

Unraveling Biostimulation of Decolorized Mediators for Microbial Fuel Cell-Aided Textile Dye Decontamination

Pei-Lin Yueh, Bor-Yann Chen, Chuan-Chung Hsueh

Abstract—This first-attempt study revealed that decolorized intermediates of azo dyes could act as redox mediators to assist wastewater (WW) decolorization due to enhancement of electron-transport phenomena. Electrochemical impedance spectra indicated that hydroxyl and amino-substituent(s) were functional group(s) as redox-mediator(s). As azo dyes are usually multiple benzene-rings structured, their derived decolorized intermediates are likely to play roles of electron shuttles due to lower barrier of energy gap for electron shuttling. According to cyclic voltammetric profiles, redox mediating characteristics of decolorized intermediates of azo dyes (e.g., RBu171, RR198, RR141, RBk5) were clearly disclosed. With supplementation of biodecolorized metabolites of RR141 and 198, decolorization performance of could be evidently augmented. This study also suggested the optimal modes of microbial fuel cell (MFC)-assisted WW decolorization would be plug-flow or batch mode of operation with no mix. Single chamber-MFCs would be more favourable than double chamber MFCs due to non-mixing contacting reactor scheme for operation.

Keywords—Redox mediators, dye decolorization, bioelectricity generation, microbial fuel cells.

I. INTRODUCTION

REGARDING wastewater treatment, biological decontamination is usually considered as one of top-priority alternative(s) due to its environmental friendliness [1]. Among all means of bioremediation, microbial fuel cell (MFC) was found to be more promising for pollutant degradation as simultaneous wastewater bioremediation and energy recycling can be taken place [2]. Therefore, using MFC as mode of operation, this feasibility study chose reductive decolorization of azo dye(s) as the model reaction for biological decolorization via energy conversion. As known, azo linkage(s) (-N=N-) bearing dyes (i.e., azo dyes) as the most popularly used textile dyes (>70%) in industry, are originally designed to be recalcitrant toward biodegradation. Inevitably, significant amount of residual dyes could be discharged in such industrial effluents to impact the environment. Degradation of residual azo dyes will thus be urgently needed due to suspected mutagenicity of dyes and derived intermediates (e.g., aromatic

amines) [3]. Moreover, as electron-withdrawing azo bonding(s) should be decolorized via reduction, azo dyes should be decomposed via anaerobic biodegradation. In fact, MFCs can extract biomass-based energy via oxidation of organic matter in diverse wastewater (WW) by using anode as the electron acceptor for bioelectricity generation [4]. Due to external circuit to direct electron transfer in MFCs as a driving force, pollutant degradation could be effectively stimulated [5], [6]. That is, MFCs could simultaneously implement WW treatment and bioelectricity generation for sustainable development [2], [6]. In fact, electrochemical characteristics of microorganisms [7] significantly influenced the performance of bioelectricity production in MFC through at least three mechanisms: electron shuttling of cell-secreting mediators (e.g., phenazine, quinones), membrane-associated redox proteins (e.g., mobile electron carriers such as cytochromes), and conductive nanowires (e.g., wired communities of *Geobacter sulfurreducens*, *Shewanella oneidensis*) [7], [8]. To promote bioelectricity-generating capability of MFCs for WW treatment, one of the most intriguing alternatives prevailing recently was exogenous supplementation of electron shuttles (ESs) with low toxicity potency [9]. Recently, decolorized intermediate(s) of azo dye(s) were found to act as electron shuttles in dye-bearing MFC to enhance the performance of simultaneous color removal and power generation [2], [6]. As a matter of fact, additional supplementation of textile dyes and derived intermediate(s) as ESs to wastewater treatment is technically not allowed due to introduction of secondary pollutant and further decontamination cost. As ESs could be generated from dye decolorization, understanding optimal accumulation of such intermediate(s) will be crucial for maximal performance of MFC-assisted WW decolorization. Chen et al. [10] and Hsueh et al. [11] showed that biodegraded intermediates of reactive blue 160 (RBu160) and reactive green 19 (RG19) could express electron-mediating characteristics to enhance power generation and reductive decolorization in MFCs. However, due to commercial interests involved, dyestuffs intermediates are usually not available for public uses, thus single benzene ring and bicyclics-based compounds were chosen herein for feasibility assessment to decipher how ES-laden WW treatment was performed. This comparative study indicated that using MFC as operation strategy could effectively stimulate pollutant degradation in WW treatment due to autocatalysis of electron shuttle(s) generated. Thus, with supplementation of decolorized metabolites (DMs) of RG19 and RBu160 by *Enterobacter cancerogenus* BYm30 and by

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Proteus hauseri ZMd44, respectively to electrochemically active bacteria (e.g., *Shewanella* sp. WLP72)-seeded MFCs, the stimulating effects upon dye decolorization and power generation were quantitatively revealed.

To disclose the mysteries of electron-shuttling phenomena in MFCs, six model mediators and two types of azo dyes (i.e., naphthol type (RG19) and non-naphthol type (RBu160)) and decolorized intermediates were used for comparison. This study also conducted electrochemical inspections upon such compounds and explored the interactive effect of different mediators on power generation and reductive decolorization in MFCs. Moreover, this work revealed that the formation of decolorized intermediates and $-OH$ and $-NH_2$ -containing chemicals could act as ESs for WW decolorization in MFCs. According to [12], no mixing-contacting pattern in reactor operation is the most favorable for irreversible reactions in series (i.e., bacterial decolorization). That is, MFCs in batch or plug flow-type reactor apparently are optimal modes of operation for WW decolorization due to effective accumulation of decolorized intermediate(s) to stimulate WW decolorization.

II. MATERIALS AND METHODS

A. Chemicals, Bacterial Strains and Culture Conditions

The model redox mediators including 2-aminophenol (2AP), 4-aminophenol (4AP), benzene-1,2-diol (B12d), benzene-1,4-diol (B14d), 1-amino-2-naphthol (1A2N) and 1-amino-4-naphthol (1A4N) were purchased from Sigma-Aldrich Inc. Seawater-cultured *Shewanella* sp. WLP72, *Exiguobacterium* sp. K2, freshwater-originated *Proteus hauseri* ZMd44, and *Enterobacter cancerogenus* BYm30 isolated from northeast Taiwan were used for study. Bacteria were cultured in Luria-Bertani (LB) broth medium with tap water (or deep seawater if marine bacteria were used). Decolorization experiments were carried out as follows: first, one loopful of colony of streak plates was inoculated for 12 h preculture in 50-mL LB broth at 30°C, 125 rpm using a water-bath shaker and then 1% (v/v) pre-cultured broth was used for dye-bearing cultures. After 6 h aerobic cultures, decolorization was conducted in static incubation to prevent introduction of air through shaking.

B. Double Chamber-Microbial Fuel Cell (DC-MFC)

DC-MFC construction and inoculation had been described elsewhere [13]. Seed bacteria of DC-MFCs were inoculated after the cells achieved the stationary phase. DC-MFCs used in the study comprised of two cuboid chambers made by transparent poly-acrylic plastic (i.e., Length \times Width \times Height = 11 \times 9 \times 2 cm). The working volume for each chamber is 200 mL and two compartments were separated by proton exchange membrane (Nafion 211; American Du Pont Inc.). The electrodes and membrane were pre-treated before use. The pre-cultured cells were centrifuged and re-suspended with 200 mL sterilized medium. Potassium hexacyanoferrate (III) together with 0.5 M KH_2PO_4 buffer were used as an electron acceptor in the cathode compartment. Prior to construction of MFC, 3.0% H_2O_2 was completely filled in the chamber for

1 day "static" sterilization [14]. Then, H_2O_2 was completely rinsed and washed out by sterilized deionized-and-distilled water after 1 day static incubation prior to study. For cell propagation in MFCs, cell culture of bacterial strain (e.g., WLP72) was first carried out as described elsewhere [14].

C. Electrochemical Measurements

- Electrochemical Impedance Spectroscopy (EIS) (HIOKI 3522-50, Japan) measurement was carried out via steady-state open circuit potential distributed with amplitude of 10 mV. The frequency range was 104 to 5×10^{-2} Hz. Data was collected and analyzed using the software for Nyquist plot (Zview 2.6b, Jiehan Tech.) [15].
- Power Generation Measurement: Cell voltage was automatically measured (set at one data point per minute) using a data acquisition system (DAS 5020; Jiehan Technology Corporation) through external resistance Rout = 1 K Ω . Note that a relatively high resistance (1000 ohms) was intentionally used in order to compare with prior results [16], [17]. The power densities (P) and current densities (I) of MFCs were determined using linear sweep voltammetry (LSV) measurement and the corresponding voltages were recorded using a multimeter. The power density (P) and current density (I) were calculated by the formulae and, respectively, where V is the voltage across the external resistor, R is the resistance of each external resistor, and A is the surface area of the anode. All MFCs were operated at 25 °C.
- Cyclic Voltammetry of different model intermediates was performed using an electrochemical workstation (Jiehan 5600, Taiwan) at 1 mV s $^{-1}$ scan rate. The working, counter, and reference electrodes were a glassy carbon electrode (0.07 cm 2), platinum electrode (6.08 cm 2), and a Hg/Hg $2Cl_2$ electrode filled with saturated KCl(aq), respectively. The glassy carbon electrode (GCE, ID = 3 mm; model CHI104, CH Instruments Inc., USA) was successively polished with 0.05 μ m alumina polish and then rinsed with 0.5 M H_2SO_4 and deionized water before use. The experiments were performed in phosphate buffer solutions (pH = 7.0) at 0.1 M and the solutions were purged with nitrogen for 15 min prior to analysis. The scanning rate was 1 mV s $^{-1}$ over the range from 0.4 to -0.6 V [8]. The redox potentials recorded as Hg/Hg $2Cl_2$ reference electrode were corrected by 0.241 V (i.e., E 0 of Hg/Hg $2Cl_2$) to the standard hydrogen electrode (SHE).

D. Decolorization Profile Analysis

The model azo dyes- reactive blue 171 (RBu171), reactive red 198 (RR198), reactive red 141 (RR141), reactive black 5 (RBk5) (purchased from Everlight Chemical Ltd., Taipei, Taiwan) were used to evaluate color removal capability of *Shewanella* sp. WLP72. A loopful of seed colony in streak culture was taken for cell culture and dye decolorization in shake-flask cultures. The experiments were implemented at 200 mg L $^{-1}$ RBu 171, RR198, RR141 or RBk5 bearing LB medium with supplemented decolorized intermediates of RR141 and RR198 at 30°C, 125 rpm [18]. Specific growth rate

(SGR) and specific decolorization rate (SDR) were determined via time-series profiles of microbial growth and dye decolorization [19] as described in [6].

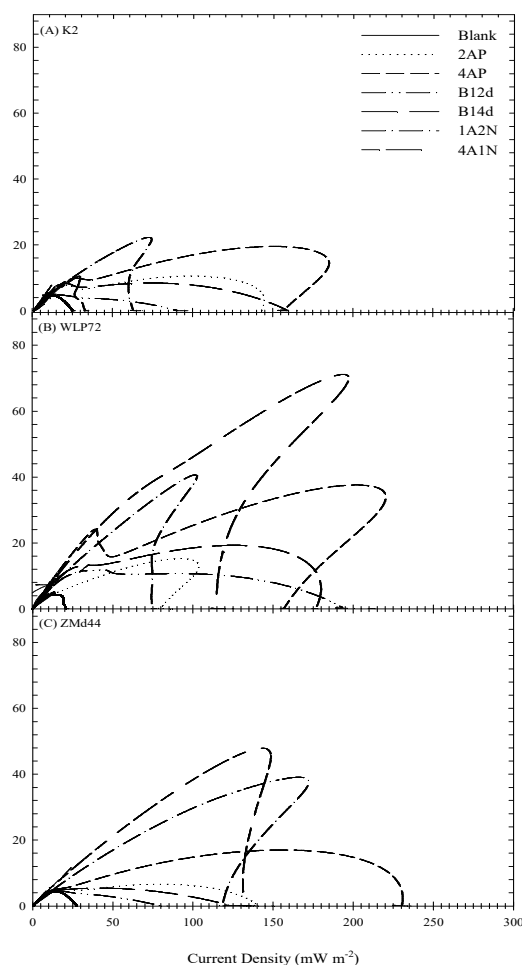


Fig. 1 Comparison upon power density profiles of (A) *Exiguobacterium* sp. K2, (B) *Shewanella* sp. WLP72, and (C) *Proteus hauseri* ZMd44-seeded DC-MFCs using different model intermediates at 40 mg L⁻¹

III. RESULTS AND DISCUSSION

A. Effects of Supplemented Ess

Prior studies indicated that decolorized intermediate(s) of azo dyes via microbial decolorization could act as ESSs[9], [16], [20], [21]. These test ESSs included mono-benzene ring based 2AP and isomeric 4AP, and bicyclics 1A2N and isomeric 4A1N generated from Acid Orange 20 (Orange I; OI), Acid Orange 7 (Orange II; OII), respectively (B12d, B14d as controls). These could effectively augment efficiency of bioelectricity generation in MFCs. However, due to suspected biotoxicity potency and effects of chemical structures of such ESSs, bioelectricity-generating capabilities of electrochemically active microbes (EAMs; e.g., nanowire-generating bacteria *Shewanella* sp. WLP72-seeded MFC, *Exiguobacterium* sp. K2, non-nanowire-generating bacterium *Proteus hauseri* ZMd44)

might be essentially reduced. Thus, this feasibility study conducted quantitative assessment upon such ESSs at 40 mg L⁻¹ to reveal whether such intermediates could stimulate power-generating capabilities in ZMd44-seeded MFCs. As Fig. 1 indicated, for *Shewanella* sp. WLP72-seeded DC-MFC with supplementation of various ESSs at 40 mg L⁻¹, power density increased from 4.43 mW/m² (Blank) to 15.26 mW/m² (increased 344% for 2AP), 37.59 mW/m² (increased 848% for 4AP), 15.86 mW/m² (increased 358% for B12d), 19.27 mW/m² (i.e., 434% increase for B14d), 40.65 mW/m² (ca. increase 9 fold for 1A2N) and 71.17 mW/m² (ca. increase 16 fold for 4A1N). Using different bacteria-seeded MFCs, the rankings of power-densities of different ESSs were showed as follows:

- *Exiguobacterium* sp. K2 (unit: mW/m²): Blank (4.62) < b12d (5.13) < b14d (8.42) < 4A1N (10.47) < 2AP (10.60) < 4AP (19.54) < 1A2N (22.28);
- *Proteus hauseri* ZMd44 (unit: mW/m²): Blank (4.35) < b12d (4.60) < b14d (5.39) < 2AP (6.61) < 4AP (16.97) < 1A2N (39.09) < 4A1N (47.94).

Note that significant reduction of power density after addition of 4A1N was associated to its biotoxicity potency to probing microbes, leading to inhibitory suppression onto power generation (data not shown). These showed that supplementation of ESSs below threshold levels of biotoxicity apparently would not resist electron transfer characteristics for promising power-generating capabilities. These comparisons also revealed that bicyclics or multiple benzene rings-based intermediate(s) (e.g., 1A2N and 4A1N) at non-toxic levels seemed to be more promising as ESSs to stimulate electron transfer performance. In addition, since azo dyes used in textile dyeing industry are complicated structures containing several benzene-rings, generated intermediate(s) are suspected to be more efficient ESSs to stimulate WW decolorization. That is, using MFC as operation strategy for wastewater decolorization could effectively augment treatment efficiency due to accumulated azo dyes and generated intermediate(s). In particular, autocatalysis of pollutant(s) and intermediate(s) could efficiently assist redox decontamination due to stimulation of electron transfer efficiency. These also suggest that using energy-recycling electrochemical methods (e.g., MFCs) to degrade dye-bearing WW seemed to be cost-effective.

B. Assessment Upon Model Ess

As aforementioned, mono-benzene ring-based 2AP, 4AP and bicyclics derived decolorized intermediates 1A2N, 4A1N and B12d, B14d all were confirmed to have electron-shuttling capabilities. Hydroxyl (-OH) and amino (-NH₂) substituent(s) were confirmed to be candidate functional group(s) for electron-shuttling [9], [16], [21]. In particular, cyclic voltammetric profiles indicated that *para*-substituent structures (e.g., 4AP, 4A1N, B14d) owned stronger redox capabilities than their corresponding isomers (e.g., 2AP, 1A2N, B12d) [16], [20], [21]. Therefore, it was suspected that *para*-isomer(s) should be more promising to be ESS(s) than other *ortho*-isomeric compound(s). The detailed reasonings were stated as follows:

(a) HOMO-LUMO analysis: According to HOMO-LUMO

theory [9], [20], [22] bicyclics-structured compounds would have lower barrier of electron energy gap and activation energy to effectively trigger electron transfer, thereby showing larger absorption wavelengths (ca. 338, 335 nm for 1A2N, 4A1N, respectively). Bicyclics aromatics 1A2N, 4A1N have one more benzene ring than 2AP, 4AP; thus, the rankings of maximal absorption wavelength in UV-Vis spectra were 1A2N(338nm) \approx 4A1N(335nm) > 2AP(295nm) \approx 4AP(284nm). According to Planck-Einstein relation: $E = \frac{hc}{\lambda} = h\nu$, it was concluded that higher wavelengths for light absorption of bicyclics structure could significantly reduce energy requirement to overcome such energy gaps. That is, the most promising ES of this study would be bicyclics 1A2N and 4A1N. This also indirectly explained why several benzene ring-related compounds (e.g., methylene blue, riboflavin, 2-hydroxy-1,4-naphthoquinone (HNQ)) were listed as ESs [23]. However, when the number of benzene-rings of an organic chemical increased (i.e., more hydrophobic due to increased organic portion), its solubility would evidently decrease to resist electron-transfer in aqueous phase. That is, when such a chemical was considered as candidate ES, not only the problem of toxicity potency, but also the solubility should be first considered to overcome.

- (b) Steric effects of ESs: As [24] indicated, the presence of electron-shuttling group(s) near azo bond(s) could assist azo dyes to be in high electrophilicity favorable to reductive decolorization. Since the *ortho* substituent caused steric hindrance in the proximity of azo linkage(s), azo dyes with *para* substituent could be more thermodynamically favorable than *ortho* substituent for azo reduction. Moreover, [15] pointed out that bacterial decolorization and bioelectricity generation are both competitive to each other for electron transfer in MFCs. That is, decolorized intermediates (e.g., aromatic amines) also could express similar *ortho* or *para* effect of redox-mediating in either bioelectricity generation or color removal. These also suggested that *para*-substituent is thermodynamically favorable to *ortho*-substituent for electron shuttling.
- (c) Toxicity potency comparison: As low solubility of chemicals with multiple-benzene rings would significantly reduce flux of electron transport due to mass transfer resistance, how to overcome solubility limitation(s) in aqueous media would apparently be critical to practical applicability. Moreover, to increase operation performance in MFCs, toxicity potency of candidate ES to receptor microorganism(s) should be top-priority concern for MFC-assisted bioremediation. For instance, as [16] pointed out, 4AP at 200 mg L⁻¹ seemed to be highly toxic to *Proteus hauseri* ZMd44. However, 4AP at same dosage was not inhibitory to *Aeromonas hydrophila* NIU01 and *Exiguobacterium* sp. K2 (data not shown). That is, toxicity potency of chemical(s) is apparently bacterial species-dependent likely due to phenotypic diversity of cellular tolerance to counteract hostile environments. This point also supported that 4A1N is toxic to

Exiguobacterium sp. K2, but not to *P. hauseri* ZMd44 (i.e., species-dependent). Of course, biotoxicity would strongly affect biodegradability of chemical(s) to biodegraders as [6] mentioned. Although ESs might be inhibitory to microbial activities, ESs supplemented at non-toxic levels would effectively augment power density to *Shewanella* sp. WLP72, *Proteus* sp. ZMd44 and *Exiguobacterium* sp. K2. As [16] mentioned, 200 mg L⁻¹ 4AP would completely inhibit cell viability of *Proteus* sp. ZMd44. Thus, this study selected 40 mg L⁻¹ as the concentration for comparative study of myriads of ESs.

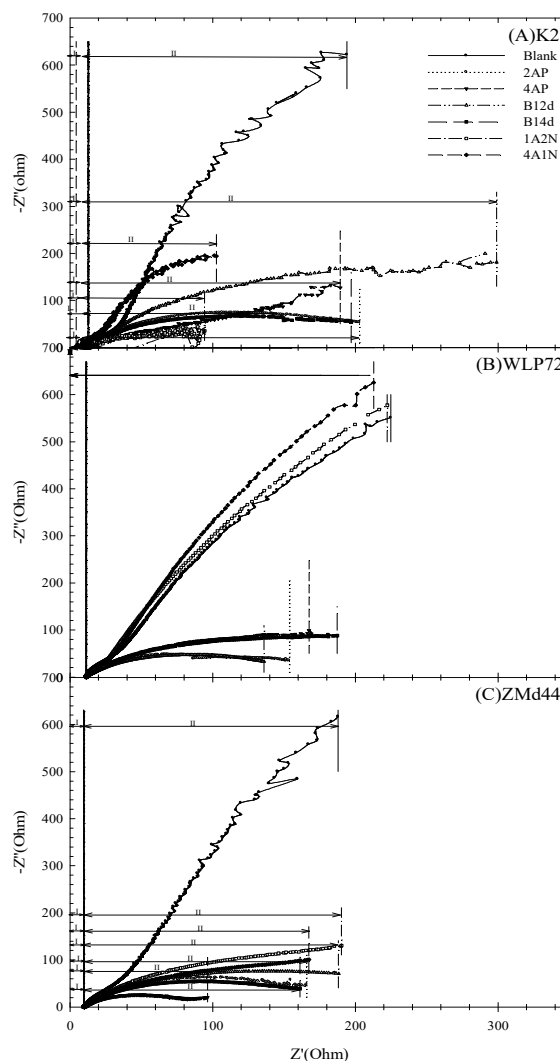


Fig. 2 Nyquist plots of electrochemical impedance spectra by (A) *Exiguobacterium* sp.K2, (B) *Shewanella* sp. WLP72, (C) *Proteus hauseri* ZMd44 DC-MFCs supplemented with various model ESs at 40 mg L⁻¹ (I: Electrolyte Resistance; II: Kinetic and Diffusion Resistance)

C. Electrochemical Impedance Spectra Analysis

As [25] mentioned, electrochemical impedance spectroscopy (EIS) measurements can be used to reveal characteristics of

charge transfer and ion transport in the anode and cathode of fuel cells (e.g., DC-MFCs). Theoretically, EIS deals with the variation of total impedance in a complex plane (i.e., Nyquist plot). When the internal resistance of MFC gradually decreased, Nyquist plot with impedance vector ($\text{Re } Z$ vs. $-\text{Im } Z$) would progressively show a well-defined semicircle with gradually-decreased radius with supplementation of model ESs (Fig. 2). In addition, decreases of semi-circle radii in Nyquist plot in EIS indicated significant reduction of internal resistance for power generation. These were all in parallel with increases in power-generating capabilities as aforementioned. However, to grasp more conclusive remarks of MFC-assisted bioremediation, detailed mechanism of electron transfer and mass transport phenomena in EIS (e.g., ohmic losses, anode activation losses, cathode activation losses) should be disclosed in follow-up investigations for system optimization [9].

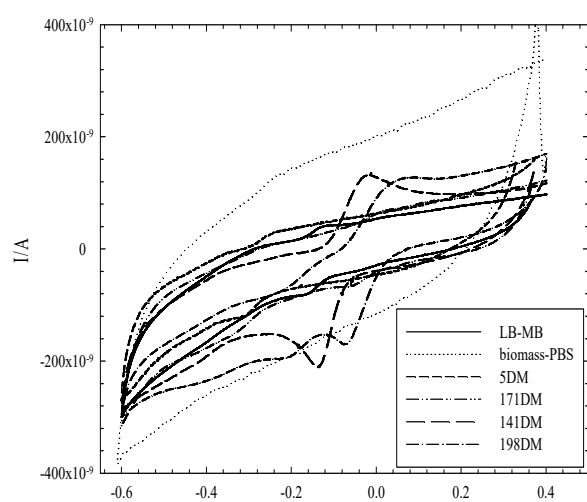


Fig. 3 Cyclic voltammetric profiles of decolorized intermediates of azo dyes RBk5 (5DM), RBU171 (171DM), RR141 (141DM), RR198 (198DM) (LB-MB and Biomass+PBS denoted cultured LB medium broth and cultured *Shewanella* sp. WLP72 in PBS solution, respectively) (Scan rate 1 mV s^{-1})

D. Cyclic Voltammetric Evaluation

As studies [16], [20], [21] revealed, model intermediates 2AP, 4AP, 1A2N, 4A1N, B12d, B14d and decolorized intermediates of azo dyes RBu160, OI, OII could act as ESs to assist electron-transfer capabilities in MFCs. In particular, bicyclics 1A2N and 4A1N could have higher electron-mediating capabilities than others. That is, if azo dyes can be decolorized to be bicyclics or higher-cyclics intermediates in soluble forms, these intermediates could be effective stimulating agents for color removal. As indicated in cyclic voltammetric profiles of some azo dyes RBu171, RR198, RR141, RBk5 (Fig. 3), significant redox potential peaks were revealed at the oxidation peak potential (E^{ox}) and reduction peak potential (E^{red}) of ca. -0.1769V , -0.0129V for RR141 (top one significant) and at ca. -0.1079V , 0.05014V for RR198 (rank two significant), respectively. Although less significant E^{ox} and E^{red} of intermediates of RBu171 and RBk5

were likely due to not at optimal dosages, electron-shuttling characteristics of decolorized intermediates might still be anticipated.

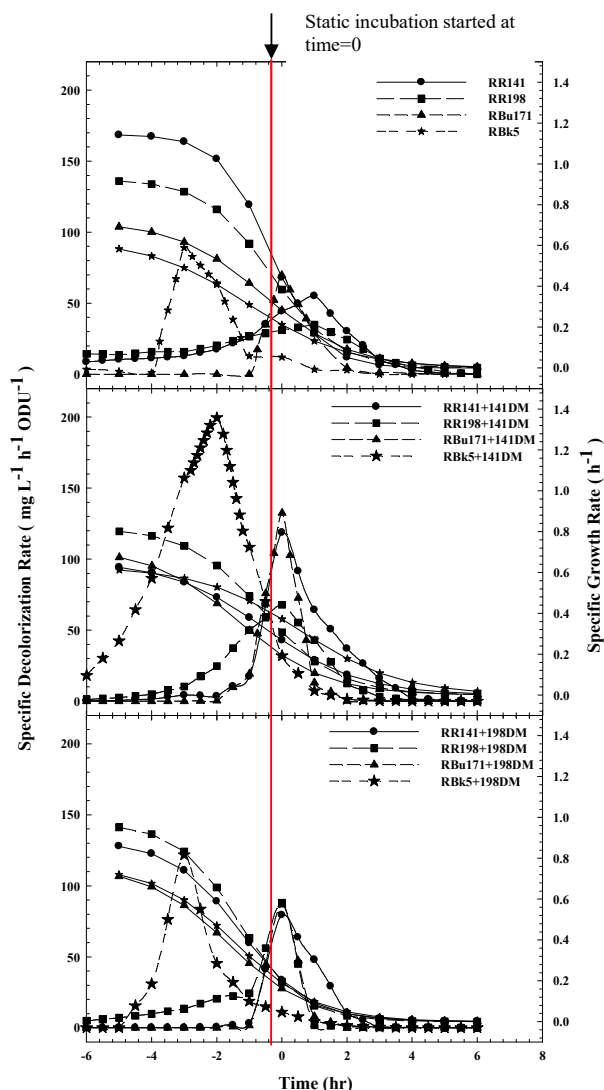


Fig. 4 Decolorization performance comparison of supplementation of decolorized metabolites of RR141 (141 DM) and RR198 (198DM)

E. Decolorization Performance Analysis

As Chen et al. [2] revealed, decolorized intermediates were suspected to enhance performance of bioelectricity generation and reductive decolorization. As aforementioned in CV profiles, DMs of RR141 and RR198 were more promising to express electron-shuttling capabilities, these decolorized intermediates were supplemented at 100 mg L^{-1} to different dye-bearing cultures for confirmation of such phenomena. As shown in Fig. 4 (time-course data not shown), the findings directly supported that these decolorized intermediates were ESs. The rankings of maximal specific decolorization rates of such DM-stimulating tests (unit: $\text{mg L}^{-1} \text{h}^{-1} \text{ODU}^{-1}$) were $\text{RBk5}+141\text{DM} (199.49) > \text{RBU171}+141\text{DM} (132.5) > \text{RBk5}+198\text{DM} (121.7) > \text{RR141}+141\text{DM} (118.8) > \text{RBk5}$

(89.12) > RR198+198DM (88.00) > RBu171+198DM (86.81) > RR141+198DM (79.44) > RBu171 (69.70) > RR198+141DM (67.90) > RR141(blank) (55.00) > RR198(blank) (34.96) (Fig. 4), indicating that decolorized intermediates of RR141 were evidently more promising ESs to enhance electron transfer assisted decolorization of textile dyes (RBu171, RR198, RR141, RBk5). In fact, decolorization performance was significantly increased ca. 120% to 250% (Table I), suggesting that augmentation of decolorized metabolites was crucial to optimization of WW decolorization.

TABLE I
COMPARATIVE LIST OF MAXIMAL SPECIFIC DECOLORIZATION RATES (SDRS) OF RR141, RR198, RBu171, RBk5 WITH (w/) AND WITHOUT (w/o) SUPPLEMENTATION (SUPPLE.) OF DM OBTAINED FROM 100 MG L⁻¹ RR141 AND RR198*

Azo dye	w/o supple.	w/ supple (141DM) (increased %)	w/ supple (198DM) (increased %)
RR141	55.00	118.8 (216.0%)	79.44(144.4%)
RR198	34.96	67.90 (194.2%)	88.00(251.7%)
RBu171	69.70	132.5 (190.1%)	86.81(124.5%)
RBk5	89.12	199.5 (223.9%)	121.7(136.6%)

*unit: mg L⁻¹ h⁻¹ ODU⁻¹

F. Optimal Strategy of MFC Operation

According to [12], for irreversible reactions in series (i.e., reductive decolorization as stated herein) “the maximal production (or accumulation) of intermediates can be achieved if fluids of different compositions and at different stages of conversion are not allowed to mix”. That is, plug flow reactor and batch modes of operation without mixing would be best to significant accumulation of intermediates. Regarding MFC mode of operation, single chamber MFCs (SC-MFCs) seemed to be more promising to double chamber MFCs (DC-MFCs) due to non-mixing contacting patterns of concentration gradient for proton diffusion in SC-MFCs.

IV. CONCLUSION

Supplementation of decolorized intermediates (DIs) of azo dyes apparently stimulated the performance of WW decolorization. As azo dyes are usually in complicated structures, reduced intermediates were very likely to be ESs due to much lower energy barriers for electron transport (ET). Electrochemical activities of DIs directly affected ET-associated bioelectricity generation and reductive decolorization. In addition, using MFC with no mix (i.e., SC-MFCs) as operation strategy would be very promising to reductive decolorization for dye-laden WW treatment due to significant accumulation of decolorized intermediates for enhancement of ESs.

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