Effect of external voltage on \textit{Pseudomonas putida} F1 in a bio electrochemical cell using toluene as sole carbon and energy source

Hen Friman,\textsuperscript{1,2} Alex Schechter,\textsuperscript{3} Yeshayahu Nitzan\textsuperscript{2} and Rivka Cahan\textsuperscript{1}

\textsuperscript{1}Department of Chemical Engineering and Biotechnology, Ariel University Center, Ariel 44837, Israel
\textsuperscript{2}The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel
\textsuperscript{3}Department of Biological Chemistry, Ariel University Center, Ariel 44837, Israel

A bio electrochemical cell (BEC) was constructed as a typical two-chamber microbial fuel cell (MFC), except that it was operated under external voltage instead of constant resistance as in an MFC. The anode chamber contained a pure culture of \textit{Pseudomonas putida} F1 grown in a minimal medium containing toluene as the sole carbon and energy source. Operating the BEC under external voltages of 75, 125, 175, 250 and 500 mV (versus an Ag/AgCl reference electrode) led to increased bacterial cell growth to an OD\textsubscript{600} of 0.62–0.75, while the control BEC, which was not connected to external voltage, reached an OD\textsubscript{600} of only 0.3. Examination of the current generated under external voltages of 75, 125, 175, 250 and 500 mV showed that the maximal currents were 11, 23, 28, 54 and 94 mA m\textsuperscript{2}, respectively. Cyclic voltammetry experiments demonstrated an anodic peak at 270 mV, which may imply oxidation of a vital molecule. The average residual toluene concentration after 147 h in the BEC operated under external voltage was 22 \%, whereas in the control BEC it was 81 \%. Proteome analysis of bacterial cells grown in the BEC (125 mV) revealed two groups of proteins, which are ascribed to charge transfer in the bacterial cells and from the cell to the electrode. In conclusion, operating the BEC at 75–500 mV enabled growth of a pure culture of \textit{P. putida} F1 and toluene degradation even in an oxygen-limited environment.

INTRODUCTION

There is growing interest in the utilization of a low-voltage electrical field in ‘electro-bioremediation’, a hybrid technology of electrokinetics and bioremediation, for the treatment of soil pollutants.

Electrokinetic technology for contaminant removal is based on electro-osmosis and ionic migration. The electrokinetic technique has been used successfully for removing more than 90 \% of heavy metals from clay, peat and argillaceous sand (Hamed \textit{et al.}, 1991; Yeung \textit{et al.}, 1997). Several studies have demonstrated improved removal of organic pollutants such as gasoline hydrocarbons, aromatic compounds, herbicides and trichloroethylene through electric fields applied to soil (Yeung \textit{et al.}, 1997). Electro-osmosis has been demonstrated to efficiently remove water-dissolved phenol, o-nitrophenol (Pazos \textit{et al.}, 2006), hexachlorobenzene (Kim \textit{et al.}, 2005), benzene, toluene, ethylene and xylene, hexane, isooctane and trichloroethylene from clay

\textbf{Abbreviations:} BEC, bio electrochemical cell; CV, cyclic voltammetry; MFC, microbial fuel cell; Omp, outer-membrane protein.

(Desarnais & Lewis, 2002; Wick \textit{et al.}, 2007). However, removal of petroleum hydrocarbon pollutants via electrokinetic technology is limited due to the low solubility of these compounds (Reddy & Cameselle, 2009).

Bioremediation methods for removal of toxic organic compounds are an attractive alternative to the conventional chemical or physical techniques. Bioremediation methods use micro-organisms to reduce the concentration and toxicity of various chemical pollutants such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phthalate esters, nitroaromatic compounds, industrial solvents, pesticides and metals (Vidali, 2001). Bacteria that degrade toxic monoaromatic hydrocarbon compounds have been isolated from sub-zero as well as from extremely hot environments (Aislabie \textit{et al.}, 2000; Chen & Taylor, 1997). Bacteria can utilize toxic organic compounds (monoaromatic hydrocarbons, polycyclic aromatic hydrocarbons and alkanes) as a source of energy and carbon (Chakraborty \textit{et al.}, 2005; Coates \textit{et al.}, 2001). For example, \textit{Pseudomonas putida} F1 can utilize toluene. In such bacteria, the \textit{tod} genes encode
enzymes involved in the catabolic pathway of toluene (Zylstra & Gibson, 1989). The toluene dioxygenase in *P. putida* F1 has a broad substrate specificity that enables it to oxidize a variety of substituted aromatic compounds such as nitrobenzene (Haigler & Spain, 1991). Stimulation of bioremediation by electrokinetic processes has been reported in a series of studies (LeDuc & Terry, 2005; Owstianiak et al., 2009; Pandey et al., 2009). Optimal biodegradation activity requires that the direct current does not have any negative effects on the physiology of the degrading bacteria. ‘Electro-bioremediation’ uses relatively weak electric fields (0.2–2 V cm⁻¹) (Schwartz et al., 1997; Wick et al., 2007); a higher-voltage electrical field has been reported to have a detrimental impact, leading to cell death (Aronsson et al., 2005; Schoenbach et al., 2000). The present research focuses on the effect of low voltage on a pure culture of *P. putida* F1 grown in a bio electrochemical cell (BEC), using toluene as the sole carbon and energy source. The BEC apparatus was designed as a typical two-chamber microbial fuel cell (MFC) (Allen & Bennett, 1993; Wingard et al., 1982), but was operated under external constant voltage. The influence of an applied low external voltage on the growth, electron transfer, toluene degradation and proteome of *P. putida* F1 was examined.

The BEC described herein shows potential for electro-bioremediation of toluene in industrial wastewater under conditions of limited dissolved oxygen.

**METHODS**

**Media.** The mineral medium (MM) used in the present study comprised (per litre): 2.44 g Na₂HPO₄, 1.52 g KH₂PO₄, 0.5 g (NH₄)₂SO₄, 0.2 g MgSO₄·7H₂O, 0.05 g CaCl₂·2H₂O and 10 ml trace element solution SL-4. pH was adjusted to 7 by addition of HCl (0.5 M) or NaOH (0.5 M). Trace element solution SL-4 comprised 0.5 g EDTA, 0.2 g FeSO₄·7H₂O, in 900 ml plus 100 ml trace element solution SL-6. Solution SL-6 comprised (per litre): 0.1 g ZnSO₄·7H₂O, 0.03 g MnCl₂·4H₂O, 0.3 g H₂BO₃, 0.2 g CoCl₂·6H₂O, 0.01 g CuCl₂·2H₂O, 0.02 g NiCl₂·6H₂O and 0.03 g Na₂MoO₄·2H₂O.

Mineral medium containing toluene as the sole carbon source (MMT) was composed of MM plus 100 mg toluene ⁻¹. All reagents and chemicals were of analytical grade and were purchased from Sigma-Aldrich.

**BEC set-up.** The BEC consisted of a dual-glass chamber separated by a proton-selective membrane (Nafion 115, Ionpower). Each chamber contained approximately 450 ml of medium. The anode chamber had four ports on the top screw cap for solution sampling, feeding a 3 mm diameter graphite rod working electrode (Graphite Engineering and Sales) and an Ag/AgCl reference electrode (CH Instruments). The cathode top had one port for the counter electrode (2 cm × 2 cm carbon cloth ELAT-LT-1400 W, ETEK International). This electrode was brush-coated with a catalyst composite layer of 0.5 mg Pt m⁻² (Johnson Matthey). The catalyst is used to reduce residues of oxygen in the solution and enhances the counter electrode current. This electrode is also used as a cathode in MFCs. The catalyst layer was prepared from slurry containing a weight ratio of 8:1:1 Pt/Nafion [5% (w/w) solution, longpower]carbon (Vulcan XC72, Cabot). The two electrodes were connected by a copper wire lead and the junction was protected from corrosion by embedding in a commercial silicone paste. All parts were autoclaved prior to each experiment, except for the reference electrode, which was rinsed with 70% ethanol followed by sterile water. Each of the BEC chambers was filled with 450 ml sterile medium containing 350 ml MM and 100 ml phosphate buffer, pH 6.9. The BEC was placed in a thermostatic bath at 26 °C and the anode chamber was agitated slowly (100 r.p.m.) using a magnetic stirrer bar. A computer-driven CH760 potentiostat (CH Instruments) was used. The anode working electrode was polarized to the constant voltage of choice: 75, 125, 175, 250 or 500 mV (versus an Ag/AgCl reference electrode). Cyclic voltammetry (CV) was performed at the beginning and end (150 h) of each experiment. These measurements were carried out by introducing a Pt wire counter electrode that replaced the carbon cloth electrode, to avoid irreversible damage to the carbon cloth electrode at high potentials. A parallel identical control BEC was constructed in each experiment, which was maintained under the same conditions but was not connected to the power supply. Furthermore, an abiotic BEC (without bacterial cells in the anode compartment) was operated with or without external voltage. Toluene (100 mg l⁻¹) was added to the anode chamber in the BEC, control BEC and abiotic BEC. Samples for optical density measurements and toluene concentration were taken from the BEC every 24 h.

An MFC was designed as for the BEC, but was connected to an external resistor of 1 kΩ (Resistance Decade Box 72-7270, Tenma) instead of an external power source. The cathode chamber was aerated through a 0.45 µm pore-size filter (Whatman) to maintain an oxygenated environment. Bubbling with oxygen in the cathode chamber and stirring in the anode compartment were kept to a minimum to prevent excessive exchange of oxygen across the membrane separating the two chambers.

**Bacterial strain and growth conditions.** A pure culture of *P. putida* F1 (6899) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. The bacterial cells were grown in MMT in a sealed bottle at 26 °C with agitation at 100 r.p.m. The BEC or MFC was inoculated with an exponential phase culture of *P. putida* F1 (OD₆₀₀ 0.15). The bacterial concentration was measured using a spectrophotometer (GENESYS 10S UV-Visible, Thermo Scientific) at 600 nm.

**Toluene bioremediation experiments.** Toluene (100 mg l⁻¹) was added to the BEC at 0, 39, 68, 112 and 143 h, and its concentration was measured in a BEC operated at external voltages of 75, 125, 175, 250 and 500 mV (versus Ag/AgCl), a control BEC and an abiotic BEC. Toluene concentration in the BEC was measured by GC/MS (see below). The percentage of the toluene remaining in the BEC was calculated from the accumulated toluene concentration added until the point of measurement.

**Toluene concentration measurement.** For determination of toluene concentration was determined by GC/MS analysis. Liquid samples (1 ml) were collected simultaneously from the BEC, the control BEC and the abiotic BEC. Toluene was extracted from the cultures using 500 µl hexane, dried over analytical grade Na₂SO₄ and diluted 10-fold in acetonitrile. The toluene solution in hexane was analysed using a Varian MS-Saturn 2100T spectrometer coupled to a Varian 3900 GC equipped with a cool-on-column inlet and cp8944 capillary column (30 m × 0.25 mm). The GC protocol was as follows: helium carrier flow 1 ml min⁻¹ at a pressure of 127 558 Pa, and injection split ratio of 20:1. The initial temperature was 30 °C for 3 min followed by a ramp of 20 °C min⁻¹ to a final temperature of 250 °C for a total run time of 16 min. A toluene concentration calibration curve was constructed from a toluene solution with a pre-defined concentration (0–100 mM) and calculation of the corresponding peak area using GC/MS analysis.

**Preparation of cell extracts.** For proteome analysis, *P. putida* F1 was grown in the BEC under an external voltage of 125 mV and in the
control BEC (without external voltage). Cultures were harvested at the late-exponential phase, washed three times by centrifugation with PBS (pH 7), and resuspended in the same buffer. The suspended cells (1 ml) were sonicated (at 70% power, in cycles of 30 s followed by intervals of 60 s for 5 min on ice; Heat Systems, Misonix). The broken cell suspension was centrifuged at 10 000 g for 10 min to fully precipitate cell debris and intact cells. The supernatant (soluble proteins) was collected and the protein concentration was determined by the method described by Bradford (1976), with BSA (Sigma-Aldrich) as a standard.

**SDS-PAGE.** Protein homogeneity and molecular mass were determined by SDS-PAGE in 4% stacking and 12.5% separating polyacrylamide gels, using Dalton Mark VII-L mixtures (Sigma) as standards. Electrophoresis was carried out at 35 mV using a running buffer containing 0.025 M Tris/HCl, pH 8.3, 0.192 M glycine and 0.1% SDS. After electrophoresis the proteins in the gels were stained with 0.025% Coomassie brilliant blue R-250 in a solution of 40% methanol and 7% acetic acid.

**Proteolysis and MS analysis.** The Coomassie blue-stained band in-gel was reduced with 10 mM DTT, incubated at 60°C for 30 min, alkylated with 10 mM iodoacetamide at room temperature for 30 min, and proteolysed overnight at 37°C using modified trypsin (Promega) at a 1:100 enzyme-to-substrate ratio. The tryptic digest peptides were resolved by reversed-phase chromatography on 0.13 mm fused silica capillaries (J&W, 100 μm internal diameter) packed with POROS R2-10 reversed-phase material (Applied Biosystems) and eluted using an 80 min linear gradient of 5-95% acetonitrile with 0.1% formic acid in water at a flow rate of about 0.3 ml min⁻¹. MS (DecaXP, Thermo) was performed in the positive mode using a repetition of the full MS scan followed by fragmentation of the three most dominant ions selected from the first full MS scan. The MS data were compared via simulated proteolysis and collision-induced dissociation of the proteins in the NR-NCBI database using Sequest software (J. Eng and J. Yates, University of Washington; Finnigan) and Pep-Miner software (L. Beer, IBM).

**Statistics.** Each experiment was performed at least in triplicate. All primary data are presented as means ± SD of the mean or as an average.

**RESULTS AND DISCUSSION**

**MFC (operating under constant resistance)**

Operating the MFC under a constant external resistance of 1 kΩ with a pure culture of *P. putida* F1 in the anode chamber using MMT as the sole carbon and energy source demonstrated almost no change in the anode equilibrium potential. The potential remained at −0.05 to −0.07 V (versus Ag/AgCl), indicating that no direct charge transfer occurred between the bacteria and the electrode over 150 h. Thus, *P. putida* F1 cannot be categorized as exoelectrogenic under these conditions. Furthermore, bacterial growth in the anode compartment reached an OD₆₀₀ of only 0.3 after 150 h, probably as a result of low electron transfer in the absence of electron acceptors in the anode compartment.

**Bacterial growth in a BEC (operating under constant potential)**

The BEC apparatus in this research was built as a conventional two-compartment MFC. The main difference between this BEC and the two-compartment MFC is that the BEC operates as a bio electrochemical reactor cell by applying constant external voltage between the reference and anode electrodes. The growth curve of *P. putida* F1 with MMT in the anode chamber in the BEC measured under external voltages of 75, 125, 175, 250 and 500 mV is shown in Fig. 1. In the BEC operated under an external voltage, there was an increase in the bacterial cell concentration compared with the control BEC (operated without external voltage). Under an external voltage of 75, 125, 175, 250 and 500 mV (versus Ag/AgCl), the cultures reached a maximum OD₆₀₀ of 0.62–0.75, while in the control BEC the cultures reached OD₆₀₀ values of 0.3.

The external positive potentials applied to the BEC anode of 75–500 mV (versus Ag/AgCl) promoted bacterial growth even under limited dissolved oxygen conditions (0.78 mg O₂ l⁻¹). We assume that the external voltage allowed the anode to accept electrons generated in the bacterial electron transport chain.

**Current production in the BEC**

The BEC was operated under external voltage using *P. putida* bacterial cells in the anode compartment and MMT as the sole carbon source. The currents generated under

---

**Fig. 1.** Growth curve of *P. putida* F1 in a BEC. The cultures were grown in the anode chamber with toluene (100 mg l⁻¹) as the sole carbon source under constant external voltages of 75 (○), 125 (●), 175 (●), 250 (●) and 500 mV (▲), and without potential (★) versus an Ag/AgCl reference electrode.
external voltages of 250 and 500 mV were 54 and 94 mA m$^{-2}$, respectively, while in the BEC operated under external voltages of 175, 125 and 75 mV the currents were 28, 23 and 11 mA m$^{-2}$, respectively (Fig. 2). In electrodes conditioned at the highest potential, 500 mV, the current reached a maximum after 90 h and a plateau region was observed from 90 to 150 h. However, at the lower potentials, when the electrode was polarized to only 75, 125, 175 and 250 mV (versus Ag/AgCl), a moderate slope was observed until the end of the experiment.

The cyclic voltammetric behaviour of the graphite anode polarized to 125 mV (versus Ag/AgCl) at the beginning and end (150 h) of the experiment is presented in Fig. 3(a). At the end of the experiment, CV revealed relatively higher anodic currents above 0.2 V (versus Ag/AgCl). The increase in the anodic current cannot be attributed to toluene electro-oxidation, as an insignificant current was measured on the same electrode in sterile MMT, indicating that toluene oxidation is negligible (data not shown). Therefore, we assumed that electro-oxidation above 0.2 V (versus Ag/AgCl) can be attributed to active molecular species accumulated in the medium during prolonged polarization. Previously, it has been shown that toluene oxidation occurs at a potential of 1.5 V (versus Ag/AgCl) in acetonitrile using a glassy carbon electrode; at potentials below 1 V (versus Ag/AgCl) there was no current, which suggests that toluene is stable electrochemically under these conditions (D’Elia & Ortiz, 2005).

We assumed that one of the active molecular molecules was 3-methyl catechol, a compound in the metabolic degradation pathway of toluene. It has been shown that electro-oxidation of catechol derivatives has an oxidation peak potential between 0.27 and 0.32 V (versus Ag/AgCl). Catechol tends to oxidize to o-benzoquinone at potentials above 220 mV (Hüsken et al., 2001; Zeng et al., 2009).

The voltammetric behaviour of the graphite rod electrode in methyl catechol solution is shown in Fig. 3(b). Two consecutive cycles are presented, beginning from the anodic potential direction. A clear oxidation wave is observed from 0.18 to 0.45 V, with a peak at 0.37 V and no reduction current in the backwards potential scan, corresponding to an irreversible redox reaction. The peak decreases on the following cycle. This indicates a diffusion control reaction of soluble species. Interestingly, the shape and position of the methyl catechol oxidation peak are similar to those seen in the BEC after 150 h (Fig. 3a). However, further investigation is required to isolate the electrochemically active molecule from the bacteria anode solution, which is beyond the scope of the present study.

The current generated in the BEC after 150 h may be attributed to biofilm redox formation on the anode or to soluble natural mediators. To examine these possibilities, anodes that were operated under 250 and 500 mV for 150 h were exchanged by sterile electrodes (without biofilm) and the current was measured in these BECs (Fig. 4).

In a separate experiment, the anode covered with the biofilm was inserted into a sterile medium of another BEC to measure the contribution of planktonic bacterial cells to the current. The current density measured from the planktonic bacterial cells with a fresh electrode under external voltages of 250 or 500 mV was 28 or 58 mA m$^{-2}$, respectively, whereas the current obtained with biofilm-covered electrodes in sterile medium was 10 or 14 mA m$^{-2}$, respectively. These results show that both planktonic bacterial cells and biofilm contributed to the overall BEC current, but that the majority of the current is obtained from the former. The higher currents of the planktonic bacterial cells with the sterile electrode may be attributed to a soluble mediator in the solution.

A biofilm current associated with direct electron transfer has also been found in selected bacterial strains such as Geobacter sulfurreducens, Shewanella oneidensis MR-1 and Anaeromyxobacter dehalogenans (Bond & Lovley, 2003; Strycharz et al., 2010). An electrode with G. sulfurreducens bacterial cells in an MFC using acetate as a carbon source produced a current of 65 mA m$^{-2}$ at the electrode surface, while in an MFC under a poised potential of 200 mV it produced a current of 163–1143 mA m$^{-2}$ (Bond & Lovley, 2003). S. oneidensis MR-1 produced electrically conductive pilus-like appendages termed bacterial nanowires in direct response to electron-acceptor limitation (Gorby et al., 2006). In comparison, the lower current obtained in our study may be attributed to the non-mature biofilm on the anode, as the experiment was operated for only 150 h.

In addition to direct electron transfer, mediated charge transfer has been suggested as an alternative mechanism in MFCs (Chaudhuri & Lovley, 2003). Classical mediated

Fig. 2. Current formation in a BEC with *P. putida* F1 grown in minimal medium with toluene (100 mg l$^{-1}$) under applied constant voltages of 75 (○), 125 (□), 175 (●), 250 (■) and 500 mV (▲) versus an Ag/AgCl reference electrode.

http://mic.sgmjournals.org
charge transfer is ascribed to natural redox or artificial molecules that can undergo reversible reduction and oxidation, e.g. phenazine 1-carboxamide, pyocyanine and 2-amino-3-carboxy-1,4-naphthoquinone (Hernandez & Newman, 2001; Pham et al., 2008). Primary mediators are metabolites resulting from anaerobic respiration and fermentation associated with oxidative substrate degradation. In MFCs, these materials are useful only if they can undergo anodic reaction at negative potentials (Schröder, 2007). However, it appears that these molecules can easily undergo oxidation at potentials above 200 mV (versus Ag/AgCl) in our BEC.

![Graph showing charge transfer](image)

**Fig. 3.** (a) CV (graphite working electrode versus an Ag/AgCl reference electrode, 5 mV s⁻¹) immediately after adding *P. putida* F1 to a control BEC (thin lines) and after 150 h growth in a BEC with an external voltage of 125 mV (thick lines). The inset shows a magnification of the relevant potential range. (b) CV of 10 mM methyl catechol in MM solution, measured at 20 mV s⁻¹ and potential range of 0–500 mV versus an Ag/AgCl electrode. The arrows indicate the potential scan direction.

![Graph showing current](image)

**Fig. 4.** Current obtained with biofilm alone, plankton alone and plankton with biofilm of *P. putida* F1 in a BEC operated at 250 (grey bars) or 500 mV (black bars) (versus an Ag/AgCl reference electrode) for 150 h.
Toluene degradation in the BEC

Toluene degradation by *P. putida* F1 in the BEC under external voltages of 75, 125, 175, 250 and 500 mV was examined and compared with results from a control BEC (Fig. 5). Toluene (100 mg l$^{-1}$) was added to the anode compartment at 0, 39, 68, 112 and 143 h. The residual toluene concentration in the BEC was examined 4 h after the last toluene addition (143 h). The average residual toluene concentration at the control BEC was 80%. However, in the BEC operated under external voltages of 75, 125, 175, 250 and 175 mV, this value was 22%.

The time dependence of the residual toluene concentration in a BEC operated under an external voltage of 125 mV was compared with a control BEC (Fig. 6). The choice of 125 mV stems from the results for bacterial growth (Fig. 1). The residual toluene concentrations were measured 4 h after adding toluene (0, 39, 68, 112 and 143 h). The results indicate that the residual toluene concentration in the control BEC was 80–85%, while in potential poised BEC the residual toluene at 43 h was 51% and decreased to 20% after 147 h. Toluene biodegradation in the poised BEC is thus time-dependent and was assumed to be correlated with bacterial concentration. The residual toluene concentration in the abiotic BEC was about 90%. Thus, 10% of the residual toluene concentration is related to general toluene losses such as evaporation. The results shown in Figs 5 and 6 indicate that biodegradation of toluene under limited oxygen concentration and low poised anodic potentials in a BEC provides an efficient method for treating this contaminant. It has been shown that *G. metallireducens* growing on a graphite anode, serving as the sole electron acceptor, enables the degradation of toluene to carbon dioxide (Zhang et al., 2010). Inserting a graphite anode into heat-killed sediments resulted in an initial phase exhibiting a decrease in toluene, which was assumed to be related to toluene adsorption by the graphite electrode. However, in live sediments with an electrode connected to a potentiostat poised at 300 mV (versus Ag/AgCl), toluene continued to be removed after the initial adsorption phase. This serves as an additional indication that toluene has no electrochemical activity on graphite, and that degradation of toluene occurs only via bacterial-assisted oxidation (Zhang et al., 2010).

Bioremediation of other aromatic hydrocarbons in MFCs has also been reported. Inoculation of *G. metallireducens* into a vessel containing benzoate as the electron donor and a graphite electrode as the only electron acceptor led to current production and benzoate oxidation. Benzoate (0.48 mM) was completely oxidized to CO$_2$ (Bond et al., 2002). A pure culture of *A. dehalogenans* in an MFC, operating with electrodes poised at low potentials which served as an electron donor, enabled the dechlorination of 2-chlorophenol to phenol (Strycharz et al., 2010). In the present study, *P. putida* F1 did not act as an exoelectrogen in the typical MFC. However, under external voltage in the BEC, *P. putida* F1 did behave as an exoelectrogen and degraded toluene, which served as the sole carbon source.

Proteomic analysis in a BEC

Cultures grown in the BEC under an external voltage of 125 mV and in a control BEC were harvested in the exponential phase to investigate the response of *P. putida* F1 to external voltage. The cell sediments were sonicated and the lysates were prepared for SDS-PAGE. As shown in Fig. 7, two protein bands appeared in the BEC lane (to which a lysate of the culture grown in a BEC under an external voltage of 125 mV was applied) but not in the control BEC lane (to which a lysate of the culture grown in a BEC without external voltage was applied). The two protein bands from the BEC lane (cut from the gel) were subjected to total tryptic digestion, and the fragments were resolved by reversed-phase HPLC followed by MS. Comparing the MS data with the NR-NCBI database revealed several proteins, details of which are summarized in Table 1. These proteins were divided into two groups according to their potential activity.

![Fig. 5. Bioremediation of toluene in a BEC under constant external voltages of 75, 125, 175, 250 and 500 mV (versus an Ag/AgCl reference electrode) (black bars), and in a control BEC operated without external voltage (white bars). The measurements were performed 4 h after the last addition of toluene (143 h).](http://mic.sgmjournals.org)
The first group comprised two proteins that play an important role in the respiratory electron transport chain. The first protein consists of 57 aa and is identical to parts of the 197 aa ubiquinol-cytochrome c reductase, iron–sulfur subunit. The second protein is cytochrome c oxidase, cbb₃-type, subunit III, and consists of 70 aa that are identical to parts of the 313 aa ubiquinol-cytochrome c reductase, iron–sulfur subunit. Ubiquinol-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV), the last enzyme in the respiratory electron transport chain, are adjacent components. The electrons from ubiquinol-cytochrome c reductase are transferred to cytochrome c oxidase by cytochrome c. Cytochrome c oxidase transfers the electron to an oxygen molecule, while also generating an electrochemical potential which is linked to ATP synthesis (Froud & Ragan, 1984). Aerobic and facultatively aerobic bacteria usually contain multiple respiratory oxidases that facilitate their adaptation to different environmental O₂ concentrations. The cbb₃-type cytochrome oxidases (cbb₃-Cox) are the second most abundant oxidases and constitute more than 20 % of the haem oxidase superfamily genome sequences (Pitcher & Watmough, 2004). In Gram-negative bacteria, the c-type cytochromes are typically located in the cytoplasmic membrane or the periplasm (Beliaev et al., 2001). An unusual feature of organisms such as Shewanella and Geobacter that enables these species to access insoluble metal electron acceptors is the production of high-molecular-mass c-type cytochromes reported to be associated with the outer membrane (Gaspard et al., 1998; Lloyd et al., 2003). It has been suggested that cytochromes MtrC and OmcA may be intermediate electron carriers that directly ‘functionalize’ electrically conductive nanowires in S. oneidensis MR-1 (Bretschger et al., 2007; Gorby et al., 2006). A mutant strain of G. sulfurreducens, in which the gene for OmcF (a monohaem outer-membrane c-type cytochrome) was deleted, was defective in electricity production in an MFC (Kim et al., 2008). We assume that the appearance of ubiquinol-cytochrome c reductase, iron–sulfur subunit, and cytochrome c oxidase, cbb₃-type, subunit III, in P. putida F1 grown under external voltage in a BEC indicates the importance of these proteins in the electron transfer to the anode.

The second group comprised three proteins that belong to a family of outer-membrane proteins (Omps) that are widespread in Gram-negative bacteria. The first protein
Effect of external voltage on *Pseudomonas putida*

**Table 1. Summary of the MS analysis of protein bands present in the gel (Fig. 7) from an extract of a culture grown in a BEC under an external voltage of 125 mV**

<table>
<thead>
<tr>
<th>Band</th>
<th>Protein</th>
<th>Molecular mass (Da)</th>
<th>Match to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Omp H1</td>
<td>21 536</td>
<td>gi148546458</td>
</tr>
<tr>
<td></td>
<td>OmpW</td>
<td>24 175</td>
<td>gi148545789</td>
</tr>
<tr>
<td></td>
<td>Ubiquinol-cytochrome c reductase, iron–sulfur subunit IV</td>
<td>21 070</td>
<td>gi26988052</td>
</tr>
<tr>
<td>b</td>
<td>OmpF</td>
<td>37 217</td>
<td>gi26988814</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c oxidase, cdby-type, subunit III</td>
<td>34 122</td>
<td>gi26990944</td>
</tr>
</tbody>
</table>

consists of 82 aa that are identical to parts of the 227 aa OmpW from *P. putida* F1. The precise functions of OmpW are unknown, but recent data suggest that it may be involved in the protection of bacteria against various forms of environmental stress and may be involved in the transport of small hydrophobic molecules across the bacterial outer membrane (Hong et al., 2006). The second protein consists of 271 aa that are identical to parts of the 344 aa OmpF. OmpF is a cation-selective porin (Koebnik et al., 2000). It was suggested that OmpF is involved in the control of the penetration of antibiotics such as β-lactams and fluoroquinolones through the enterobacterial outer membrane (Dupont et al., 2007). The third protein consists of 150 aa that are identical to parts of the 201 aa Omp H1 from *P. putida* F1. Omp H1 is believed to act by replacing divalent cations at binding sites on lipopolysaccharides, thereby preventing disruption of the sites and subsequent self-promoted uptake of antibiotics (Bell & Hancock, 1989).

Several studies have emphasized the role of non-electron transport proteins, such as Omps, in electron transfer to a variety of electron acceptors. OmpJ is a putative porin found in the outer membrane of the metal reducer *G. sulfurreducens*. OmpJ was found to be required to maintain the integrity of the periplasmic space necessary for proper folding and functioning of periplasmic and outer-membrane electron transport components (Afkar et al., 2005). The increase in Omps found in the present study may be linked to the function of OmpJ found in *G. sulfurreducens*. Previous studies have suggested that Omps may be required for proper localization of outer-membrane c-type cytochromes and if not properly localized and folded, cytochromes may be proteolytically degraded (Myers & Myers, 2002). Furthermore, Omp35, which is a putative porin in the outer membrane of *S. oneidensis* MR-1, has an indirect effect on an electron acceptor, and was found to be important for the ability of this strain to reduce insoluble metal oxides (Myers & Myers, 2002). We assume that the Omps OmpW, OmpF and Omp H1 found in *P. putida* F1 grown in a BEC under an external voltage of 125 mV have some effect on the electron transport chain, which was forced to be more active under the external voltage.

**Conclusions**

Operating a BEC with *P. putida* F1 in the anode under external anodic potentials led to intensive bacterial culture growth, current production and toluene degradation compared with a control BEC. The anode electrode in the BEC served as an electron acceptor, as there was limited dissolved oxygen, thereby enabling the bacterial cells to generate ATP, which stimulated culture growth. The results of the CV experiment suggest that a soluble molecule may be involved in the charge transfer mediation, and this was released during toluene degradation. This molecule was oxidized on the anode above 200 mV (versus Ag/AgCl), and we suggest that it may be 3-methyl catechol. The residual toluene concentration in the BEC was significantly lower than in the abiotic BEC. Examination of the proteome of bacterial cells grown in a BEC operated at 125 mV revealed two groups of proteins which can be ascribed to electron transfer to the electrode.

In conclusion, the BEC described here has potential for electro-bioremediation of toluene in industrial wastewater under limited dissolved oxygen.

**ACKNOWLEDGEMENTS**

This research was supported in part by the Samaria and Jordan Rift Valley Regional R&D Center, the Research Authority of the Ariel University Center and the Rappaport Foundation for Medical Microbiology, Bar-Ilan University (to Y.N.).

**REFERENCES**


Edited by: D. J. Arp