglae and anoxygenic phototrophic bacteria

Xubin Zhang, Jian Sun, Mengmeng Zhao*

Guangzhou Key Laboratory of Environmental Catalysis and Pollution Control, Guangdong Key Laboratory of Environmental Catalysis and Health Risk Control, School of Environmental Science and Engineering, Institute of Environmental Health and Pollution Control, Guangdong University of Technology, Guangzhou 510006, China

ARTICLE INFO

Article history:
Received 1 June 2021
Revised 14 July 2021
Accepted 14 July 2021
Available online 1 August 2021

Keywords:
Photoelectrogenic biofilm
Microalgae
Anoxygenic phototrophic bacteria
Photosynthetic current
Antibiotic

ABSTRACT

High efficient removal of antibiotics during nutriments recovery for biomass production poses a major technical challenge for photosynthetic microbial biofilm-based wastewater treatment since antibiotics are always co-exist with nutriments in wastewater and resist biodegradation due to their strong biotoxicity and recalcitrance. In this study, we make a first attempt to enhance metronidazole (MNZ) removal from wastewater using electrochemistry-activated binary-species photosynthetic biofilm of *Rhodopseudomonas Palustris* (*R. Palustris*) and *Chlorella vulgaris* (*C. vulgaris*) by cultivating them under different applied potentials. The results showed that application of external potentials of -0.3, 0 and 0.2 V led to 11, 33 and 26-fold acceleration in MNZ removal, respectively, as compared to that of potential free. The extent of enhancement in MNZ removal was positively correlated to the intensities of photosynthetic current produced under different externally applied potentials. The binary-species photoelectrogenic biofilm exhibited 18 and 6-fold higher MNZ removal rate than that of single-species of *C. vulgaris* and *R. Palustris*, respectively, due to the enhanced metabolic interaction between them. Application of an external potential of 0V significantly promoted the accumulation of tryptophan and tyrosine-like compounds as well as humic acid in extracellular polymeric substance, whose concentrations were 7.4, 7.1 and 2.0-fold higher than those produced at potential free, contributing to accelerated adsorption and reductive and photosensitive degradation of MNZ.

Introduction

Simultaneous pollutant removal and biomass harvesting for production of high-value products make photosynthetic microorganism-based technology as one of the most promising technologies for resourceful treatment of wastewater. Different from the respiratory metabolism of non-photosynthetic microorganisms, photosynthesis, which enables phototrophs to harvest light to drive chemical reaction and gain energy, help them take advantages in this technical field. Based on the specificity of the photosynthetic metabolism mode, photosynthetic microorganisms are mainly divided into two categories: oxygenic photoautotrophs (e.g. cyanobacteria, algae)

* Corresponding author.
E-mail: sunjian472@163.com (J. Sun).

https://doi.org/10.1016/j.jes.2021.07.016

and anoxygenic photohetertrophs (e.g. purple non-sulfur photosynthetic bacteria, photosynthetic green sulfur bacteria) (Hamilton, 2019; Sasikala and Ramana, 1997; Su, 2021). Oxygenic and anoxygenic photosynthetic organisms frequently live together (as in sewage pond water) or in close proximity (oceanic particulate matter, microbial mats).

These environments are dominated by anoxygenic purple non-sulphur bacteria (mainly *Rhodopseudomonas*) and green unicellular algae (Chandaravithoon et al., 2018). Previous studies have also shown that the microalgae and anoxygenic photosynthetic bacteria are the dominant microbial populations in various type of wastewaters treated by photosynthetic microorganisms, and the photosynthetic metabolism is tightly related to pollutant removal efficiency and biomass accumulation (Lu et al., 2019; Martins et al., 2021; Wang et al., 2020; Yarnold et al., 2019). For example, photosynthetic assimilation is the main approach for recovery of nutriments for biomass production (Puyol et al., 2017). Moreover, secondary metabolites from the photosynthesis, like synthetic pigments, enzymes or photosensitizers, can induce extracellular degradation of contaminants in multiple ways (Tian et al., 2019; Wei et al., 2021). The driving forces of the above-mentioned series of biochemical processes come from the adenine nucleoside triphosphate (ATP) synthesis triggered by the intracellular photosynthetic electron flow coupled with the electrochemical gradient of transmembrane protons (Mullineaux, 2014; Yamamoto et al., 2021). Therefore, photosynthetic electron flow is regarded as the core biochemical process that driving photosynthesis and secondary metabolism of photosynthetic microorganisms and thus affects their performance of wastewater treatment. So far, the wastewater treatment that using photosynthetic microorganisms occurred mainly in wastewaters containing excess nutrients such as urban sewage, livestock and poultry farming wastewater, manure fermentation biogas slurry and fish pond farming wastewater and so on (Chen et al., 2020; Hulsen et al., 2014; Sepúlveda-Muñoz et al., 2020). Antibiotics as emerging pollutant are always co-exist with nutrients in these wastewaters. Removal of antibiotics from wastewater aroused a worldwide attention because antibiotics can induce expression of resistant gene at very low concentrations which pose a significant threat to public water security and human hearth (Zhang et al., 2019). Since great efforts have been undertaken to optimize the performance of photosynthetic microorganisms for nutrients recovery and biomass production, rather less attention was given to antibiotic removal from wastewaters. Metronidazole (MNZ) is among the most widely used veterinary drugs in animal husbandry or in aquaculture and is also the drug of choice for clinical treatment of infectious diseases caused by anaerobic bacteria and protozoans in humans. MNZ is frequently detected in sewage treatment plants at the concentration levels of μg/L-mg/L (Ahmadzadeh et al., 2018). Due to its high solubility in water and low biodegradability, MNZ may enforce severe toxic effects and undesirable disturbance on aquatic ecosystems that results in developing antibiotic resistance (Zhao et al., 2018). Therefore, great attempts have been made recently for efficient removal of MNZ from wastewater before discharging it into the environment.

It is well established that some non-photosynthetic microorganisms in environment can transfer their intracellular respiratory electrons to extracellular solid electron acceptor through conductive outer membrane, self-secreted redox active substances or exogenous electron-shuttling compounds, (Liu and Li, 2020; Zhao et al., 2020). Studies have shown that extracellular extraction of respiratory electrons by electrode can enhance the degradation capability of the non-photosynthetic microorganisms for removal of organic pollutants in water. Tahir et al. (2019) found that the removal efficiency of carbamazepine reached 84% when an external potential of 0.4V was applied, while less than 20% was observed under potential free conditions. You et al. (2021) proved that an external potential can regulate the extracellular electron transfer path and thus affect the degradation rate of toluene. As an important component, extracellular polymeric substances (EPS) play an important role in mediating the extracellular electron transfer (EET) of non-photosynthetic microorganisms and enhancing the extracellular degradation of pollutants. Firstly, the cytochromes and other substances in EPS can mediate the transfer of intracellular respiratory electrons of non-photosynthetic microbe from the cell surface to the extracellular electron acceptors to enhance degradation of pollutants (Sathishkumar et al., 2020; Zhuang et al., 2020). Secondly, proteins and other components in EPS can bind to organic contaminants so that contaminants can be quickly removed from water (Hou et al., 2016; Qiu et al., 2020; Xu et al., 2013; Xu et al., 2018). What's more, EPS can induce indirect photolysis of organic matter (Yang et al., 2018). However, the mechanism of photosynthetic metabolism is entirely different from the respiratory of non-photosynthetic microbes. At present, the research on the photosynthetic electron extraction from photosynthetic microorganisms and its resultant environmental effects is still in its infancy.

In this study, *Rhodopseudomonas Palustris* (*R. Palustris*) and *Chlorella vulgaris* (*C. vulgaris*) were selected as model anoxygenic photoherertroph and oxygenic autotrophy, respectively, and MNZ was used as a model antibiotic. We conducted a systematical investigation on the effect of application of applied external potentials for photosynthetic electrons extraction from the photosynthetic binary-species biofilm of *R. Palustris* and *C. vulgaris* on MNZ removal, with a special emphasis on the role of the applied external potentials in strengthening interaction between them and regulating EPS synthesis for enhancing removal of MNZ. The results obtained here could contribute to the technical progress of the photosynthetic biofilm-based wastewater treatment regarding of the refractory toxic organic pollutants removal with simultaneously biomass recovery to cater to the sustainable utilization of water resources and construction of circular bioeconomy.

1. Materials and methods

1.1. *Reactor configuration*

The three-electrode electrochemical device was constructed in a single cylindrical glass chamber with 144 mL total volume, where graphite plates (3 × 1.5 × 0.4 cm), titanium wire (0.6 mm diameter) and saturated calomel electrode (SCE) were used as the working, counter and reference electrodes, respectively. Before being used, the working and counter electrode were placed in an ultrasonic water bath for 45 min and then assembled in reactor for sterilization (Appendix A Fig. S1).

1.2. Culture and operation

C. vulgaris and *R. palustris* were isolated from the South China Basin and were used as model oxygenic photoautotroph and anoxygenic photohetertroph, respectively. The two phototrophic microorganisms were transferred successively to reactors containing sterilized growth medium in an aseptic manipulation box for biofilm formation with 20% inoculation density. The reactors were run in repeat-batch mode at 27 ± 2°C, and potentiostat (CHI 1000C, CH Instrument, Shanghai, China) was used to control the working electrode potential. The growth medium contained (per liter): 1 g NaAc, 7.76 g K_2HPO4, 0.006 g Ferric citrate, 0.006 g Citric acid, 0.001 g EDATA-Na_2, 0.31 g NH_4Cl, 0.13 g KCl, 12.5 mL of mineral solution and 12.5 mL of vitamin solution (Sun et al., 2020). After the startup and acclimation stage, the MNZ was added to reactors to provide a final concentration of 1 mg/L. In order to investigate the externally applied potential on MNZ removal by the photoelectrogenic biofilm, the potentials of the working electrode were set to -0.3, 0 and 0.2 V (vs. SCE), with aseptic electrochemical reactor and the biofilm operated under potential free or dark condition as controls. During the whole experimental period, a cold white fluorescent lamp (3000 Lux) was used as light source to simulate the sunlight for photosynthesis, with the distance between light source and reactor set as 5 cm.

1.3. Contribution of photoelectrogenic biofilm-associated EPS to MNZ removal

In order to investigate the contribution of the photoelectrogenic biofilm-associated EPSs to MNZ removal, the EPS extracted from the photoelectrogenic biofilms grown under different externally applied potentials were added to the aseptic growth medium with additionally supplement of 1 mg/L MNZ. The removal of MNZ was monitored in real-time.

1.4. Analytical methods

1.4.1. Electrochemical measurements

The photosynthetic electron extracted from the photoelectrogenic biofilm of *R. Palustris* and *C. vulgaris* was transformed to electrical signal which was recorded every 5 min using a multi-channel potentiostat (CHI 1000C, Chenhua, China). Cyclic voltammograms (CV) and electrochemical impedance spectroscopy (EIS) were performed by a single-channel potentiostat (CHI 660, Chenhua, China) to test the electrochemical activity and photosynthetic electron transfer rate of the photoelectrogenic biofilm, respectively. The parameters of CV were set with a scan rate of 5 mV/s from −0.6 to 0.5 V. EIS was measured over a frequency range of 0.1 to 5 mHz with a perturbation amplitude of 5 mV.

1.4.2. Chemical analysis

The concentrations of MNZ were measured by using high performance liquid chromatography (HPLC, LC-16, Jiangsu, China) equipped with a C18 column (5 μm; 4.6×150 mm, Phenomenex, CA, USA) at the column temperature of 30°C. The mobile phase is consisted of methanol: 0.1% formic acid (ultrapure water) = 20:80 (V/V). The analysis was started by injected the sample (20 μL) into the column where mobile phase flowed at 1 mL/min. The wavelength for MNZ detection was 318 nm.

To identify the degradation intermediates of MNZ by the photoelectrogenic biofilm, Ultra performance liquid chromatography Q-Exactive Orbitrap mass spectrometry (Thermo Fisher Scientific, USA) with Hypersil GOLD C18 (100×2.1 mm, 1.9 μm) was carried out. Gradient LC elution was performed with 0.1% formic acid in ultrapure water as mobile phase A and methanol as mobile phase B. It was carried out as followed:2 min with 98% A, 13 min with 98% to 5% A, 2 min with 98% A.

1.4.3. EPS analysis

The EPSs of the photoelectrogenic biofilm were extracted following a method described previously (Zimu Xu1, 2017). In brief, the working electrodes covered with photoelectrogenic biofilm were suspended in 4 mL of NaCl solution (0.9%), and further diluted with 4 mL of NaCl solution (0.9%) that had been preheated to 70 °C to maintain a temperature of 35 °C in the final solution. The solution was then sonicated for 2 min for the separation of EPS. The bare electrode was removed from the solution and discarded. The resulting suspension was sheared by a vortex mixer for 2 min and then centrifuged at 5000 × g for 15 min. The supernatant was filtered through a 0.22 μm membrane filter and the organic matter in the supernatant was collected as the loosely bound EPS (LB-EPS).

The remaining cells in the centrifuge tube were again resuspended in 10 mL of 0.9% NaCl solution and heated at 50°C for 30 min. The heated solution was then centrifuged at 5,000 g for 20 min and filtered through a 0.22 μm membrane filter. The liquid finally obtained contained mainly the heat extract-able microbial product which might roughly represent the tightly bound EPS (TB-EPS) (Wang et al., 2016).

The protein in the collected EPS were determined by a BCA protein concentration determination kit (Coolaber, SK1070). The polysaccharides and humic acid were measured by the anthrone method (Yu Qi, 2017) and a modified Lowry method (Fr¢lund et al., 1995) respectively.

The components of EPS were analyzed using a three-dimensional fluorescence spectrometer using a luminescence spectrometer (FLS1000, Edinburgh Co., UK). The excitation wavelength was from 220 to 450 nm, and the emission spectrum was from 250 to 700 nm at 5 nm increments. The concentration of different components of EPS were expressed as a function of the dry cell (mg/g).

A high-resolution confocal laser microscope (LSM 800 with Airscan, Carl Zeiss) was used to observe the spatial distribution of proteins, α-polysaccharides and β-polysaccharides of the EPS within the photoelectrogenic biofilm grown under different externally applied potentials. 5 g/L fluorescein isothiocyanate (FITC) was used to stain proteins and amino sugars, indicated in green. 0.25 g/L concanavalin A (Con A) was used to bind α-polysaccharides, indicated in red. The β-polysaccharide was stained with 0.3g/L Calcofluor white M2R (CW), which was indicated in blue. The excitation wavelengths

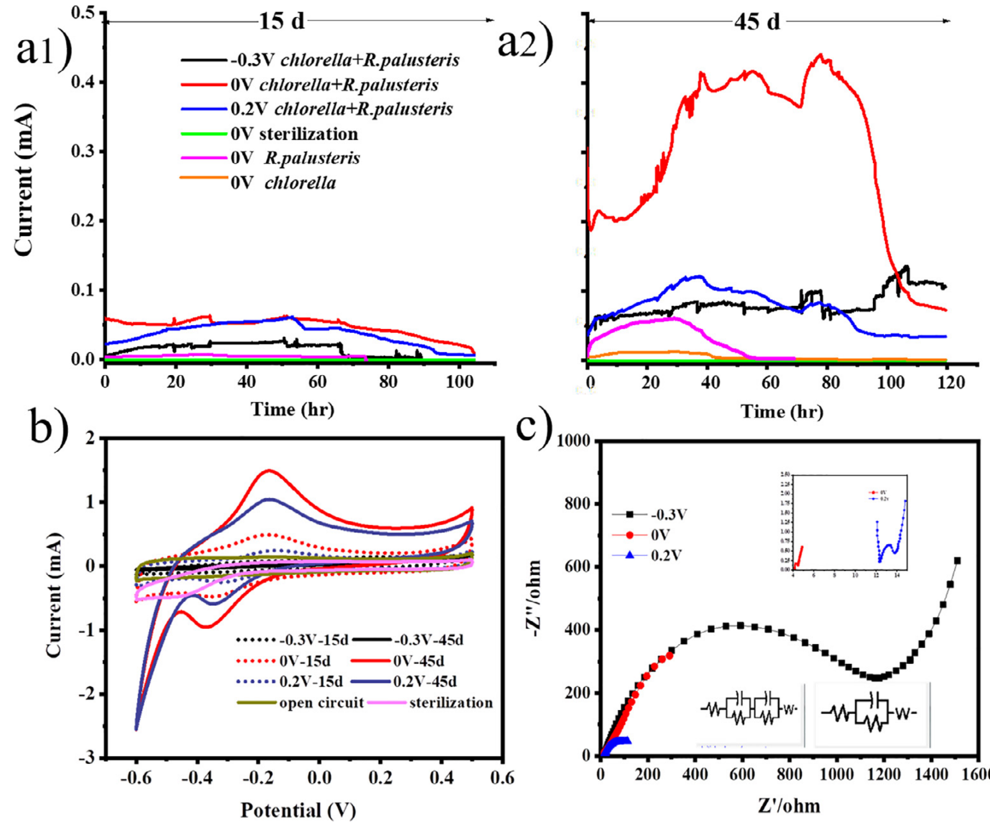

Fig. 1 – Current-time curves of the photosynthetic biofilms cultivated under different conditions at 15 and 45 days (a); cyclic voltammograms (b) and electrochemical impedance spectroscopies (c) of the binary-species photoelectrogenic biofilms cultivated at different applied potentials.

of protein, α-polysaccharide and β-polysaccharide were 488, 543 and 400 nm, respectively.

Firstly, 0.1 mol/L sodium bicarbonate buffer was added to the sample to keep the amine group in a protonated form. FITC solution was added to the sample and then let it stand for 1 hr in the dark. Next, added the Con A solution to the sample, and let it keep for 30 min in the dark. Then, the sample was dye with CW solution and stood for 30 min in the dark. After each staining, the samples were washed three times with phosphate buffered saline (PBS) to remove excess staining. The spatial structure of biofilm EPS was observed using a 5 × water mirror.

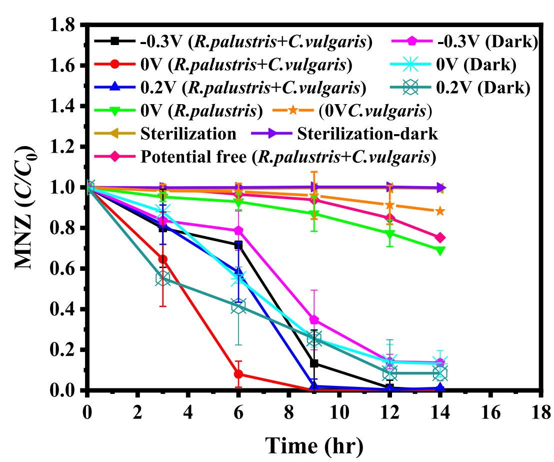

Fig. 2 – The removal rate of MNZ by the electrochemical device operated under different conditions

2. Results and discussion

2.1. *Externally applied potentials driven photosynthetic electrons extraction from the binary-species photoelectrogenic biofilm of* R. Palustris *and* C. vulgaris

Photosynthetic current can intuitively reflect the amounts of photosynthetic electrons extracted from the photoelectrogenic biofilm. Fig. 1a showed exemplary photosynthetic current cycles produced by the photoelectrogenic biofilm cultivated under different externally applied potentials at 15 (Fig. 1, a1) and 45 (Fig. 1, a2) days. The photosynthetic current was found to be potential-dependent. A maximum photosynthetic current of 0.06 mA was observed simultaneously for the photoelectrogenic biofilm cultivated at 0 V and 0.2 V after 15 days cultivation. However, the photoelectrogenic biofilm cultivated at 0 V yielded a photosynthetic current intensity of 0.44 mA at 45 days, which was much higher than that yielded by the photoelectrogenic biofilm cultivated at 0.2 V (0.12 mA) and -0.3 V (0.13 mA). It is concluded that 0 V is a favorable potential for extraction of photosynthetic electrons from the binary-species photoelectrogenic biofilm. In addition, the stable photosynthetic current generated by the photoelectrogenic biofilm cultivated at -0.3 V can be sustained more longer than that generated by the photoelectrogenic biofilm cultivated at 0 V and 0.2 V, indicating a slow photosynthetic electrons extraction rate at -0.3 V.

Similar current responses were observed from the CV scans under turnover conditions (Fig. 1b). A set of redox peaks were found at -0.17 V and -0.32 V for the photoelectrogenic biofilm cultivated at 0 V and 0.2 V, while it was disappeared completely for the photoelectrogenic biofilm cultivated at -0.3 V. The redox peak is closed to characteristic peaks of outer membrane cytochromes (OmcA) according to previous studies (Edwards et al., 2017; Venkidusamy and Megharaj, 2016). The results of CV indicated that an applied potential of 0 V can promote the secretion of OmcA. Therefore, the highly efficient photosynthetic electron extraction at 0 V might be attributed to high level expression of OmcA.

Fig. 1c presented the EIS of the photoelectrogenic biofilm under non-turnover conditions. The solution resistance (Rs), charge transfer resistance (Rct) and Warburg's diffusion element were obtained by simulating the Nyquist plot with an equivalent circuit (an inset in Fig. 1c). As shown in Appendix A Table S1, the photoelectrogenic biofilm cultivated at 0 V exhibited a Rct of 4 Ω which was much smaller than that of the photoelectrogenic biofilm cultivated at -0.3 V (692 Ω) and 0.2 V (45 Ω), indicating a extremely low extracellular photosynthetic electrons transfer resistance at 0 V.

2.2. *Performance of the photoelectrogenic biofilm for metronidazole (MNZ) removal*

As shown in Fig. 2. under illumination, the binary-species photoelectrogenic biofilm achieved better MNZ removal performance than that of pure culture of *R. palustris* and *C. vulgaris*. No matter how much the potential is applied, the binary-species photoelectrogenic biofilms showed incredible ability to remove MNZ completely within 12 hr with a removal rate of 0.494 hr^{-1} at 0 V, 0.396 hr^{-1} at 0.2 V and 0.271 hr^{-1} at -0.3 V, respectively, while barely 0.021 hr^{-1} and 0.008 hr^{-1} were observed for the pure culture of *R. palustris* and *C. vulgaris*. These results indicated that the synergy of *C. vulgaris* and *R. palustris* enhanced MNZ removal which might be due to the enhanced aerobic biodegradation of MNZ by *R. palustris* in the presence of photosynthetic oxygen released by *C. vulgaris* (Wang et al., 2020). In addition, the removal of MNZ by the no-photoelectrogenic biofilm cultivated at potential free significantly slower than that achieved by the photoelectrogenic biofilms, indicating a major contribution of photosynthetic electrons extraction to MNZ removal. It was well documented that the conductive solid matrix with applied potential can act as an electron acceptor for accelerating oxidation of electron donors (H_2O for photosynthesis of *C. vulgaris* and organic carbon for photoheterotrophic metabolism of *R. palustris*), which provides more electrons for reductive degradation of metronidazole (Wen et al., 2011). Fig. 2 also showed that the removal of MNZ was significantly slowed down when the illumination was cancelled due to lack of photosynthesis (Sun et al., 2020).

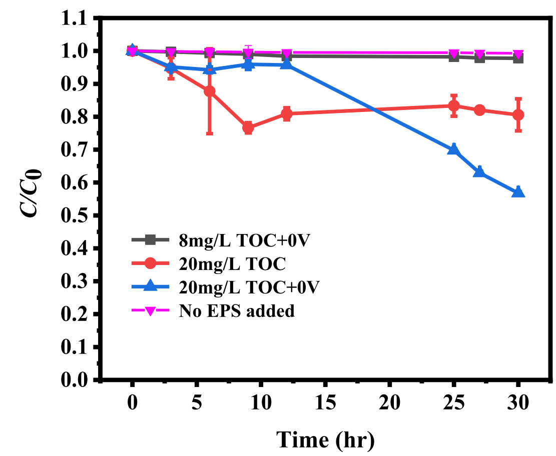

Fig. 3 – Comparison of MNZ removal by the EPS extracted from the binary-species photoelectrogenic biofilm at different EPS concentrations.

The EPSs extracted from the binary-species photoelectrogenic biofilms were put into sterile reactors containing 1mg/L MNZ to investigate the contribution of EPS to MNZ removal. As shown in Fig. 3, the removal performance of MNZ by the EPS extracted from the binary-species photoelectrogenic biofilms was concentration-dependent. No obvious removal of MNZ was observed at EPS concentration of 8 mg TOC/L under potential free condition. However, addition of 20 mg TOC/L of EPS resulted in a fast removal of MNZ within 8 hours but no further removal was observed as time goes by. It is the electrostatic interactions and hydrophobicity that makes combination between antibiotics and EPS happened, however, MNZ is hydrophilic and easier to release in water again (Xu et al., 2013; Zhu et al., 2020). The combination of addition of 20 mg TOC/L EPS and application of an external potential of 0V resulted in fast removal of MNZ after 12 hr with a high total MNZ removal efficiency of 54% which might be attributed to the EPS-induced photosensitive degradation of MNZ (Li and Hu, 2016; Xu et al., 2011). Obviously, the removal of MNZ by the EPS of the photoelectrogenic biofilms showed obvious dosage effect. It has been reported that the pollutants removal efficiency increases as the concentration of EPS increased due to the increases in the number of binding sites available for the pollutants (Nouha et al., 2016). In addition, high concentration of EPS contains more photo-active substance which could induce faster photosensitive degradation of antibiotic pollutant than that with addition of low concentration of EPS.

LC/MS analysis was performed to identify the intermediate products of MNZ, which were shown in Appendix A Fig.S2 and Table S2. Four possible degradation pathways were proposed based on intermediate products analysis and shown in Fig. 4. In pathway 1, the hydroxyl of MNZ is oxidized to carboxyl and then nitro is replaced by hydroxyl, while the nitro is firstly attacked to form a *m/z*=127 compound in pathway 2 (Mai et al., 2019). The formation of 2-methyl, 5-hydroxy-imidazole in both pathway1 and 2 was decomposed to oxalic acid. Pathway 3 represents the indirect electric reduction of MNZ. MNZ turned to nitroso compound (*m/z*=156) at first by capturing $2e^-$, and then the nitroso turned to hydroxylamine

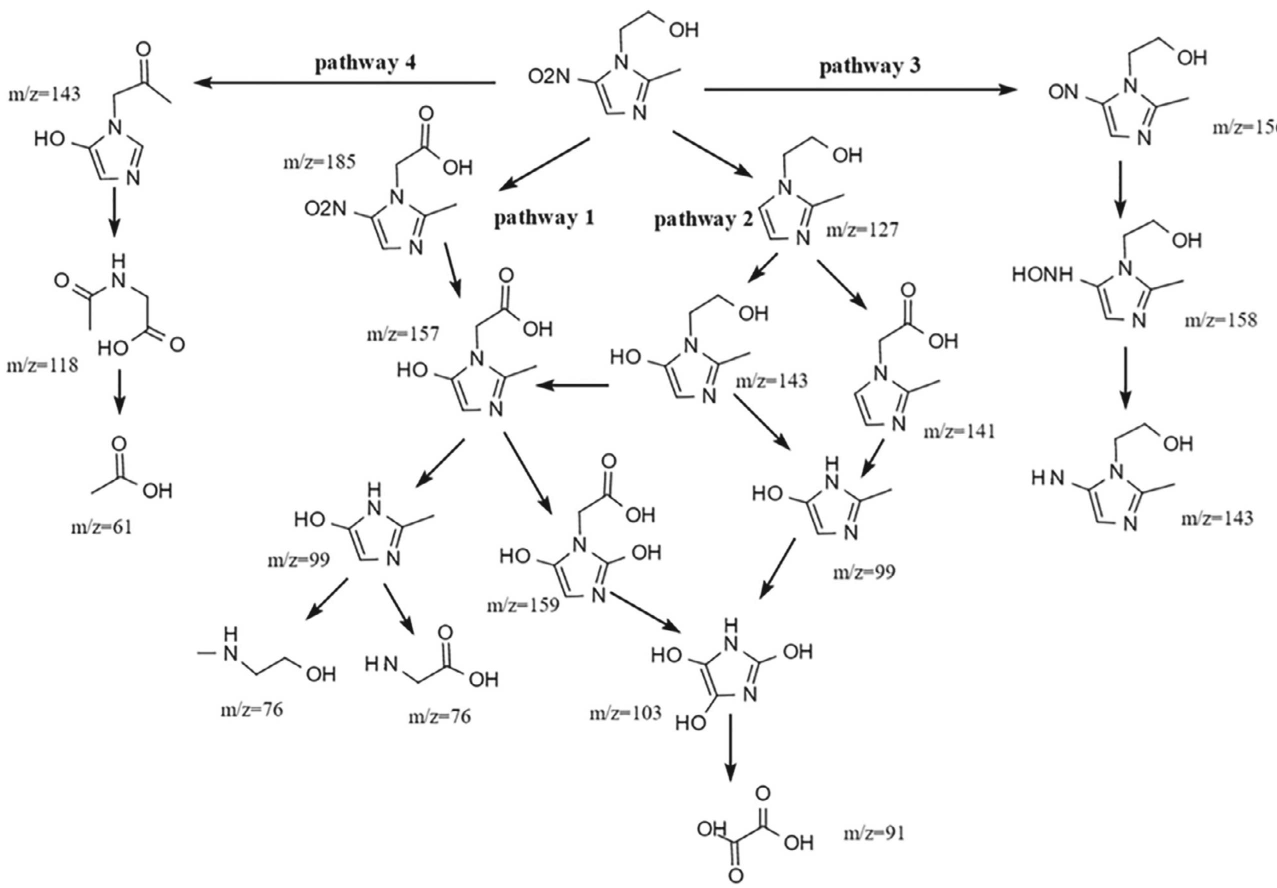

Fig. 4 – The possible degradation pathway of metronidazole by the binary-species photoelectrogenic biofilm of *C.vulgaris* and *R. palustris*.

azanol (*m*/z=158) by capturing another 2e⁻ to forming amide (m/z=143) (Kong 2015; Saidi, 2016). In pathway 4, nitro was replaced by H^+ leading to the decomposition of C=N and the formation of glycine (*m*/z=118), and finally become acetic acid (Dai et al., 2016). Based on the analysis of the intermediate products, the reductive denitration followed by dissociation of aromatic ring structure to form organic acid was demonstrated. There are three possible mechanisms that account for the degradation of MNZ by the photoelectrogenic biofilm: (1) a portion of photosynthetic electrons extracted by the electrode was transferred to MNZ for its reductive denitration (Sun et al., 2020); (2) the reductive denitration products was further degraded by extracellular enzymes produced by the photoelectrogenic biofilm (Hena et al., 2020); (3) the EPS can play as biophotosensitizer for inducing indirect photolysis of MNZ (Wei et al., 2021).

2.3. *Mechanism of the photoelectrogenic biofilm EPS-induced MNZ removal*

2.3.1. *EPS production regulated by externally applied potentials*

Exogenous stress/induction have a great influence on the microbial EPS synthesis through regulation of the physiological metabolism of cells (Zhu et al., 2020; Liu et al., 2016). It can be seen from Fig. 5, an application of an external potential of 0 V and 0.2 V obviously promoted protein synthesis which may contain cytochrome C, leading to high electron transfer rate and enhanced co-metabolic degradation of MNZ (Yang et al., 2019). In contrast, the protein content in EPS did not undergo significant increases under an external potential of -0.3 V. Moreover, the application of external potential greatly increased the content of polysaccharides and humic acid-like substrate in EPS when compared to the EPS obtained under potential free condition. It is reported that humic acid plays an important role in microbial extracellular electron transfer (Xiao et al., 2017), redox degrading pollutants (Young et al., 2006) and inducing photosensitive degradation of organic pollutants (Tenorio et al., 2017). These results could partly explain the mechanisms of enhanced MNZ removal by the photoelectrogenic biofilm (Figs. 2 and 3). The polysaccharides in EPS have a strong adsorption capability on aromatic hydrocarbons and can combine with antibiotics to form complexes (Li et al., 2021; Pan et al., 2010; Xu and Sheng, 2020).

The cultivation period has also a great influence on the EPS components of the photoelectrogenic biofilm. Comparing with 15-day cultivation, the content of protein, polysaccharides and humic acid-like substrate increased significantly in LB-EPS but decreased in TB-EPS after 45 days cultivation (Fig. 5d and 5e). It is well documented that the microbial cells are embedded by EPS, which can be divided into the tightly-bound EPS (TB-EPS) located in inner layer of biofilm and the

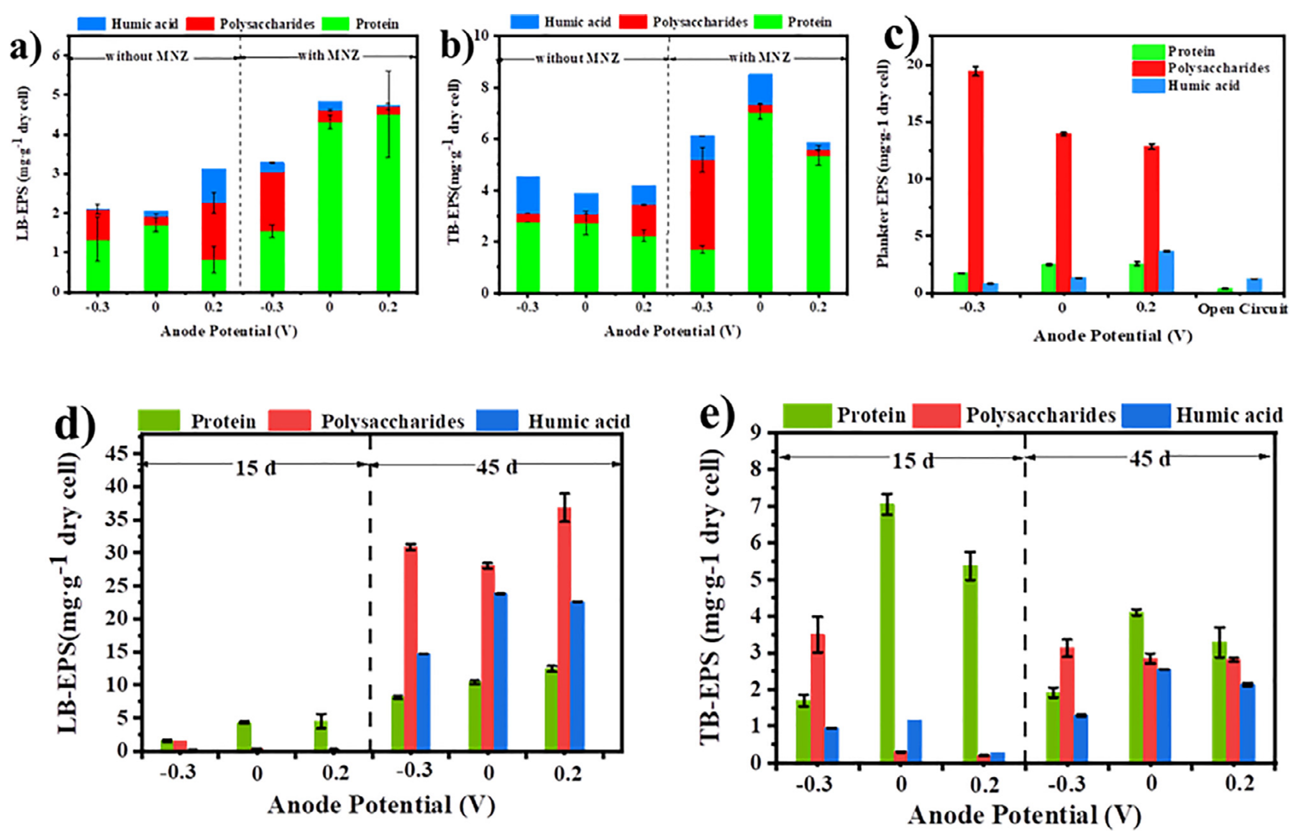

Fig. 5 – Comparison of functional substance contents in loosely bound extracellular polymer components (a) and tightly bound extracellular polymer components (b) of the EPS produced at different applied potentials; Comparison of functional substance contents in the EPS of plankter produced at different applied potentials (c); Effect of cultivation periods on functional substances accumulation in loosely bound extracellular polymer components (d) and tightly bound extracellular polymer components (e) of EPS produced at different applied potentials.

loosely-bound EPS (LB-EPS) existing in outer layer of biofilm. The loosely bound EPS can directly interact with pollutants in wastewater and thus plays a more important role than tightly bound EPS in pollutants removal. In this study,

In sum, application of external potentials posed a positive influence on extracellular removal of MNZ by the photoelectrogenic biofilm. On the one hand, the increase of EPS can impede diffusion of MNZ in biofilm and protect microbial cells from toxicity induced by MNZ (Li et al., 2021). On the other hand, the accumulation of protein, polysaccharides and humic acid-liked photoactive substance in EPS could promote the adsorptive, reductive and photosensitive removal of MNZ (Xiao and Zhao, 2017).

2.3.2. *Spatial distribution of EPS in the photoelectrogenic biofilm*

The spatial distribution of protein and polysaccharide of EPS in the photoelectrogenic biofilms was analyzed by fluorescent staining. At a specific excitation wavelength, the fluorescence of protein is green, α-polysaccharide is red and β-polysaccharide is blue. As is shown in Fig. 6, tight and homogeneous biofilms were formed under different applied potentials (100-160 μm in thickness). Moreover, protein accounts for a large portion of the EPS cultivated at 0 V, while excessive polysaccharides are accumulated in the EPS formed at 0.2 V. Coupling with the result of CV, we already known that an applied potential of 0 V and 0.2 V can promote the secretion of cytochrome for enhancing extracellular photosynthetic electron transport which explained why there is a tiny difference in photosynthetic current intensity between the photoelectrogenic biofilm cultivated at 0 V and 0.2 V when their contents of protein were similar after 15 days cultivation. However, nonconductive polysaccharides had increased with time passing at 0.2 V, which could dissipate electroactivity of the biofilm (Kitayama, 2017). As indicated by CV results, the photoelectrogenic biofilm cultivated at -0.3 V shows low electroactivity, despite the average thickness of the biofilm was 160 μm. When analyzed each layer of EPS, it was found that the EPS was mainly accumulated in the middle layers of the biofilm, because either the toxicity of antibiotics or the absence of light source leads to less biomass on outer or inner membrane. High content of redox proteins in EPS accumulated in the middle layer could beneficial to photosynthetic electrons transfer from outer biofilm to solid matrix. Besides, there is an increasing tendency for β-polysaccharide towards to the outer biofilm which could contribute to form a firm biofilm (Adav, 2008).

Three-dimensional EEM fluorescence spectroscopy can give a spectral information to investigate the changes of

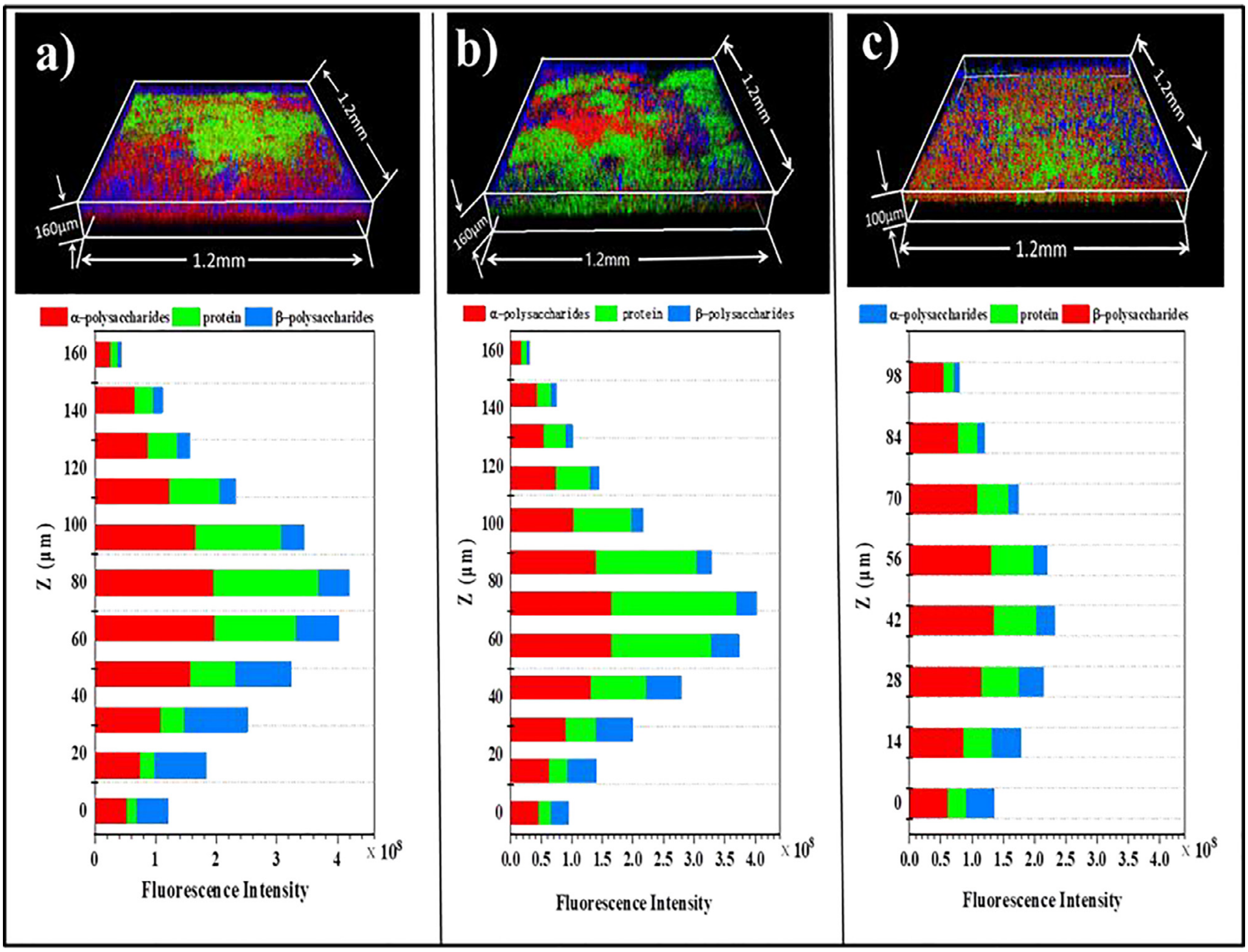

Fig. 6 – The spatial distribution of EPS produced at -0.3V (a), 0V(b) and 0.2V (c). The upper part is a three-dimensional view of the space, and the lower part is the content distribution of each component of extracellular polymer substrate in different layers of the biofilm according to the biofilm thickness.

organic component in the EPS of the photoelectrogenic biofilm cultivated at different external potentials. As shown in Fig. 7. characteristic peaks A (Ex/Em:225-230/320-335) and B (Ex/Em:280-290/335-345), which represented tyrosine-like and tryptophan-like substances respectively (Lai et al., 2018), were significantly enhanced along with increase of external potentials. High intensities of peak A and B in all stages implied that tyrosine and tryptophan protein might be the main component of proteins in EPS. The highest intensities of peak C (Ex/Em:260-275/440-480) and D (Ex/Em:345-350/430-440) which belongs to humic acid-like substances (Peiris et al., 2011) were achieved for the EPS of the photoelectrogenic biofilm cultivated at 0 V. Previous studies have proved that tyrosine-like substance, tryptophan-like substance and humic acid can interact with antibiotics through C=O bonding site (Li et al., 2020; Lin et al., 2010; Wang et al., 2018) and the humic acid can also function as photosensitizer to promote photosensitized degradation of antibiotics (Wei et al., 2021). An external potential of 0.2V mainly promoted the accumulation of protein in EPS, while an external potential of 0 V promoted the accumulation of both protein and humic acid which could explain the best removal performance of MNZ by the photoelectrogenic biofilm cultivated at 0 V. In comparison, the intensities of peak A and B in EPS produced at -0.3 V were significantly lower than that observed for EPSs produced at 0 V and 0.2 V, leading to a poor performance in MNZ removal.

The EPSs extracted from the planktonic microorganisms were also analyzed. As is shown in Figure 7, characteristic peak E (Ex/Em:244/440) and peak F (Ex/Em:325/375) were detected only in plankter but absent in biofilm. Peak E belongs to fulvic-like substances, and the existence of which may contribute to the blue-shift of humic acid (Li et al., 2021). Besides, characteristic peak of tryptophan-like was not observed in plankter. Tryptophan usually acts as a reducing agent in biochemical reactions. It only needs very low energy (~4 eV) to excite it to lose electrons and combine with organics, resulting in fluorescence quenching (Callis, 2014). Popova et al., (2010) proposed that nitroso compounds tend to combine with tryptophan to form complexes, so it can be speculated that nitro compounds is the main reason of fluorescence quenching of tryptophan-like substances. And it also inferred that the

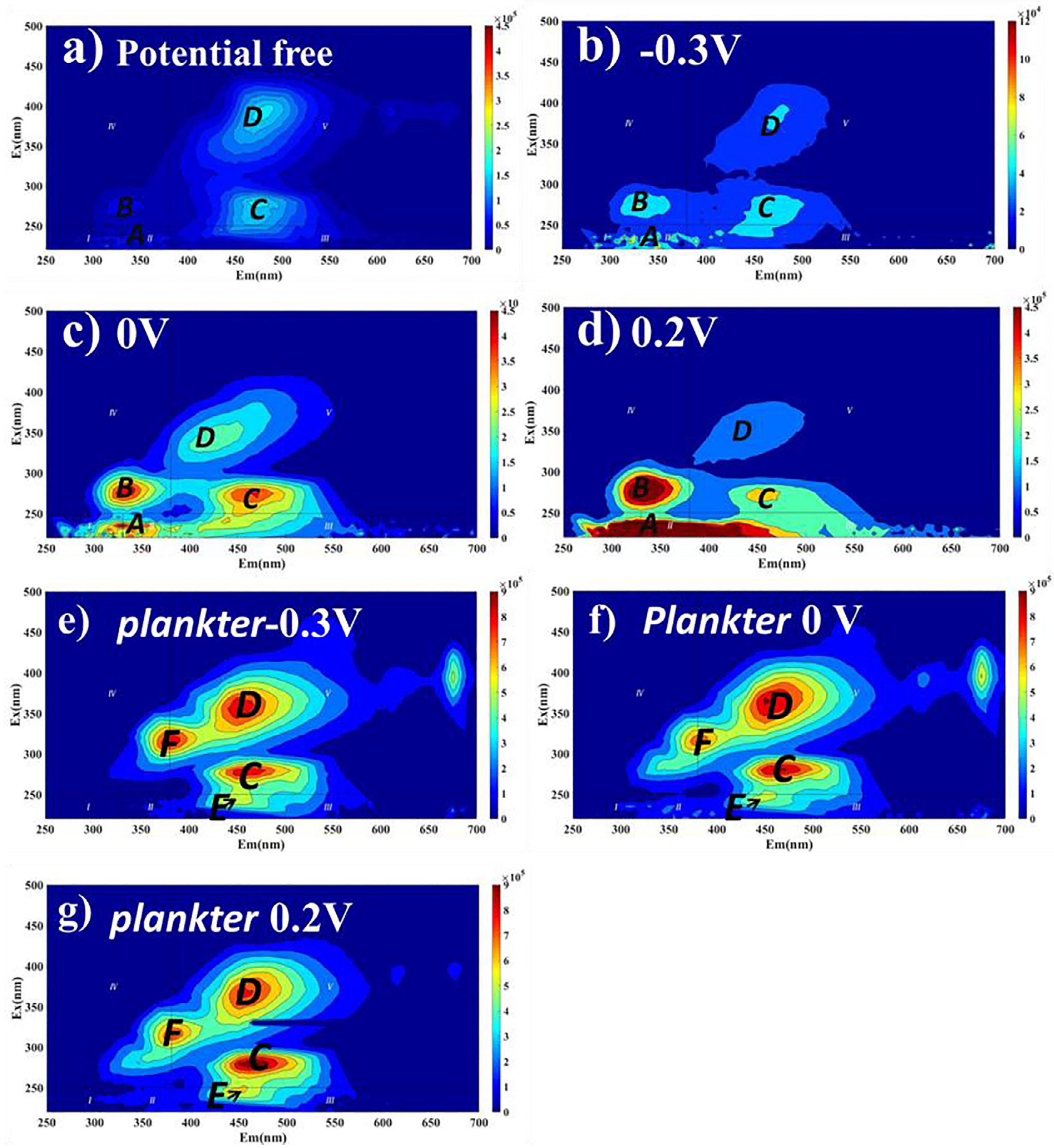

Fig. 7 – The three-dimensional fluorescence of the EPSs produced at potential free (a), -0.3 V, (b) 0 V (c) and 0.2 V (d), and the EPS of plankter cultivated at -0.3 V, (e) 0 V (f) and 0.2 V (g).

presence of tryptophan-like substances in biofilm can induce reductive degradation of MNZ.

2.3.3. *Proposed mechanisms of photoelectrogenic biofilm for MNZ removal*

Based on the obtained results, we proposed the mechanisms of the enhanced MNZ removal by the binary-species photoelectrogenic biofilm of *C. vulgaris* and *R. Palustris* through potential-dependent photosynthetic electron extraction (Fig. 8). The externally applied potential provides a drive force to extract photosynthetic electron within the microbial cell, which simultaneously promotes the photoautotrophic metabolism of *C. vulgaris* and photoheterotrophic metabolism of *R. Palustris*, leading to enhancement in exchange utilization of their respective metabolites and EPS synthesis which enhanced a series of process for MNZ removal including adsorption, biological oxidation/reduction and photosensitive degradation.

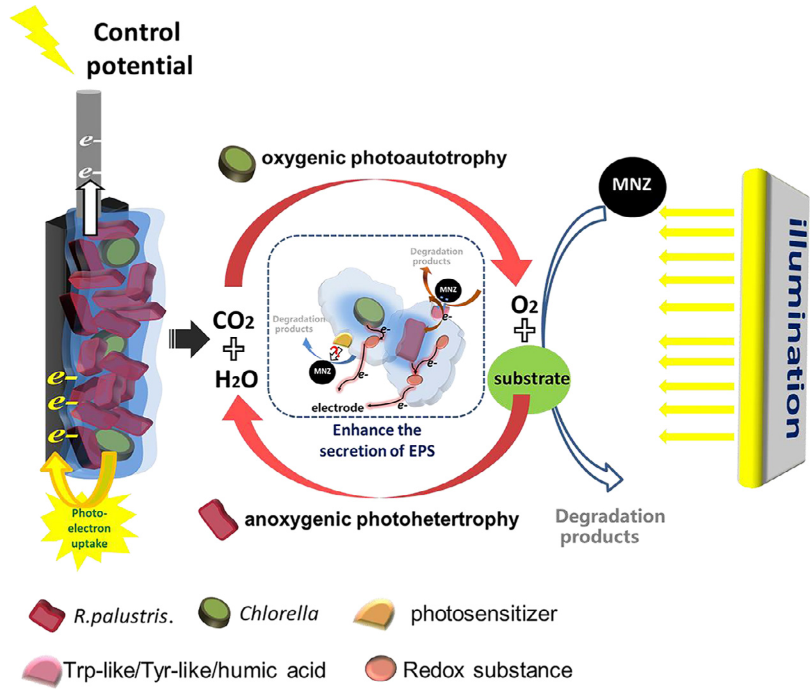

Fig. 8 – The proposed degradation mechanisms of MNZ by the binary-species photoelectrogenic biofilm of *C. vulgaris* and *R. palustris*.

3. Conclusions

Use of a solid matrix with applied potentials for photosynthetic electrons extraction demonstrated an efficient and convenient approach to promote the cooperation between microalgae and anoxygenic photosynthetic bacteria and regulate their EPS metabolism towards enhancing removal of antibiotic from wastewater. The external potential of 0V exhibited the best performance for photosynthetic electrons extraction which significantly promoted the accumulation of tryptophan and humic acid protein, polysaccharide and humic acid-liked photoactive substances in EPS of the binary-species of *C. vulgaris* and *R. Palustris*, leading to multipath removal of MNZ though adsorption, biological oxidation/reduction and photosensitive degradation. These results could contribute to the technical progress of the photosynthetic microorganism-based resourceful treatment of wastewater regarding as highly efficient toxic organic micro-pollutants removal coupling with microbial biomass recovery.

Acknowledgments

The authors thank the financial support provided by the National Natural Science Foundation of China (No. 51108186), the Tip-top Scientific and Technical Innovative Youth Talents of Guangdong Special Support Program, China (No. 2015TQ01Z039) and the Natural Science Research Project of Higher Education Institutions of Guangdong Province (No. 2015KTSCX025).

Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2021.07.016.

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