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Abstract

BACKGROUND: Many approaches have been employed to increase the understanding and consequently the performance of Microbial Fuel Cells to obtain simultaneous power production and biodegradation. This study uses recombinant *Escherichia coli* K-12 with *MtrA*, *MtrC* and *MtrCAB* inserts previously prepared using synthetic biology to evaluate the involvement of each of these genes in bioenergy production and biodegradation of Congo red using a double chamber microbial fuel cell.

RESULTS: *MtrC* was the key gene required for energy production corresponding to an average voltage of 360 mV (external resistance 1 K&) and power density of 59 mW/m^2, while *E. coli* with *MtrCAB* insert showed the highest decolourisation which reached 80% in 36 h under microbial fuel cells conditions. Coloumbic efficiency was 1.2% for *E.coli* with *MtrCAB* compared to 2.5% and 2.3% for *Mtr*C and *Mtr*A inserts, respectively. Riboflavin seems to be involved in the electron transferring, its concentration was highest for *E.coli* with *MtrA* insert despite its poor performance in both bioenergy production and dye degradation.

CONCLUSION: This study suggests that electrons are mutually exclusive between electricity production, dye degradation and other cellular activities. This study helps us improve our understanding of the dual bioenergy/decolourisation process taking place in MFCs in order to maximize the outcome.

Key words: Synthetic Biology; electron shuttling; decolourisation; energy production; biofilm formation; *Mtr* pathway genes.

Introduction

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Environmental pollution and energy shortage are two of the critical problems on the increase worldwide. The search for a technology, by which both problems are solved simultaneously, is considered a pertinent solution. Microbial fuel cells (MFC) are bioelectrochemical systems (BES) that utilise electrochemically active microorganisms (exoelectrogens) e.g. *Shewanella, Geobacter, Rhodoferax, yeasts etc.* to catalyse an oxidation and reduction reaction at an anode and cathode electrode respectively and can produce electricity when connected to a load/resistor via an external circuit [6.] Microbial Fuel Cells (MFCs) have drawn the attention of researchers because of their ability to simultaneously decolorize dyes while generating electricity. The process takes place in different reactors such as single chamber[1], double chamber[2], constructed wetland[3] or in combination with electrolysis cell[4]. The simultaneous processes are enhanced by studying bacteria in anode[5] electrode[7].

Congo red is a recalcitrant azo dye that is commonly used in dyeing textiles. About 10-15% of the used dye is released back in the produced waste water at the end of the dyeing process. The following table represents some values of textile waste water content as reported by Fernando [14]

Textile waste water characteristics	
pH	7-9
Biological Oxygen Demand (BOD)	80-6000 µg/l
COD	150-10000 µg/l
TSS	15-8000 µg/l
TDS	2900-3100 µg/l
Chlorine	1000-1600 µg/l
Total Kjeldahl N	70-80 µg/l
Colour	50-2500

Physical and chemical decolourisation methods have their drawbacks of cost and complexity, while biodegradation offers an efficient, sustainable and cost-effective solution. Azo dyes are usually transformed under anaerobic conditions which makes azo dye degradation suitable for implementation in the anodic chamber of MFCs[8]. Simultaneous azo dye decolourisation and electricity production have been studied in microbial fuel cells. Electron transfer was reported to be the key in both decolourisation and electricity production, where electrons compete for the two processes [9].

Three mechanisms have been suggested for electron transfer from exoelectrogens to the anode: via membrane bound redox proteins, electron shuttling through endogenous compounds, or by nanowires[10]. These may be categorized into direct and mediated electron transfer. Biofilms formed on the electrode are considered to aid direct electron transfer mechanism[5,11], and cytochromes play a key role in this process too. The cytochromes include periplasmic decaheme cytochrome (MtrA), the outer membrane decaheme cytochrome (MtrC), and the ß-barrel porin within the gene cluster (*MtrCAB*). This paper probes the mechanism of simultaneous decolourisation and electricity production.

Different approaches have been pursued to unveil the relationship between bioelectricity production and dye decolourisation. Bacterial acclimatization was adopted by Chen et al [10] who used wild type *Proteus hauseri* and suggested that there was a competitive process between dye decolourisation and energy production. On the other hand, Cao et al [12] used bacteria with *Mtr* mutants for *MtrA, MtrC, MtrC/OmcA, CymA* and concluded that both processes are simultaneous. Chen et al [13] stated that dye intermediate compounds act as electron shuttling compounds thus enhancing the electricity production. Considering the current state of knowledge, there is need to improve both electricity production and dye degradation in microbial fuel cells. However, the mechanisms involved in both processes are not well understood or agreed upon. One way of understanding the mechanisms is to use a bottom up approach where genes encoding for proteins involved in the EET *Mtr* pathway are expressed in a host (e.g. *Escherichia coli*) with an innate ability to decolourise azo dyes. The strains were prepared using synthetic biological approach. Therefore, aim of the present work was therefore to determine the contribution of the genes *MtrA, MtrC* and *MtrCAB,* involved in the *Mtr* pathway for EET, to dye decolourisation / electricity production in microbial fuel cells.

Material and Methods

Chemicals used

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

Microorganisms and media used

Microbial cultures used in this study were 3 strains of *E. coli*-K12 containing *MtrA, MtrC* and *MtrCAB* inserts that were kindly provided by iGEM team 2015 at the University of Westminster (http://2015.igem.org/Team:Westminster). A loopful of strain seed was pre-cultured in 50 ml LB medium spiked with Kanamycin as indicated by the iGEM team, the culture was incubated overnight at 37^{o}C and 150 rpm. About 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.

Experimental design

Set up	Rationale
Wild-type *E.coli* (WT *E.coli*)	Inefficient at producing electricity but decolourises Congo red.
E.coli with *MtrCAB* insert	Both dye decolourisation and electricity production expected to take place.
E.coli with *MtrC* insert	Both dye decolourisation and electricity production expected to take place. Tells us the contribution of *MtrC* as well as importance of *MtrAB* (by comparison with *MtrCAB*).
E.coli with *MtrA* insert	Both dye decolurisation and electricity production expected to take place. Tells us the contribution of *MtrA* and by comparison with *MtrCAB* and *MtrC*, the contribution of *MtrB*.

Reactor set up

MFC systems were H-type two chambered with two identical Duran bottles held together with external metal clamps. The electrodes were made of carbon fibre and were cut to 4x4 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 and 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[14], 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. *E.coli*–K12 with inserts *MtrA, MtrC* and *MtrCAB* were added separately to different MFC systems, another MFC system was used to inoculate using *E.coli*–K12 with no insert. External resistance for all experiments was 1000 &.

Dye decolourisation and analysis of riboflavin concentration

Decolourisation was calculated as the decrease in colour and was plotted in percentages according the following equation:

$$\text{Decolourisation (\%)} = \frac{Ai - Af}{Ai} \times 100$$

Where A_i and A_f are the initial and final dye concentrations, respectively.

Riboflavin concentration was performed according to Xu et al[15] by measuring the absorbance at 444 nm.

Electrochemical measurements

Voltage output data was collected using a Picolog ADC-24 (PicoTechnology, UK) online data logging system. Polarization tests were done by connecting different values of external resistances once voltage had stabilized. Columbic Efficiency (CE) was calculated according to Fernando et al [14].

COD tests

The chemical oxygen demand removal was determined using the closed reflux titrimetric method as described by Westwood [16]. COD was calculated as follows:

$$\text{COD (mg/l)} = (V_b - Vs) * DF * M * 3200$$

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

The percentage of COD removal was calculated as follows:

COD removal (%) = [COD_I-COD_T/COD_I]X100

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 ready for metabolite detection and run on 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.

Confocal Scanning Laser Microscopy (CSLM)

At the end of the incubation phase, the anode from each MFC system was removed, washed with phosphate buffer and washings placed in a six well plate and SPYRO fluorescent dye (excitation and emission wavelength are 495/515 nm, respectively) was added and plate was left to incubate in dark for another 45 min at 37^{o}C. Samples were washed again with phosphate buffer and images were taken 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

The experimental data represented in figures and tables are the mean value of duplicate experiments and the data plotted are the mean values.

Results and Discussion

Power generation and dye decolourisation by the different *E. coli* constructs

Figures 1, 2 and 3 represent the closed circuit voltage profiles, power density and polarization curves for the three chosen *E. coli* strains containing constructs *MtrA*, *MtrC* and *MtrCAB*, respectively. The *MtrC* construct gave the highest electrochemical performance overall. The average voltage was 360 mV for *MtrC* after 4000 min as

compared to 260 and 200 mV for *E. coli* containing constructs *MtrA* and MtrCAB, respectively. The same applies to power density where the highest value (59.5 mW/m^2) was represented by *MtrC*. This was followed by *MtrA* (30.0 mW/m^2), *MtrCAB* (26.6 mW/m^2) and the wild type *E.coli*, *E.coli* WT, (4.4 mW/m^2). On the other hand, Congo red decolourisation was highest when *E. coli* containing construct *MtrCAB* was used (82% for *E. coli* with construct *MtrCAB* after 48 h) as opposed to 55.4%, 46% and 38% for *E.coli* WT, *E. coli* containing constructs *MtrC* and *MtrA*, respectively (Fig. 4). Some researchers have suggested (Figure 5) that dye decolourisation involves electron transfer with the dye acting as a terminal electron acceptor and that the transfer, which is mediated by cytochrome c proteins and a membrane-bound azoreductase, can be coupled to energy generation i.e. growth of cells [17].

Bacterial electricity production involves electron transfer with C-type cytochromes playing an essential role although the extracellular electron transfer mechanisms are still a subject of study [18]. Mutants of *Shewanella decolorationis* S12 without cytochrome c were shown to be deficient in dye (Amaranth) decolourisation and electricity production but this deficiency could be remedied by addition of riboflavin to the mutant strain [19].

If dye decolourisation is due to reduction reaction, the reaction being mediated by azoreductases, then any pathway e.g. *MtrCAB* that improves electron flow should lead to faster and more efficient decolourisation which is what was observed here. Riboflavin self-produced by the strains can mediate in the (purely chemical) reduction reaction but the redox coupling has to be valid thermodynamically. The increase in dye decolourisation for *E. coli* with *MtrCAB* insert over the *E.coli* WT strain seems to have happened at the expense of power production (Fig 2 and low coulombic efficiency in Table 1). This is possibly because the dye used had an advantage over the electrode from a redox potential perspective. The apparent redox potential of Congo red is reported to be 0.63 – 0.67 V [20] . Comparing this to the redox potential for the anode which has been estimated using EIS to be in the region of -0.15 V (vs NHE) [21]. The *MtrC* construct performed less than *E.coli* WT probably because some of the electrons were channelled away for electricity production (Figure 2). The same argument can be made for *MtrA* as periplasmic proteins were not found to be involved in azo reduction [17] but they are thought to be involved in EET [18].

Since power production followed the order *MtrC* > *MtrA* > *MtrCAB*> *E.coli* WT and dye decolourisation followed the order *MtrCAB* > *E.coli* WT > *MtrC* > *MtrA*, it seems *MtrC* and *MtrA* are more relevant for power production than for dye decolourisation and this may be attributed to increased riboflavin concentrations (Table 1). Redox mediators e.g. riboflavin or cofactors can deliver electrons from OMCs to electrodes

and stimulate EET by up to 50-fold [22]. Since *MtrCAB* was the best construct regarding dye decolourisation, it suggests *MtrB* is involved in dye reduction and that this helps to divert electrons to mainly for dye reduction.

Biofilm studies using confocal scanning laser microscopy

In order to depict the influence of each insert on the biofilm formation, bacteria with the inserts were grown on carbon electrodes and the intensity of the fluorescent dye for each bacterial strain was used to assess the biofilm formation. Image 6c shows, through the sporadic fluorescent spots, cell deposition of *E. coli* with insert *MtrC*. Biofilm formation could be detected easily in image 6d for *E. coli* containing *MtrCAB*. *E. coli* containing insert *MtrA* did not show any cell deposition on the electrode (Fig. 6b), it looks very similar to image 6a representing negative control. The improvement by *MtrCAB* of both decolourisation efficiency and power production compared to the wild type could be attributed to increased COD reduction (Table 1) linked to growth.

Accumulated metabolites at the end of MFC operation

At the end of the experiment, riboflavin, COD and volatile compounds were analyzed. The results shown in table 1 indicates that riboflavin was 18.26 mg/ml for *E. coli* with insert *MtrA*, this was followed by that produced by *MtrC* (12.9 mg/ml), and the lowest production belonged to that containing *MtrCAB* insert and *E. coli* (6.3 and 4.2 mg/ml, respectively). These results confirms that riboflavin production is increased as a compensation to the lack of MtrC gene, as reported in our previous work[23]. Results for coulombic CE% indicate that the highest values belonged to *MtrC>MtrA>MtrCAB>* WT. As for butyric acid formed at the end of the MFC operation, the results were very close (30-31 mg/ml) for the three constructs used in this study. Butyric acid production is consistent with metabolites profiles from anaerobic oxidation. Riboflavin production has been observed by other workers previously[22] and its production is expected to play a role in extracellular electron transfer. Increased COD reduction by the mutant strains compared to the *E.coli* WT strain implies that protein expression in the mutant strains was not toxic to the cells.

Overall evaluation of the mutants and suggested mechanism

The overall contribution of the mutants to COD reduction, power production and decolourisation efficiency, which are all objectives of dye degradation in microbial fuel cells, can be estimated by using a lumped parameter, the O-index. The index is obtained from the equation O-index = %COD * P_{max} (mW/m^2) * % decolourisation /

10,000 (Table 2). Based on the table the *MtrCAB* construct emerged to be the best construct. The efficiency of dual chamber MFCs for simultaneous bioelectricity production and wastewater treatment has been questioned by researchers due to the divergence of the results. Therefore, a technique such as synthetic biology would be helpful in elucidating the role of each of the *Mtr* pathway genes. The results of our investigations show that in terms of electricity generation, the highest power density values can be correlated to the presence of *MtrC*. On the other hand, the MFC containing *MtrCAB* showed the strongest ability for decolourisation of congo red. A schematic diagram summing up all the results is presented in Fig. 7.

Conclusion

There is evidence that dyes can be used as terminal electron acceptors to support growth as indicated earlier. *MtrCAB* with the highest COD reduction gave the highest biofilm density too. There appears to be a link between dye decolourisation and COD values but the low values of the coloumbic efficiency shows that majority of electrons generated were not used for electricity production in this instance. However, the ability of the cells for biosynthesis of riboflavin increased to compensate for the absence of genes in the *Mtr* pathway. The *E. coli* WT on its own could decolorize the dye, to a moderate extent, not via the *Mtr* pathway but rather through another dye reduction pathway.

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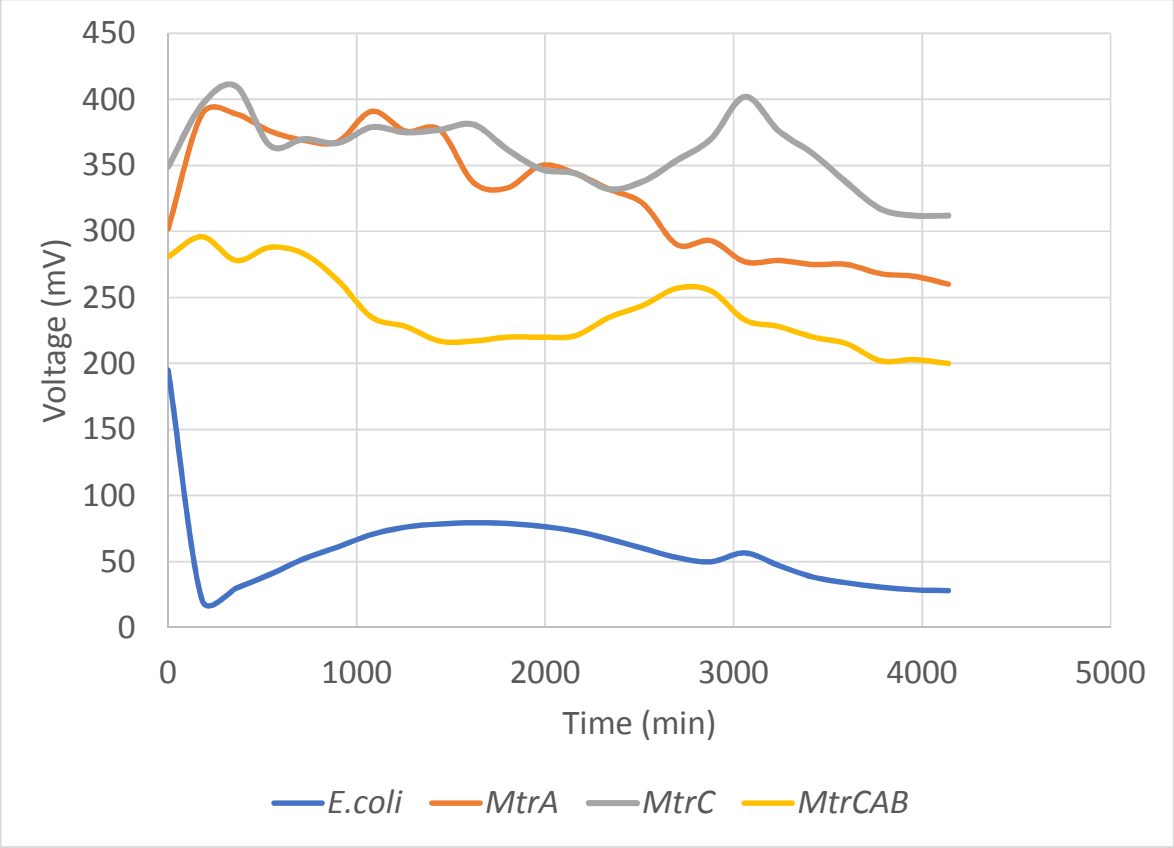

Figure 1. Voltage-time profile for MFCs containing *E. coli* -K12 with *MtrA, MtrC and MtrCAB* inserts as compared to *E. coli* with no insert.

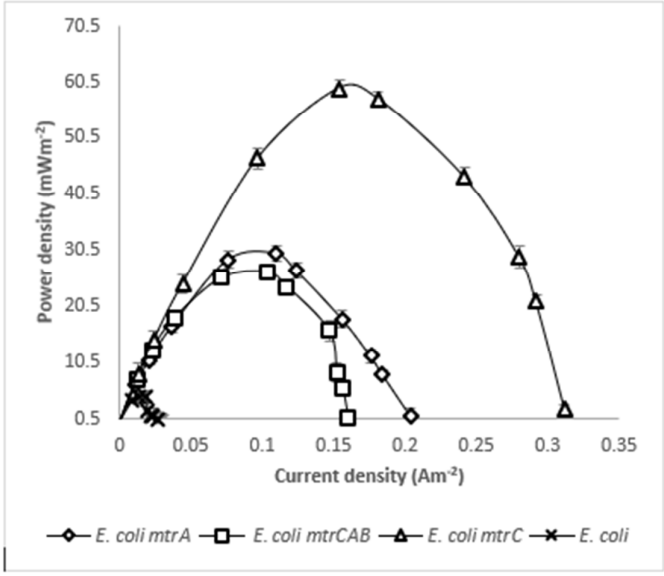

Figure 2. Power density curves for MFCs containing *E. coli* -K12 with *Mtr*A, *Mtr*C and *Mtr*CAB inserts as compared to *E. coli* with no insert. Error bars represent standard deviation from the mean.

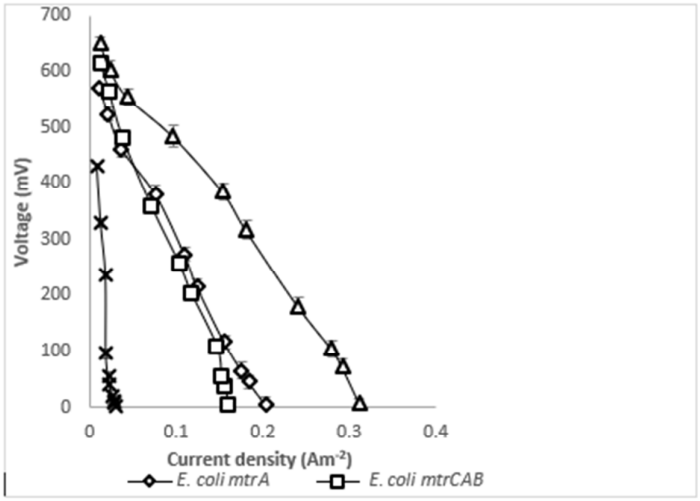

Figure 3. Polarisation curve for MFCs containing *E. coli* -K12 with *Mtr*A, *Mtr*C and *Mtr*CAB inserts as compared to *E. coli* with no insert. Error bars represent standard deviation from the mean.

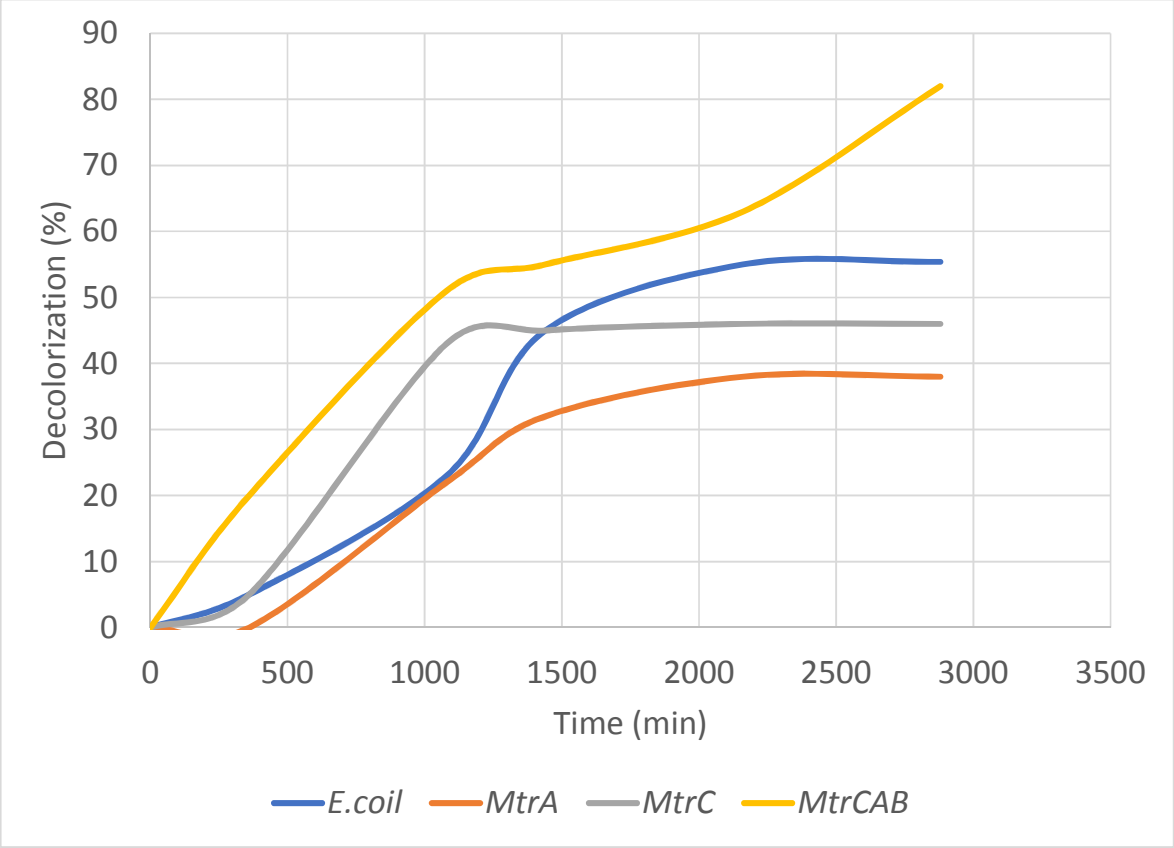

Figure 4. Congo red decolourisation profile for *E. coli* with inserts *MtrA, MtrC* and *MtrCAB* as compared to that for *E. coli* with no insert.

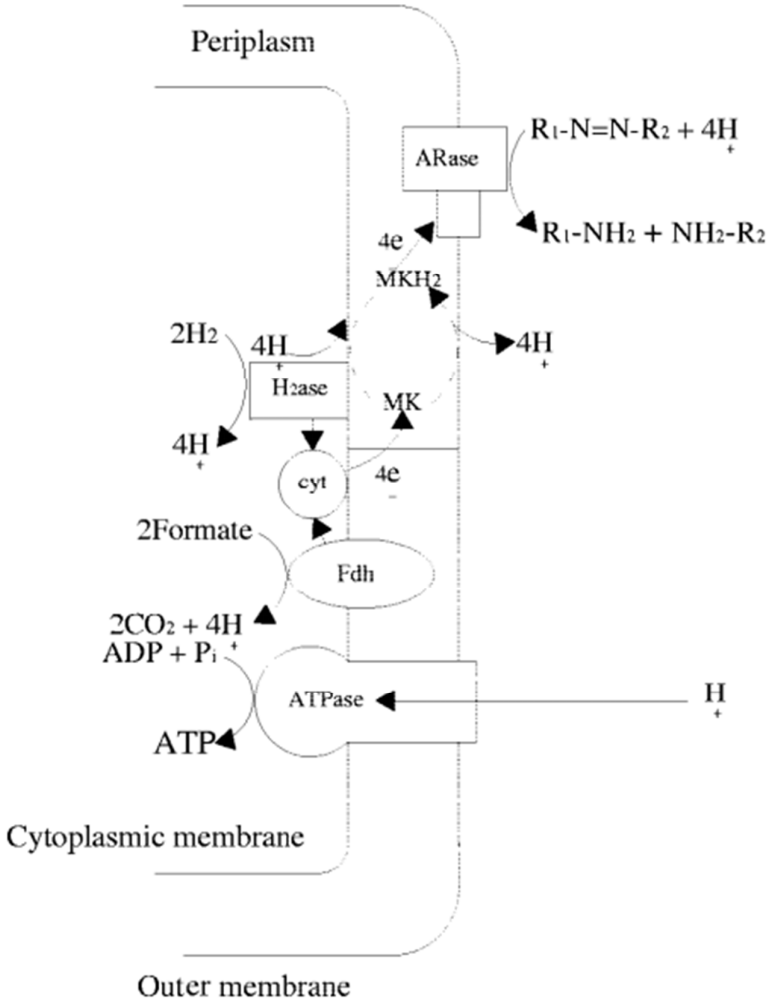

Figure 5. Putative chemiosmotic model of respiration involving azo dyes as terminal electron acceptors. Hydrogen and formate are examples of electron donors. The reduction of azo dye R_1-N=N – R_2 is mediated by a membrane-bound azoreductase (ARase). H_2ase = hydrogenase; cyt = cytochrome P450; MKH_2 = hydroquinone of menaquinol [Adapted from Hong et al., 2007).

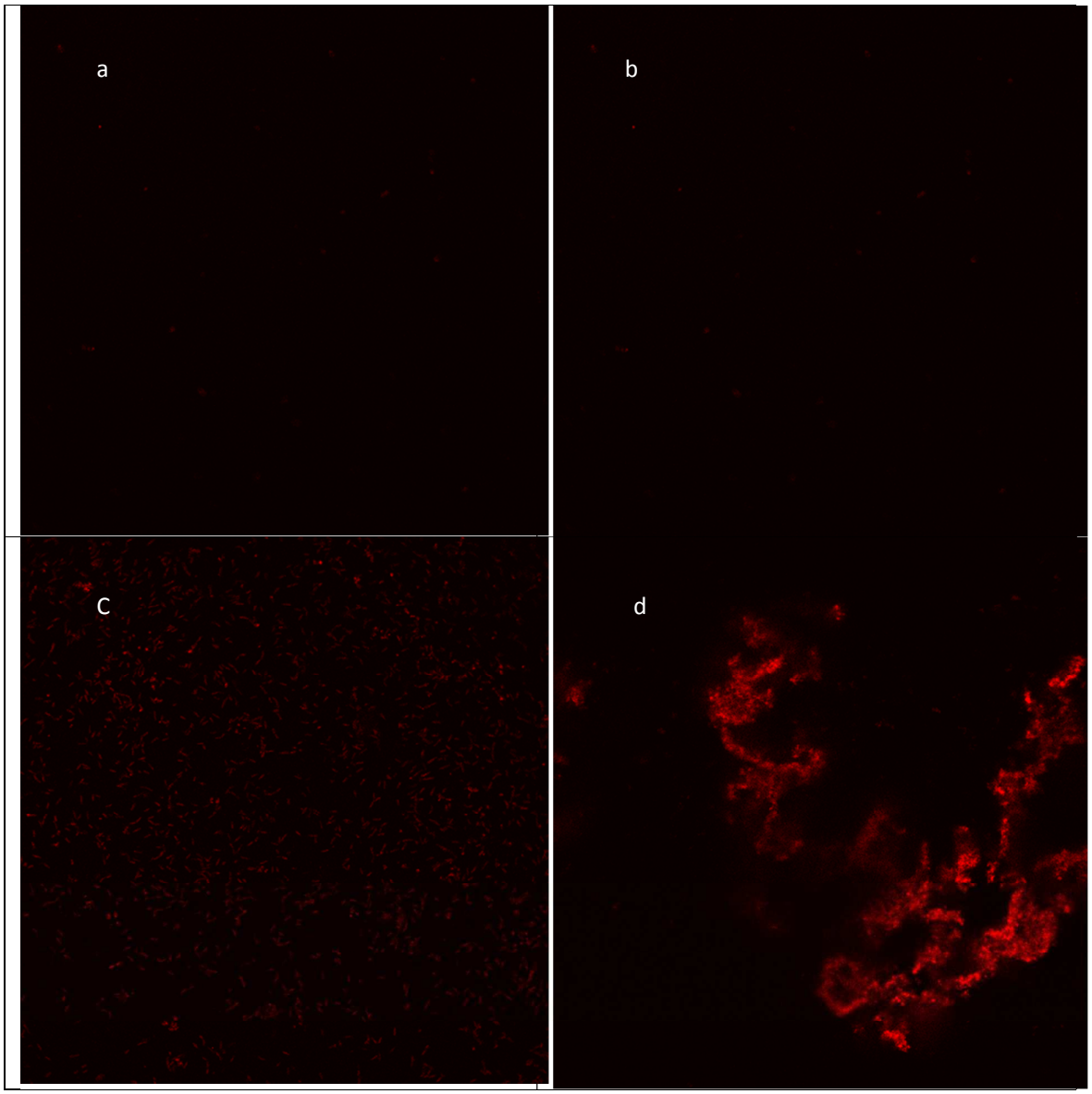

Figure 6. Confocal Scanning Laser Microscope (CSLM) images showing biofilm formation of *E. coli* with inserts *MtrA* (a), *MtrC* (c) and *MtrCAB* (d) as compared to *E. coli* with no inserts (a)

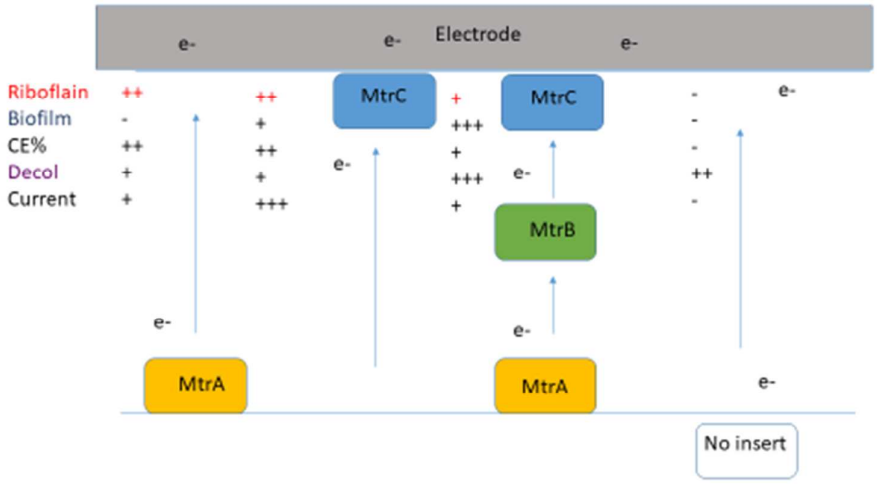

Figure 7. Schematic diagram representing the changes that took place for each of the tested parameters and its correlation to the denoted genes.

Table 1: Riboflavin, CE (%), COD and butyric acid at the end of MFC operation for *E. coli* with inserts *Mtr*A, *Mtr*C and *MtrCAB*

Insert	Riboflavin (mg/ml)	$CE_{\%}$	Butyric acid (mg/ml)	COD reduction (%)
MtrA	18.26	2.33	31.47	61
*Mtr*C	12.9	2.45	30.49	62
MtrCAB	6.3	1.2	31.79	83
no insert	4.2	0.3	30.23	28

Table 2 . Overall evaluation of the performance of the strains by lumping together decolourisation efficiency, power production and COD reduction efficiency.

	%COD	P_{max} (mW/m^2)	% decolourisation	O-index (%COD*P_{max}*% decolourisation)/10^4
MtrC	62	59.5	46	17.0
MtrCAB	83	26.6	82	18.1
MtrA	61	30.0	38	7.0
E.coli	28	4.40	55	6.8