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The role of riboflavin in decolourisation of Congo red and bioelectricity production using *Shewanella oneidensis*-MR1 under MFC and non-MFC conditions

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Abstract:

Dissimilatory metal reducing bacteria can exchange electrons extracellularly and hold great promise for their use in simultaneous wastewater treatment and electricity production. This study investigated the role of riboflavin, an electron carrier, in the decolourisation of Congo red in microbial fuel cells (MFCs) using *Shewanella oneidensis* MR-1 as a model organism. The contribution of the membrane-bound protein MtrC to the decolourisation process was also investigated. Within the range of riboflavin concentrations tested, 20 µM was found to be the best with >95% of the dye (initial concentration 200 mg/L) decolourised in MFCs within 50 hours compared to 90% in the case where no riboflavin was added. The corresponding maximum power density was 45 mW/m^2. There was no significant difference in the overall decolourisation efficiencies of *Shewanela oneidensis* MR-1 ΔMtrC mutants compared to the wild type. However, in terms of power production the mutant produced more power (P_{max} 76 mW/m^2) compared to the wild type (P_{max} 46 mW/m^2) which was attributed to higher levels of riboflavin secreted in solution. Decolourisation efficiencies in non-MFC systems (anaerobic bottles) were similar to those under MFC systems indicating that electricity generation in MFCs does not impair dye

decolourisation efficiencies. The results suggest that riboflavin enhances both decolourisation of dyes and simultaneous electricity production in MFCs.

Key words: Electron shuttling, MtrC, *Shewanella*, dye decolourisation, energy production, riboflavin

Introduction

Azo dyes have been one of many synthetic dyes used in the textile industry. To this day, more azo dyes are synthesized, not only for colouring purposes but also for biological-medical imaging and other optical applications (Mohammadi et al., 2015). Research focusing on azo decolourisation has always been in demand, focusing on novel ways to degrade this dye, the danger always lying in the azo group (–N=N-). Methods may depend on physicochemical removal such as adsorption on magnetic biocomposites (Sivashankar et al., 2015) or could rely on biological removal depending on enzyme induction such as peroxidases, azo reductases etc. Biological removal is considered more favourable for complete degradation of azo compounds (Imran et al., 2016).

Biologically, the azo bond is thought to be cleaved under anaerobic conditions leading to decolourisation but the mechanism is not clear. Since azo dyes are large and usually charged, they are likely to be reduced extracellularly. It has been suggested that the decolourisation process is a fortuitous one where azo dyes might act as an electron acceptor supplied by carriers of the electron transport chain. There are also suggestions that decolourisation is due to non-specific extracellular reactions occurring between reduced compounds of anaerobic metabolism e.g. sulphides and the azo dyes. Others suggest that anaerobic reductive cleavage of the azo bond

is aided by azoreductases, the electron shuttling being aided by soluble redox mediators e.g. flavins (Fernando 2014; Saratale et al., 2011)

Rau *et al*., 2002 observed enhanced anaerobic reduction of azo dyes using *Escherichia coli* in presence of quinoid redox mediators e.g. Anthraquinone -2-sulphonate and lawsone and attributed this to induction of azoreductases. High concentrations though of lawsone (> 200 µM) did not increase reduction rate possibly due to toxicity.

The use of microbial fuel cells (MFCs) has been considered a promising technology for both decolourisation of azo dyes and electricity production (Hou et al., 2011, Pandey et al., 2016). MFCs utilise electrochemically active bacteria to catalyse an oxidation reaction at the anode. Electrons generated are channelled via an external circuit to a cathode electrode where they recombine with protons and a terminal electron acceptor e.g. oxygen. The extracellular reduction of azo moieties in the anode chamber is likely to occur using electrons coming from a co-substrate e.g. glucose. Furthermore, the ability of MFCs to use oxygen or ferricyanide indirectly as the terminal electron acceptor also confers MFCs an additional advantage over conventional anaerobic systems in terms of faster microbial metabolic rates and growth rates that could potentially be beneficial in achieving faster azo dye degradation kinetics in MFC anodes.

Different bacteria have the ability to degrade azo dyes, among which are those belonging to family *Shewanellaceae* which is considered a model bacteria for studying electron transfer (Carmona-Martinez et al., 2011). The electron transport process in *Shewanella* is thought to be through one of two mechanisms: direct electron transfer which depends on the direct contact and facilitated electron transfer which usually takes place via electron shuttles or mediators (Chen et al., 2013). Direct electron transfer from the cytoplasm to an electron acceptor (e.g. azo dye) is

thought to be aided by a number of multi-heme cytochromes (CymA, MtrA, MtrB, MtrC, OmcA) that span the inner membrane, periplasm and outer membrane. Electron shuttles are low molecular weight organic molecules that are chemically stable, can easily diffuse into the medium to pass on the electron to external electron acceptors and thus catalyse the reduction/oxidation reactions (Velasquez-Orta et al., 2010). Some studies have suggested that 75% of the total electron transfer in *Shewanella* is due to shuttling by Flavin-based redox mediators (Kotloski and Gralnick, 2013) leaving 25% for direct electron transfer.

There are different applications for electron shuttling in biotechnology, those include azo dye reduction, enhancing electricity production in MFCs as well as halogenated organic compound degradation. The electron shuttling molecules are usually derivatives of the following compounds: Cobalamin, Phenazine, Naphthoquinone, Anthraquinone and Viologen (Watanaeb et al., 2009)

One of the electron shuttle compounds is riboflavin, which is secreted by *Shewanella oneidensis* MR-1 and is confirmed as an electron shuttle compound (Marsili et al., 2008). Flavins in general play different roles for *Shewanella*; they were reported to be released to act, not only as growth accelerators but also as electron shuttles during Fe (III) oxides reduction (von Canstein et al., 2008). Riboflavin is a commonly known redox mediator (Hsueh et al., 2014) and its presence in *Shewanella* biofilm was correlated to its electron transfer ability (Marsili et al., 2008).

It would be expected that supplementation of *Shewanella oneidensis* cells in MFCs with Riboflavin would aid decolourisation. However, the situation in an MFC anode chamber may be complicated by the presence of an anode which can also accept electrons depending on its redox potential relative to that of the dye / electron donor. Co-substrate conversion in an MFC is

however, expected to be higher than in typical anaerobic systems as oxygen (or ferricyanide) is used indirectly (in the cathode) as a terminal electron acceptor. It would also be expected that a ΔMtrC mutant of *Shewanella oneidensis* would not decolourise azo dyes to the same extent as the wild type strain because the direct electron transfer mechanism would not be in operation.

The aim of the present work was to assess the role of riboflavin in azo dye decolourisation as the main dominant form of electron shuttling in *Shewanella oneidensis* MR-1 and to determine whether the decolourisation process is competitive with electricity production. The contribution of the MtrC cytochrome in the decolourisation process was also investigated.

Material and Methods

Chemicals used

Congo red (C.I Direct red 28), riboflavin and chemicals used for GC (purity ≥ 96%) were purchased from Sigma Aldrich, UK. For the chemical oxygen demand (COD) test, Ficodox Plus^{TM} mixed reagent was purchased from Fisher Scientific UK.

Microorganisms and media used

Microbial cultures used in this study were *Shewanella oneidensis* MR-1 (ATCC 700550) and *Shewanella oneidensis* MR-1 Δ*mtrC*; the latter was kindly provided by Marcus Edwards, School of Biological Sciences, University of East Anglia.

A loopful of strain seed was pre-cultured in 50 ml of LB media (Sigma Aldrich) overnight at 37 ^{o}C and 150 rpm. Then 20% (v/v) of the pre-cultured broth was inoculated into an MFC system for cell propagation to stimulate dye decolourisation and/or bioelectricity generation.

Congo red decolourisation (50, 100, 200 and 400 mg/l), riboflavin addition (10, 20, 30, 40 µM), use of 10 mM of different inhibitors (sodium nitrate, copper sulphate, sodium azide and ferrous sulphate) and cell entrapment in alginate beads were all performed in 20 ml bottles containing 5 ml LB media. Cultures were incubated at 30 ^{o}C overnight after purging with nitrogen gas for 10 minutes through 0.22 µm pore size diameter filter. The used inoculum was 20%v/v.

OD and Riboflavin concentration measurement

The bacterial growth was monitored by measuring OD at 600 nm. Riboflavin concentration was performed according to Xu et al., (2015) by measuring the absorbance at 444 nm

Decolourisation in MFC systems

MFC systems were H-type two chambered system with two identical Duran bottles held together with external metal clamps. The electrodes were made of carbon fibre and were cut to 4 cm x 4 cm. The anode and the cathode compartments were separated with a cation-exchange membrane CMI-7000 (Membranes International USA). The anaerobic anode compartment contained 200 ml working volume was purged with nitrogen gas for 10 minutes through 0.22 µm pore size diameter filter prior to inoculation, the media used was MSM prepared according to Fernando et al., 2012, which contained the following (g/L): NH_4Cl 0.46, KCl 0.225, $MgSO_4.7H_2O$ 0.117, NaH_2PO_4 2.5, Na_2HPO_4 4.11, $(NH_4)_2SO_4$ 0.225, a vitamin mixture and trace mineral solution was added (1%), 500 mg/L casein hydrolysate and 2.2 g/L sodium pyruvate were also added. Air- saturated cathode contained 200 ml working volume of 0.1 M potassium ferricyanide in 50 mM sodium phosphate buffer (pH 7). MFC systems were incubated at 30 ^{o}C in a Stuart 160 Incubator (Fisher Scientific UK). Congo red (200 mg/l) was added to the anodic chamber for all experiments, riboflavin concentrations used for the first experiment were in the following

concentrations: 10, 20 and 40 µM. *Shewanella oneidensis* MR-1 was used to inoculate the first experiment. As for the second experiment, 4 MFC bottles were used, the first was inoculated with *Shewanella oneidensis* MR-1, the second contained *Shewanella oneidensis* MR-1 and 40 µM riboflavin, the third was inoculated with *Shewanella oneidensis* MR-1 Δ*mtrC* and the fourth (control) *Shewanella oneidensis* MR-1 and contained no dye. External resistance for all experiments was 1000 Ω.

Electrochemical measurements

Voltage output data was collected using a Picolog ADC-24 (Pico Technology, UK) online data logging system. Polarization tests were done by connecting different values of external resistances once voltage had stabilized.

COD tests

The chemical oxygen demand removal was determined using the closed reflux titrimetric method as described by Westwood (2007). COD was calculated as follows:

$$COD\ (mg/l) = (V_b - V_s) * DF * M * 3200$$

Where V_b was the ferrous ammonium sulphate titrant of the blank, Vs was the ferrous ammonium sulphate titrant of the sample, DF was the dilution factor, M was the molarity of the ferrous ammonium sulphate titrant.

The percentage of COD removal was calculated as follows:

$$COD\ removal\ (\%) = [COD_I - COD_T / COD_I\ ] * 100$$

where COD_I and COD_T were the initial and final calculated COD values, respectively.

Detection of by-products using Gas Chromatography

To detect the by-products remaining at the end of the MFC operation, samples were withdrawn from each system centrifuged at 14000 rpm for 15 min, filtered and placed in a 2 ml glass vial tube for metabolite detection using a Varian 3900 GC system. The mobile phase consisted of a carrier gas (helium) with a flow rate of 2 mL/min; the column was a 30 mm length x 0.320 mm diameter x 0.50 µm film- HP INNOWax (Agilent J&W GC columns), injector temperature was 260^{o}C. The oven was initially set at 35 ^{o}C for 5 min and then ramped up to 170 ^{o}C for the subsequent 10 minutes. Detector temperature was 250 ^{o}C. The presence of degradation metabolites ethanol, acetic acid and butyric acid was detected using the retention time of the respective standard compounds.

Decolourisation and UV-visible scans

Decolourisation of Congo red dye was calculated as follows:

Dye decolourisation (%) = $(A_I-A_F)/A_I$ x 100

where A_I is the absorbance of the dye at zero time, A_F is the absorbance of the dye at the end of the operation process. The absorption maxima of Congo Red was 497 nm. UV-Visible scans for the samples before and after decolourisation were performed using Perkin-Elmer Lambda-35 UV-Visible spectrophotometer; scans were performed within a range from 280-700 nm. Data was collected using Perkin-Elmer UV Winlab 6.0.4.

Toxicity assay

The confocal microscope was used to study the impact of using riboflavin to enhance the degradation of Congo red. A VL-17A cell line was used (HepG2 cells), About 250 µl was added to wells in a 6 well plate. DMEM media with 10% FCS (3 ml) was added to each well and incubated at 37^{o}C overnight. The media was removed and replaced with 100 µl of the following samples: 1) dye before decolourisation, 2) dye after decolourisation, and 3) positive control cells. Samples were left to incubate overnight, after which, 300 µl of freshly prepared Calcein fluorescent dye dissolved in DMSO (excitation and emission wavelength of 495/515 nm, respectively) was added and the plate was left to incubate in darkness for another 45 min at 37^{o}C. Samples were then studied using confocal scanning laser microscope (CSML). Images were acquired by sequential sequencing using Leica TCS SP2 confocal system (Leica Microsystems, Milton Keynes, UK) and a X63 ceramic dipping objective at 1024x1024 format and scanning speed of 400 Hz with a line average of 2.

Statistical analysis

All experimental data indicated on the graphs and table are the means of duplicate experiments unless otherwise stated and the error bars in the graphs represent the standard deviation of the mean (SD). Statistical analysis of data was conducted by one way analysis of variance (ANOVA) using Microsoft Excel statistics package.

Results

Effect of riboflavin concentration on decolourisation and current production

Different amounts of riboflavin were added to *Shewanella oneidensis* MR-1 culture supplemented with 200 mg/l Congo red in anaerobic serum bottles. The relationship between decolourization and growth with the different concentrations is illustrated in Figure 1. The figure

shows that both decolourisation of Congo red dye increased with the increase in riboflavin concentration; there were no changes in the decolourisation for concentrations above 40 µM. On the other hand, the addition of riboflavin enhanced the growth profile. As the concentration of riboflavin increased, the OD of bacterial growth showed an increase as well.

Fig. 2a represents the variation of current production with time in response to the addition of different riboflavin concentrations. The Figure shows that the maximal current was produced when 20 µM riboflavin was added to the MFC culture. The decrease in voltage in Figure 2a when 20 µM Riboflavin was supplemented could be due to depletion of easy to use substrate (sodium pyruvate) with the microorganisms starting to use fermentation end products e.g. butyrate. The addition of 40 µM riboflavin resulted in less current production (perhaps due to toxicity of riboflavin an high concentrations) and the minimal current production resulted after adding 10 µM riboflavin to the MFC culture, which was less than that obtained for control (no riboflavin). The same result was obtained for both the voltage and power density curves (Fig 2b&2c), where the addition of 20 µM riboflavin resulted in the highest voltage (510 mV) and power density (45 mW/m^2).

Deducing the pathway for electron transfer for decolourisation

To investigate the mechanism by which *Shewanella oneidensis* MR-1 cells transfer electrons, different approaches were tested and decolourisation was calculated. Fig 3 shows that decolourisation increased from 81% for control (freely suspended cells, no riboflavin or inhibitor) to 96.7% when riboflavin was added to the media. On the other hand, decolourisation decreased when sodium nitrate (46.8%), copper sulphate (43.7%), sodium azide (52%), ferrous

sulphate (60.8%) and when cells were entrapped in sodium alginate beads (32%). Entrapment was used to preclude direct contact between the bacteria and the azo dye in the media.

The role of MtrC in decolourisation and current production

To determine the role of MtrC, an MtrC knock out strain was used to compare its performance to that for *Shewanella oneidensis* MR-1 wild type in the presence and absence of riboflavin. The results shown in Figure 4a represent the decolourisation profile over 72 h. The figure clearly shows that the rate of decolourisation increased gradually in the first 24 h, after which there was a steady state that was maintained. Supplementation of the cultures with riboflavin gave a decolourisation efficiency of almost 100% (Figure 4a). As for the MtrC knock out, there was an initial lag in decolourisation which was maintained for the first 24 h, followed by a compensated decolourisation that was more or less the same as that for *Shewanella oneidensis* MR-1 wild type. As a negative control, the dye was added to the media without adding any microbial cells, the results show an average of 8% decolourisation which accounts for the percentage of non-biological degradation.

Fig. 4b represents the riboflavin concentration at the end of the MFC system. The figure shows that the highest riboflavin was produced by the MtrC knock out (15.1 µM) and that when the dye was added to the media there was a decrease in riboflavin production (11.9 µM).

[Figure 4a&b near here]

As for the bioelectricity production, Fig. 5a shows that the MtrC knockout exhibited the highest voltage, this was followed by that obtained when riboflavin was added to *Shewanella oneidensis* MR-1 culture. The figure also shows that the least current was obtained from *Shewanella oneidensis* MR-1 culture in the absence of riboflavin. Both voltage and power density followed

the same trend for the culture as shown in Figures 5b&c, the highest belonging to MtrC knock out (500 mV) and power density reaching 78 mW/m^2. The remaining COD was shown to be the least when riboflavin was added to the media (41%), as compared to that without riboflavin (52%) and MtrC knock out culture which showed 46% residual COD (Fig. 5d).

In an attempt to assess the decolourisation to be removal or degradation, a UV-visible scan was performed for the samples, the results show that both MtrC knock out culture and riboflavin supplemented cultures (20 and 40 µM) exhibited a complete breakdown of dye as indicated by the complete disappearance of the peak at 480 nm as compared to *Shewanella oneidensis* MR-1 without riboflavin which exhibited a decrease in the peak absorbance (Fig. 6).

Analysis of the metabolites produced

The analysis of the metabolites at the end of the MFC system showed no distinctive peaks for both acetic acid and ethanol, on the other hand butyric acid was detected in the media (Table 1). The concentrations were highest for *Shewanella oneidensis* MR-1 (88.9 mg/l) and decreased dramatically when the dye was added. The addition of riboflavin resulted in a slight increase in butyric acid concentrations from 29.7 mg/l for control samples to 32 mg/l for samples supplemented with riboflavin. On the other hand, MtrC knock out produced the lowest butyric acid concentration (9.7 mg/l).

Comparison of dye removal in the presence or absence of MFC

In order to determine if an MFC system is required to achieve better decolourisation, MFC systems were prepared as mentioned in the materials and methods section. The results shown in Figure 7 represent decolourisation in MFC and non-MFC systems in the presence and absence of

riboflavin. The results clearly show that it is not the closed MFC system that contributes to decolourisation but the presence of riboflavin in the system.

Toxicity test

To assess the effect of dye on the human Hep G2 cells, we incubated the dye before and after decolourisation with the cells and visualised the live cells using calcein dye. The images shown in Figure 8 show that the fluorescence was minimal when the dye was added before it was treated, on the other hand, the cells incubated with dye after treatment showed fluorescence almost the same as that obtained for control cells.

Discussion

Shewanella oneidensis MR-1, a classic model exoelectrogen used in bioremediation and electricity production, was used in this study and the effect of added riboflavin as electron shuttle compound was evaluated in terms of azo dye decolourisation and electricity production. The increase in both decolourisation and growth indicates that riboflavin was used by *Shewanella oneidensis* MR-1 wild type to transfer the electrons and reduce the azo dye in a matter of hours. The variation of the optimal riboflavin concentration required to achieve high decolourisation and that required to obtain highest current indicates that both decolourisation and electricity production are not necessarily a simultaneous process and that there might be a competition in both functions. There is currently no agreement on the mode of action of bacterial electron transfer. While Chen et al., (2011) and Hsueh et al., (2014) indicated that the two electron transfer processes go in parallel, our results indicate that the process is competitive. Considering that all the mentioned studies used azo dyes as a common chemical structure, this might suggest

281 that the difference could be attributed to the type of organisms used, since they are not the same.
282 Chen et al., (2011) used *Proteus hauseri,* Hsueh et al., 2014 used *Enterobacter cancerogenus*,
283 while in our study we used *Shewanella oneidensis* MR-1 wild type.

284 The electron transport process for azo reduction indicated different inhibition levels when nitrate,
285 copper, azide and ferrous ions were present. The results suggest that inhibition in decolourisation
286 may have occurred as a result of competition for electrons from electron donors. This is in
287 agreement with Hong et al., (2007) who studied the effect of electron donors to understand the
288 mechanism of bacterial anaerobic azo reduction to improve the treatment of azo dye
289 contaminated waste water.

290 'In an attempt to understand the role of direct electron transfer in the azo reduction process,
291 *Shewanella oneidensis* MR-1 was entrapped in alginate beads to preclude the direct contact. The
292 results showed that the decolourisation decreased by almost 3 fold as compared to control cells
293 which were in direct contact with the dye. This result confirms the involvement of both direct
294 and electron mediated decolourisation process. Bacterial electron transport in *Shewanella*
295 *oneidensis* MR-1 involves either direct transfer via outer membrane c-type cytochromes
296 (OMCs), nanowires or indirect electron transfer via endogenously secreted flavins (Yong et al.,
297 2013). Most studies focus on the involvement of membrane bound cytochromes in the electron
298 transfer process (Carmona-Martinez et al., 2011). MtrC is one of the membrane bound
299 cytochromes. Edwards et al., (2015) stated that redox-linked flavin sites in extracellular
300 decaheme proteins are involved in microbe-mineral electron transfer under anaerobic conditions.
301 MtrC combines with flavin mononucleotide to produce flavocytochromes, a semi-reduced flavin
302 that is located at the biofilm-electrode interface (Edwards et al., 2015). From this stand point, an
303 MtrC knock out strain was used to study azo decolourisation and electricity production. The

304 results show that initial decolourisation within the first 24 h was faster in wild types, both with
305 and without riboflavin, the latter being faster. MtrC knock out strain lagged in decolourisation
306 but the rate quickly increased to match that for *Shewanella oneidensis* MR-1 wild type (Figure
307 4a). Results showing residual riboflavin concentrations in the media revealed that riboflavins
308 were endogenously secreted in the media for the MtrC knock out strain. This result suggests that
309 in order for the cells to compensate the lack of MtrC as the end protein in the electron transport
310 chain, the cells resorted to over secretion of Flavin to act as a mediator in the process. The results
311 are the same for both decolourisation and electricity production. While *Geobacter* species were
312 reported to lack the ability to secrete flavins by themselves, *Shewanella oneidensis* MR-1 have
313 been reported to produce quinone-like compounds and flavins which act as electron shuttling
314 compounds (Watanabe et al., 2009). A study focusing on the kinetic measurements of purified
315 OmcA and MtrC revealed that those cytochromes were slower in direct reduction of metal oxides
316 and that this was rapidly increased after the addition of flavins to the medium (Ross et al., 2009).
317 Exploring metabolic pathways can shed light on optimal strategies to maximize the performance
318 of microbial fuel cells (Hsueh et al., 2014). The results obtained indicated that only butyric acid
319 was produced under the employed culture conditions. This suggests the possibility of reusing the
320 butyric acid again by the cells which sustained the electricity production. Identification of
321 metabolites and/or degraded azo dye could shed light on how to increase the decolourisation
322 process in terms of rate and efficiency (Solanki et al., 2013). The debate about MFC Vs. non-
323 MFC in rate and efficiency of decolourisation is still not concluded. Some studies confirm that
324 MFC conditions are required for faster and significant colour removal (Hsueh et al., 2014), while
325 our study indicates that the addition of riboflavin is the key to rapid decolourisation as indicated

by the complete disappearance of the dye peak. For a comparison of dye decolourisation rates in MFC and non-MFC conditions, see Fernando (2014).

In order to claim that we can re-use the treated coloured wastewater, a toxicity test was performed to assess the quality of the resulting treated water. The results obtained indicate that there is no obvious difference between treated and control cells in viability, as opposed to dead cells resulting from incubation with the dye before treatment. This is a preliminary test that proposes the possibility to reuse. However, more tests are required for confirmation.

Conclusion

Deciphering the role of electron shuttling in bioremediation and electricity production reveals information about how to enhance these processes through fine tuning of microbial metabolism. In the current study, there were two main findings; the first results showed that flavin addition plays a role in the decolourisation process which indicates that this process is mediated. The second indicates that electricity production in an MFC does not affect simultaneous dye decolourisation. The study also shows that MtrC was not the responsible protein in both electricity production and decolourisation, confirming that riboflavin is the key player. A question still outstanding is which gene is responsible for decolourisation and which one is responsible for electricity production? We are currently studying the exact role of Mtr pathway genes for both decolourisation and energy production using synthetic biology to answer this question.

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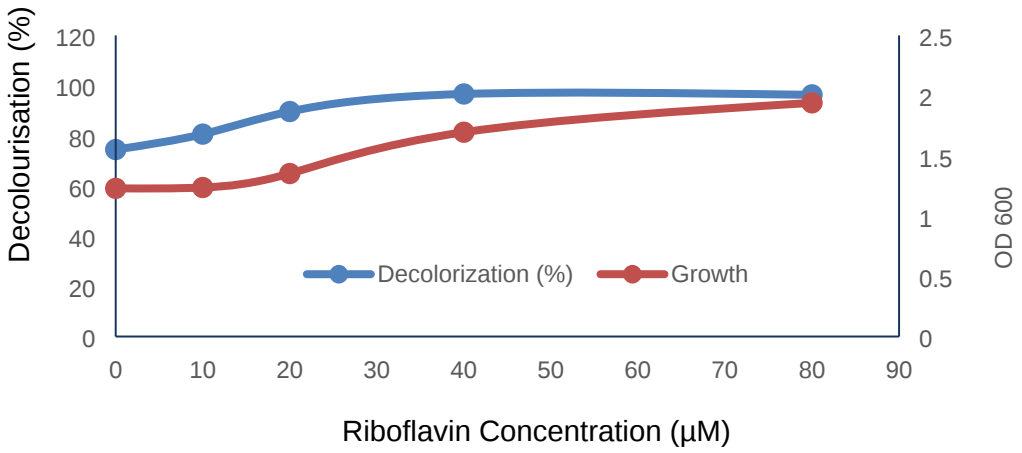


Figure (1): The effect of riboflavin concentration on the decolourisation of Congo red and growth of *Shewanella oneidensis* MR-1 under non-MFC conditions (anaerobic bottles, results taken after 24 h incubation.

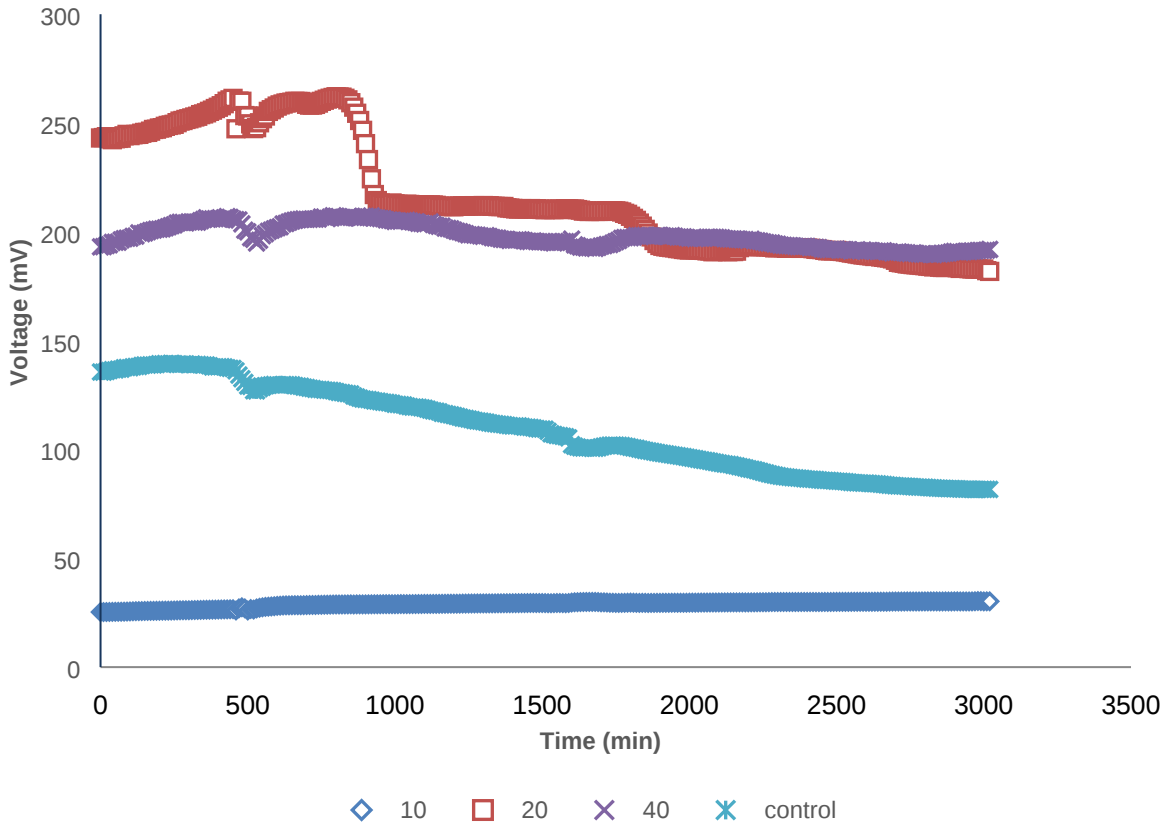
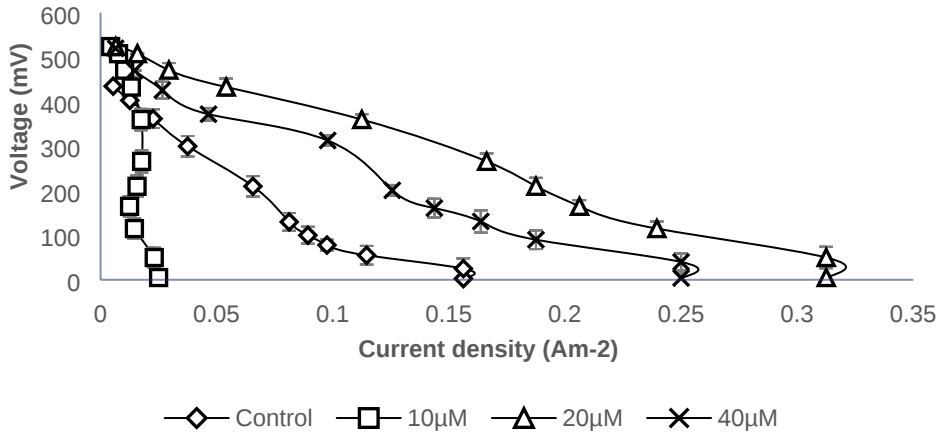


Figure 2(a): Voltage-time profiles for MFCs containing 200 mg/l Congo red and 0, 10, 20, 40 µM riboflavin.



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456 Figure 2(b): Voltage profile for MFCs containing 200 mg/l Congo red and 0, 10, 20, 40 µM

457 riboflavin.

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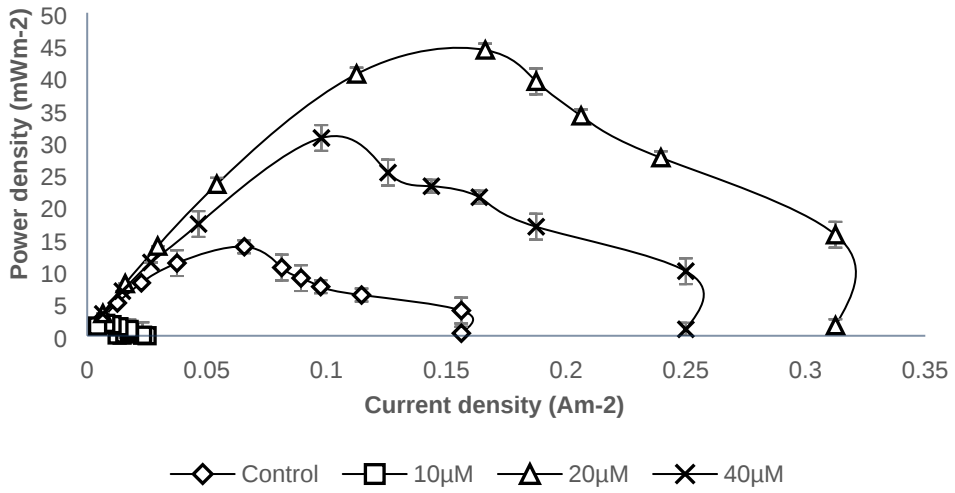

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465 Figure 2(c): Power density profile for MFCs containing 200 mg/l congo red and 0, 10, 20, 40 µM

466 riboflavin.

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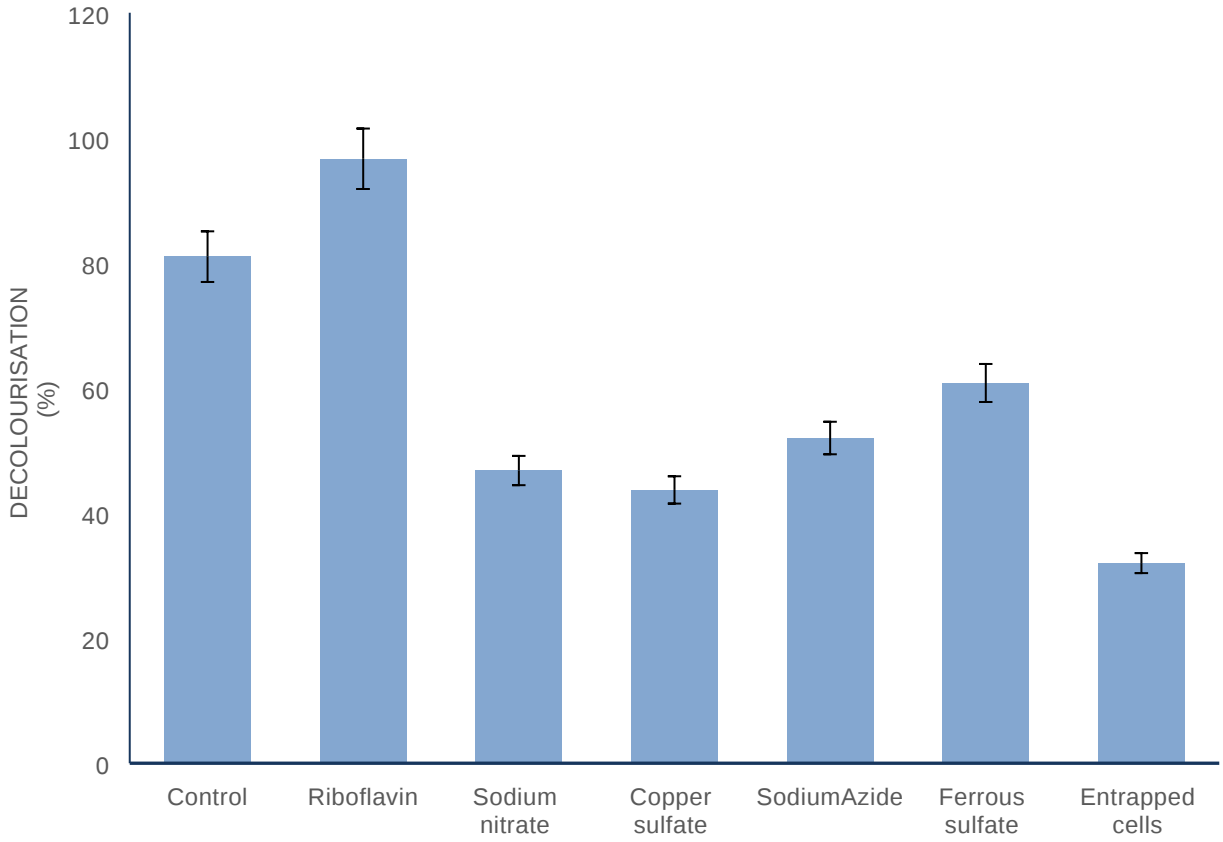
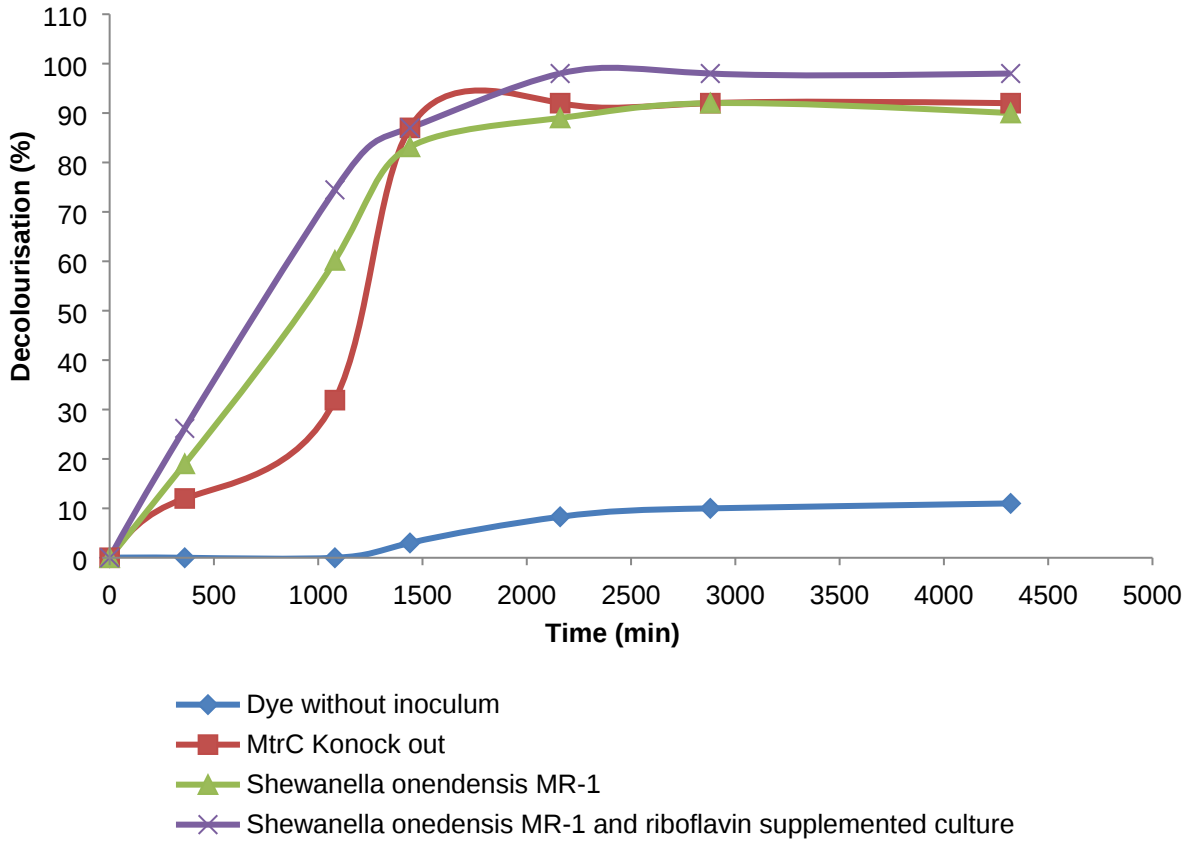


Figure (3): Decolourisation of congo red using *Shewanella oneidensis* MR-1 in non-MFC conditions (anaerobic bottles) under the following conditions: riboflavin for increasing decolourisation, sodium nitrate and ferrous sulphate for competitive terminal electron acceptor, copper sulphate and sodium azide for respiratory inhibition, entrapped cell to prevent direct electron transfer.



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480 Figure 4(a): Time course of decolorization under MFC conditions for the mentioned cultures.

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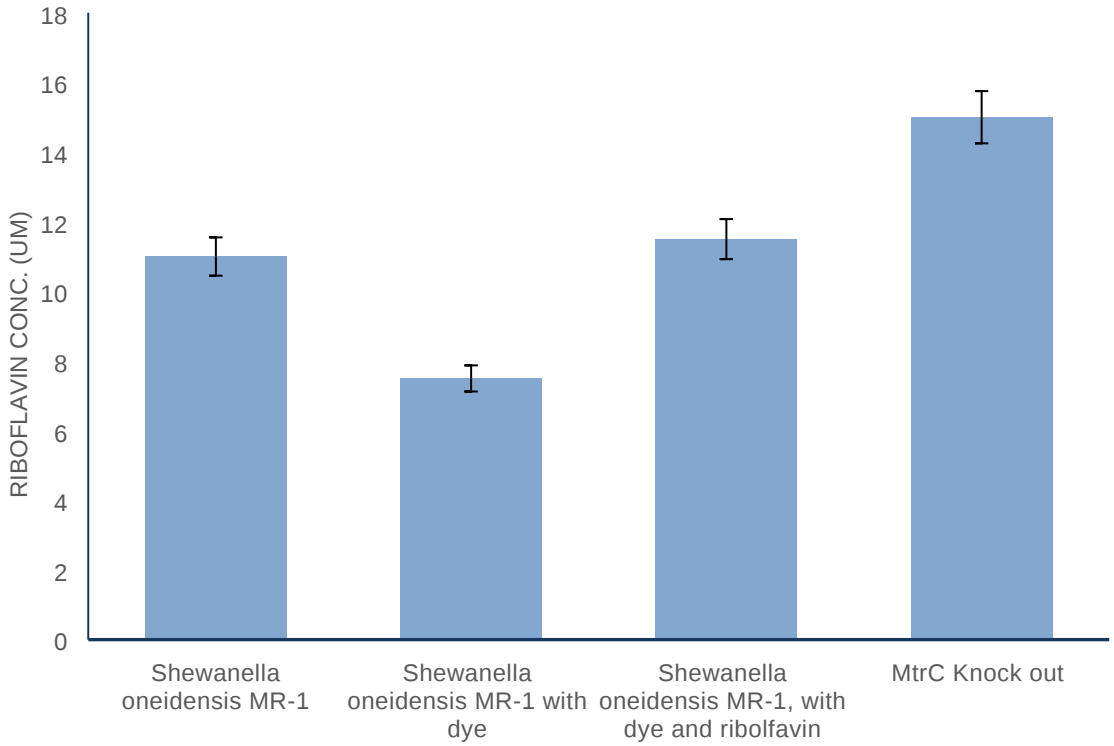

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485 Figure 4(b): The residual riboflavin at the end of MFC process

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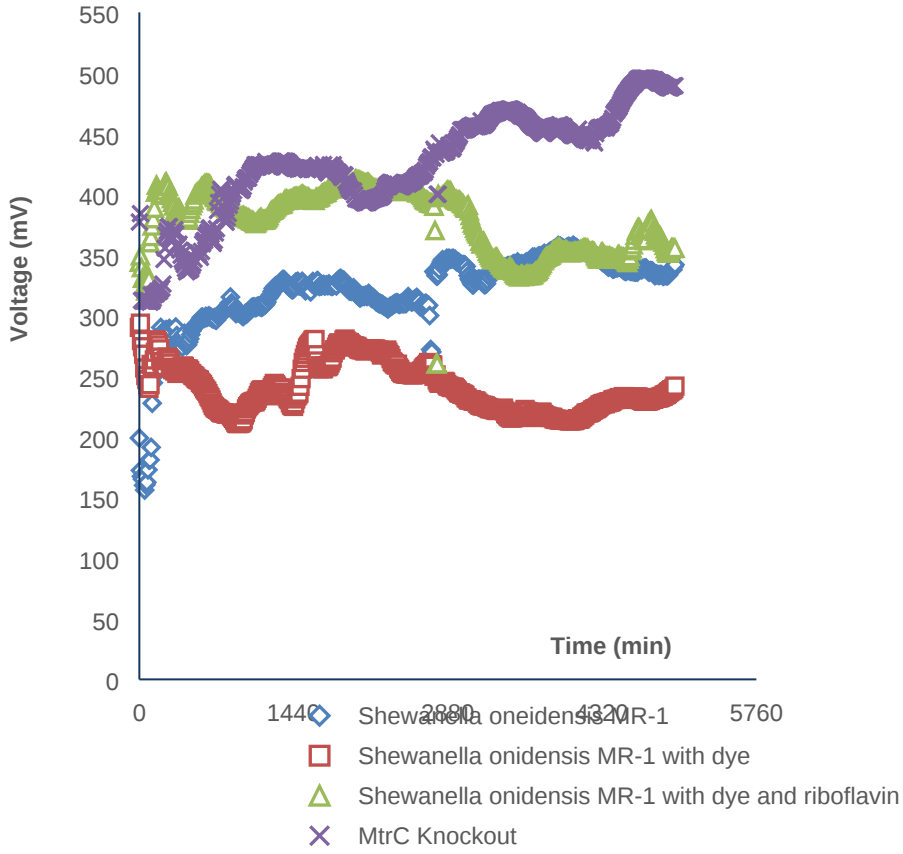

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488 Figure 5(a): Bioelectricity generation profile for MFC containing different culture conditions.

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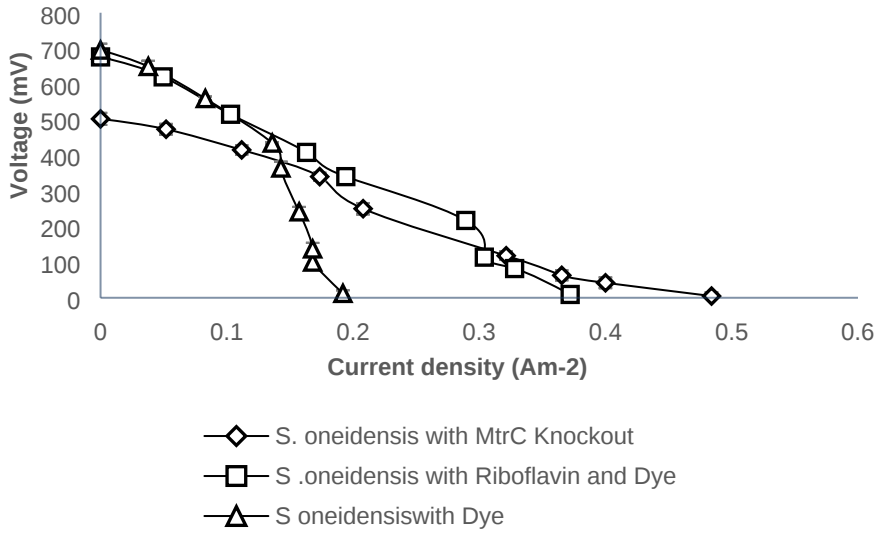

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493 Figure 5(b): Voltage profile for MFCs under different conditions.

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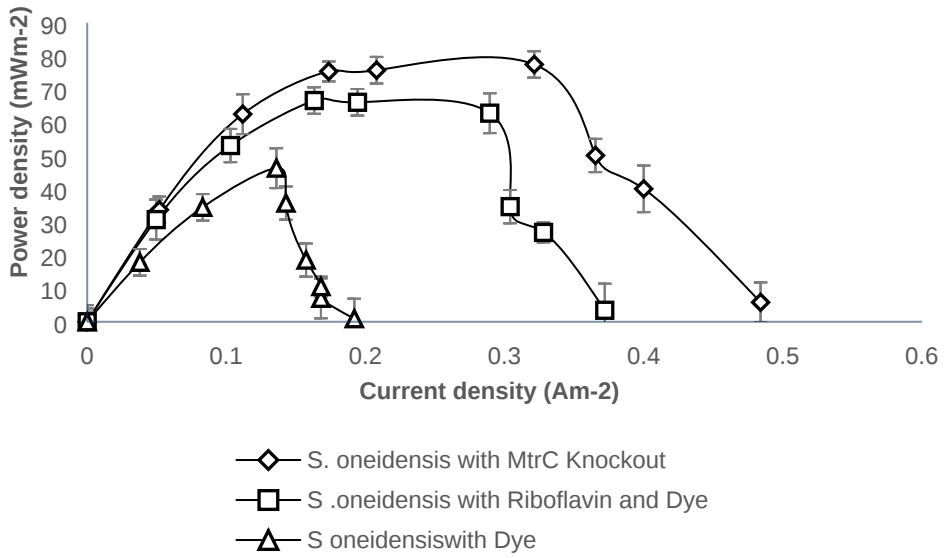

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501 Figure 5(c): Power density profile under different conditions using 20 uM riboflavin

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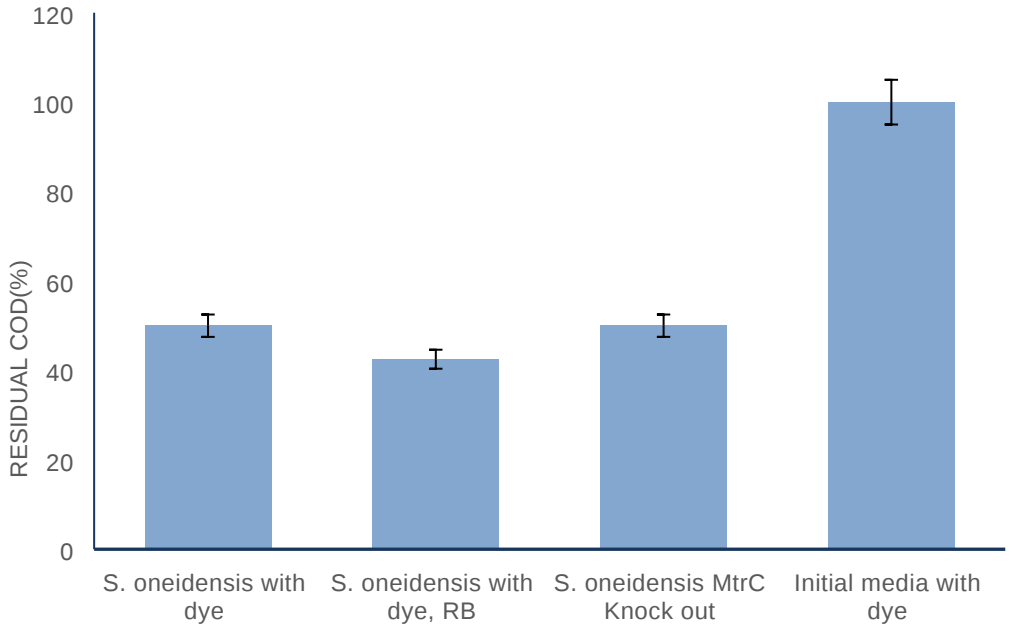

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509 Figure 5(d): Residual COD % for MFC cultures, 20uM riboflavin was added

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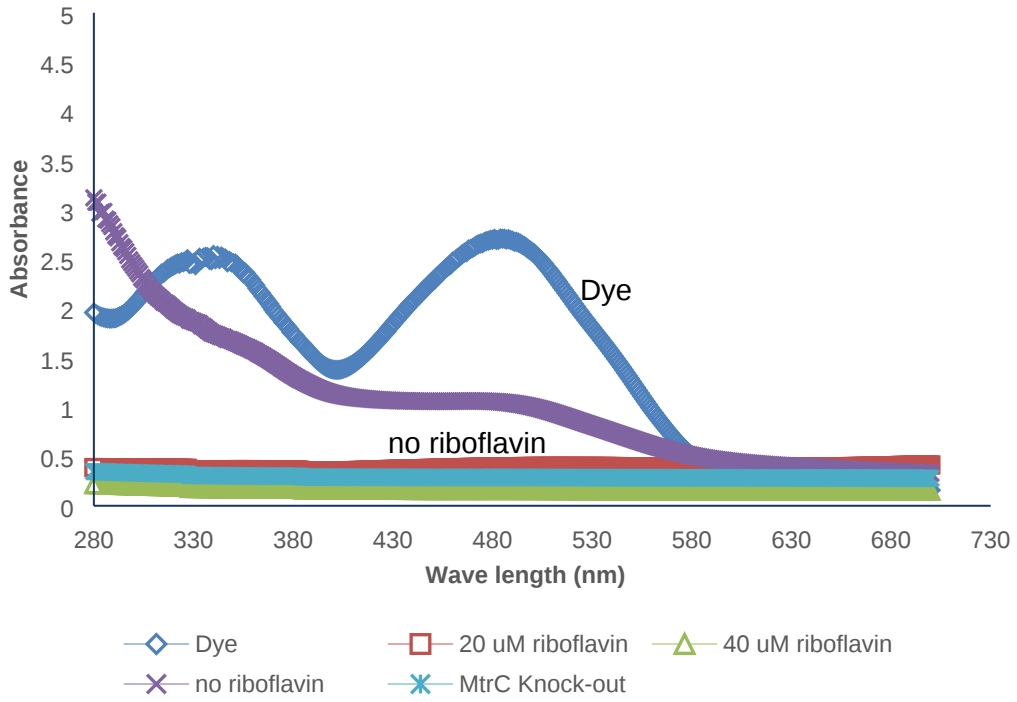

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516 Figure (6): UV-visible spectrum of congo red before and after decolourisation by *Shewanella*

517 *oniedensis* MR-1 in the absence and presence of riboflavin (20 and 40 uM) and using MtrC

518 knock out strain.

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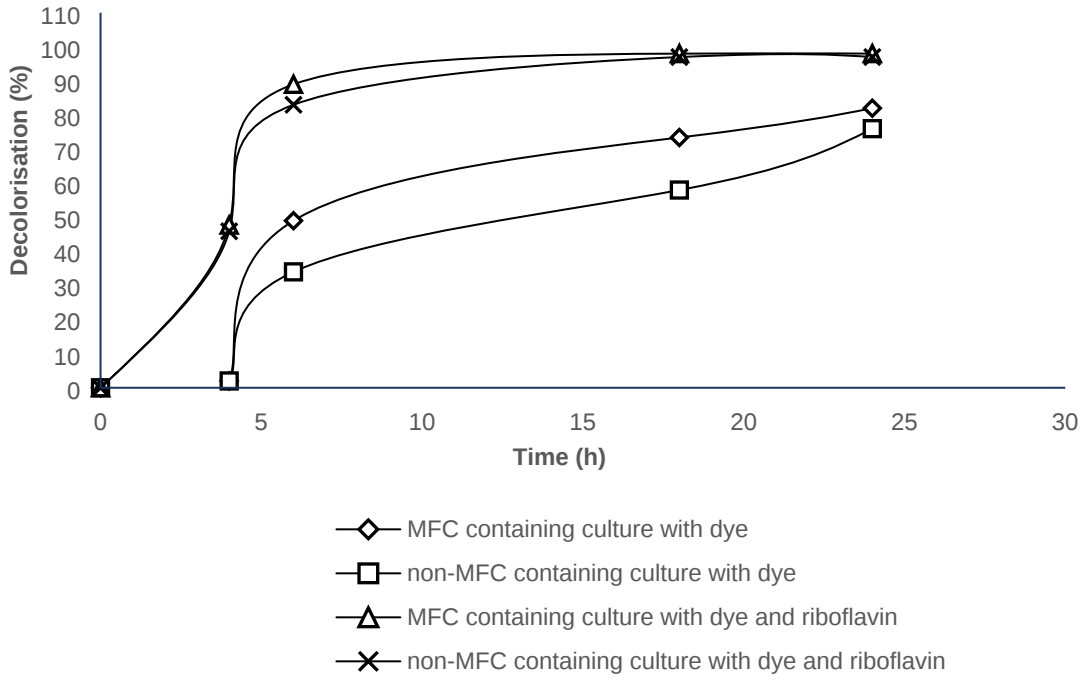

Figure (7): Decolourisation of Congo red by *Shewanella oneidensis* MR-1 in MFC and non-MFC conditions and in the presence and absence of riboflavin.

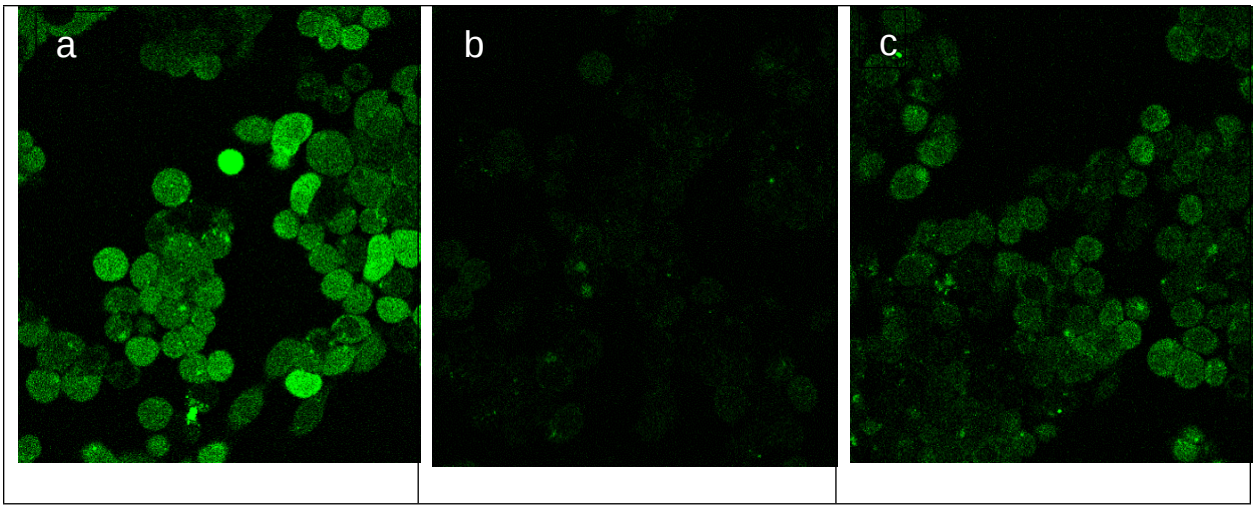

Figure (8): Images of HepG2 cells exposed to different treatments. Images are taken using confocal scanning laser microscopy (CSLM) and represent cells treated with congo red prior to decolorization (b), cells after incubation with treated dye under MFC conditions (c) as compared to control cells grown under regular conditions (a).

Table (1): The metabolites produced at the end of MFC operation

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Test	Fermentation product (mg/ml)		
	Acetic acid	**Ethanol**	**Butyric acid**
***S. oneidensis* MR-1**	ND	ND	88.92
***S. oneidensis* MR-1 with dye**	ND	ND	29.692
***S. oneidensis* MR-1 with dye and riboflavin**	ND	ND	32
***S. oneidensis* MtrC knock out with dye**	ND	ND	9.692

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