Ann Microbiol (2016) 66:695–702
DOI 10.1007/s13213-015-1152-8

ORIGINAL ARTICLE

Neutral red as a mediator for the enhancement of electricity production using a domestic wastewater double chamber microbial fuel cell

Reham Fathey[1] · Ola M. Gomaa[1] · Amr El-Hag Ali[2] · Hussein Abd El Kareem[1] · Mohamed Abou Zaid[3]

Received: 1 June 2015 / Accepted: 8 September 2015 / Published online: 29 September 2015

Abstract Neutral red is an electron transport compound that is used in bacteria as a mediator for decolorization, degradation of different organic compounds and in electricity production using microbial fuel cells (MFCs). In this study, bacterial electron transport was followed by monitoring electricity production in a double chamber microbial fuel cell, and measured as current (mA). To enhance electron transfer and consequently increase electricity production, neutral red was added as a mediator. The optimal concentration that had minimal effect on bacterial growth was 15 mM. Spiking the culture with this concentration resulted in a maximal current of 1.36 mA and a power intensity that reached 467.6 mW/m^{2}over 120 h, as compared to 0.74 mA and 209.5 mW/m^2 over a period of just 24 h as a control. This was also confirmed by the increase in initial bacterial conductivity measured in the presence of neutral red as compared to low initial conductivity in the absence of a mediator. To prove that the process of electricity production is correlated to electron transfer, azole and azide were both used to inhibit membrane cytochrome complexes. The results showed a sharp decrease in electrical current production within less than 24 h, suggesting that the process takes place via membrane protein cytochromes. The predominant microorganism present under all previously mentioned conditions was *Buttiauxella agrestis* SW-1 (GenBank ID KP770022) as identified using 16S rRNA phylogenetic analysis, this bacterium was also studied morphologically to detect micropilli. Protein profiles of the membrane showed a distinct band close to that reported for cytochrome *c*; nevertheless, further investigation is required to highlight both its nature and its role.

Keywords Electron transport · Microbial fuel cell · Domestic wastewater · Mediator · Neutral red · Electricity production

✉ Ola M. Gomaa
ola_gomaa@hotmail.com

[1] Microbiology Department, National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority (AEA), Cairo, Egypt

[2] Polymer Chemistry Department, National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority (AEA), Cairo, Egypt

[3] Microbiology Department, Faculty of Science, Ain Shams University, Cairo, Egypt

Introduction

The need for energy has been increasing exponentially with the increase in the world's population. Energy supply is dependent mostly on fossil fuels, which eventually leads to the foreseeable depletion of limited fossil energy sources, carbon dioxide emissions and global warming (Das and Veziroglu 2001). Other sources include nuclear fuel and renewable energy sources (Rahimnejad et al. 2015). Much effort has been devoted to developing alternative electricity production methods (Du et al. 2007). Microbial production of electricity may become an important form of bioenergy in future because microbial fuel cells (MFC) offer the possibility of extracting electric current from a wide range of soluble or dissolved complex organic wastes and renewable biomass (Pant et al. 2010). The simultaneous production of energy and degradation of contaminants in wastewater in MFCs can provide both economic and environmental benefits (Logan and Regan 2006; Choi and Ahn 2015).

A number of factors control the power generation efficiency of MFCs, most important of which are the structure of microorganisms present in the anodic chamber and the

mediators added to the microbial culture (Lin et al. 2014). The microorganisms used in MFCs are of great importance since they play a crucial role in bioelectricity production. Electricity can be produced via different mechanisms: membrane-bound proteins such as cytochromes, electron shuttling via redox mediators in wireless communities or via conductive pili in wired communities, also known as nanowires (Chen et al. 2011). The two main requirements for electron transfer from a microbial cell to an electrode, i.e., direct electron transfer or mediated electron transfer, are facilitated by extracellular electron transfer (Wu et al. 2014); the former involves physical contact of bacterial cells with the anode through a cell membrane or cellular membrane organelle, while the latter requires exogenous redox mediators such as potassium ferricyanide, neutral red, thionine, methylene blue or secondary metabolites such as phenazines and pyocyanine (Lin et al. 2014). Neutral red (NR) has a redox potential of −325 mV, which is close to that of NADH (−320 mV), and is highly permeable to the lipid bilayer (McKinlay and Zeikus 2004). The addition of NR to the anodic compartment is thought to increase the power output (Wang et al. 2011). In our previous work (El-Hag et al. 2015), we used a mediator-less double chamber MFC under optimized conditions to obtain current using domestic waste water consortia; however, the current obtained and longevity of the process needs to be increased. Therefore, the aim of the current study was to investigate the effect of adding NR as a mediator to increase electricity production via enhancing mediated electron transport, and proving its involvement using cytochrome inhibitors and conductivity measurements.

Materials and methods

MFC configuration and operating conditions

A double-chambered MFC was assembled as previously described by El-Hag et al. (2015). A silver electrode preparation and proton exchange membrane were both described also in the same publication. The MFC consisted of two 300 mL bottles (anode and cathode) each containing a silver electrode with a surface area of 10.7 × 5.1 cm totally immersed in the anode and cathode solution. The bottles were joined by a glass bridge held by Parafilm between the flattened ends of two glass tubes. This glass bridge contained a 3.5-cm diameter hole that was covered with proton exchange membrane (PEM) for proton transport. The anode was filled with domestic wastewater collected from Al Gabal Al Asfar stage-2 station wastewater treatment plant (WWTP), Cairo Governorate, Egypt, where wastewater aggregated from the inlet working pumping station (IWPS) stage only with mechanical treatment; the sample was taken during the month of April, representing one of the hot seasons in Egypt. The anodic chamber was anaerobic, the incubation temperature was adjusted to 37 °C, pH 9 and 5 mM sucrose was added to the domestic waste water. The cathodic chamber was filled with 50 mM potassium ferricyanide in 100 mM phosphate buffer pH 7 (El-Hag et al. 2015). A total bacterial count was determined at the end of each step; the bacterium with the highest count and that appears in all samples was chosen for subsequent experiments. Electricity production was measured as current in milli ampere (mA) against time (h) by a digital multi meter (Model number DT-3900, Digiworks Instruments, Concord, ON).

Enhancement and inhibition of electricity production in a domestic waste water consortium

To enhance electricity production, NR was added to the anodic chamber. A series of NR concentrations was used to test the tolerance of the natural microbial consortium in the domestic waste water sample to the dye: final concentrations of NR were 0.15 mM, 1.5 mM, 15 mM and 30 mM. The samples were incubated for 24 h in a static incubator at 37 °C, after which, 0.1 mL of each sample was plated onto nutrient agar plates. At the same time, each sample was scanned using a UV-visible spectrophotometer at the end of the 24 h to determine whether the dye had been degraded compared to the original samples at zero time incubation. The optimal NR concentration was used. To confirm electron shuttle is involved, azole (640 μM) was added to inhibit cytochrome complex I and sodium azide (40 μM) to inhibit bacterial respiration. Both were added simultaneously at the beginning of the experiment to avoid any false current production.

Monitoring electricity

The electric current of the MFC during each experiment was measured by a digital multi meter (Model number DT-3900). Readings were taken at regular intervals with a maximum of 120 h. Voltage was measured in mV and the power intensity was obtained in mW/m^2. All experiments were repeated at least three times to ensure consistency of reported results; the data are presented as average values.

Bacterial conductivity measurements

Bacteria were grown in LB for 24 h, harvested and suspended in 50 mL distilled water, the same concentration of NR was added, and the conductivity of the sample was assessed using dielectric measurements and compared to another sample without NR. The conductivity measurements were carried out using LCR meter type HIOKI 3531 (Hioki, Nagano, Japan). The measuring cell is a parallel plate conductivity cell with platinum electrodes, coated with a platinum black layer to reduce electrode polarization (Iwamoto and Kumagai 1998), which covers an area of 4 cm^2 and a separating distance of 2 cm. The parameters measured were capacitance C (F) at 1 kHz, and conductance G (S) at 10 kHz.

Isolation of genomic DNA and 16S rRNA identification

The predominant bacterium appearing at the end of each physiological step was submitted to 16S rRNA characterization. Genomic DNA was extracted from pure bacterial culture of 24 h grown in LB broth medium at 30 °C, harvested by centrifugation for 15 min at 6000 rpm. Bacterial lysis and DNA extraction were performed according to the manufacturer's instructions using The GeneJET™ genomic DNA purification kit (Fermentas Life Sciences, Vilnius, Lithuania). The purified DNA obtained was re-suspended in 100 μL TE buffer.

Oligonucleotide primers were used to amplify 16S rRNA. The universal primers PA forward (AGAGTTTGAT CCTGGCTCAG) and PH reverse (AAGGAGGTGAT CCAGCCGCA) were used to amplify the 16S rRNA using the following PCR program: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, annealing at 55 °C for 1 min, and 72 °C for 2 min, and final extension at 72 °C for 7 min (Edwards et al. 1989). Amplification was done using Perkin Elmer GeneAmp PCR system 2400 (Perkin Elmer, Überlingen, Germany). Amplicons were visualized by electrophoresis on a 1 % agarose gel (Sambrook and Russell 2001).

Sequencing and phylogenetic analysis

The 16S rRNA PCR product was extracted from the gel using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). DNA sequencing was conducted using an ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit according the manufacturer's instructions (PE Applied Biosystems, Weiterstadt, Germany) on an ABI Prism™ 377XL DNA Sequencer (Perkin Elmer).

The 16S rRNA DNA sequence was submitted to the National Center for Biotechnology Information (NCBI) database and the sequence was compared to other available 16S rRNA sequences representing other relevant species using an automatic alignment tool (Blastn). A phylogenetic tree was constructed by PhyML and visualized by TreeDyn using the online program http://www.phylogeny.fr. Bootstrap values were obtained by drawing a tree using a netwick-generated file using the molecular evolutionary genetics analysis program MEGA 5 (Kumar et al. 2008).

Nucleotide sequence accession number

The 16S rRNA sequence was deposited with the NCBI GenBank nucleotide sequence database under accession number KP770022.

Transmission electron microscopy

The predominant bacterium was subcultured into LB medium and incubated at 37 °C for 24 h, the culture was centrifuged at 6000 rpm for 15 min; cells were washed in saline and fixed overnight in 2.5 % glutaraldehyde, washed twice for 15 min in 0.1 M sodium phosphate buffer (pH 6), post-fixed in 2 % osmium tetroxide, then washed twice in distilled water, after which cells were dehydrated in 95 % and 100 % ethanol and pipetted onto carbon-coated copper grid (mesh ×200). Scanning was performed using a JOEL 100cx JEM transmission electron microscope at 80 kV (magnification 8300× and 33,000×). Pictures were captured using a CCD camera attached to the microscope.

SDS-polyacrylamide gel electrophoresis

For purification of membrane proteins, the predominant bacterial sample was separated by centrifugation to remove precipitated polysaccharides. The cells were washed with PBS, membrane proteins were extracted using RIPA buffer. Equal amounts (100 μg) of protein were loaded per lane along with pre-stained protein ladder (250–10 kDa). Samples were then electrophoresed at room temperature using Tris-glycine buffer (pH 8.3) at 50 V through the stacking polyacrylamide gel (6 %) and at 100 V through the resolving polyacrylamide gel (10 %) (Laemmli 1970). After electrophoresis, the polyacrylamide gels were stained with Coomassie Brilliant blue R-250; images were captured by a CCD camera, molecular weights were analyzed using AlphaImager 2200 software for protein analysis (Protein Simple, San Jose, CA). Protein concentrations were determined according to Lowry et al. (1951).

Results

Enhancing electron transfer by adding NR as a mediator

Figure 1 shows the response of the microbial consortia to the addition of different concentrations of NR. The total bacterial count remained stable at different low dose NR concentrations (30×10^6), but there was a marked decrease upon addition of 30 mM NR, with the total bacterial count being reduced to 1.6×10^6. Thus, we chose 15 mM NR as the optimal concentration for the enhancement of total domestic waste water bacteria; the figure also shows that there was no decolorization of the dye. Figure 2 shows that addition of 15 mM NR resulted in an increase in the current produced, reaching a maximum of 1.36 mA that was stable for over 100 h. The corresponding control was only 0.78 mA, thus almost double the current was produced when 15 mM NR was added. Figure 3 represents the difference in power intensity produced in the presence of an electron transfer enhancer and an electron transfer inhibitor.

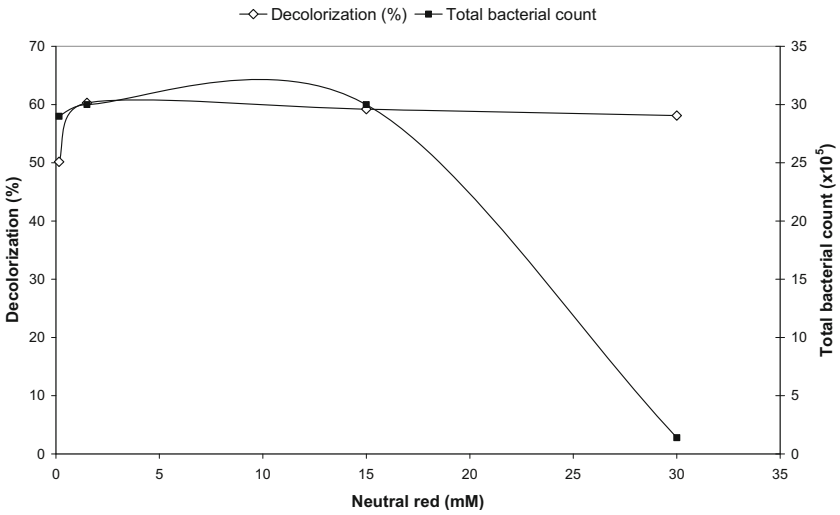

Fig. 1 Effect of adding different concentrations of neutral red (NR) on total bacterial count and decolorization of a domestic waste water consortium

The results show that the power reached 467.6 mW/ m^2 over a period of 120 h, as compared to 209.5 mW/ m^2 within 24 h of operation for control samples. On the other hand, there was a sharp increase of 174 mW/ m^2 at 6 h in samples amended with electron transfer inhibitors, which was followed by a very sharp drop to zero after 26 h. There was an initial increase in the conductivity of NR-amended samples, which took place over 60 min, as compared to a steady state for control samples (Fig. 4).

Characterization of the predominant bacteria

At the end of each MFC operation process, a sample was taken and spread onto LB agar plates after appropriate serial dilution. The most predominant bacteria were taken and identified by sequencing of 16S rRNA. The sequence was submitted to the GenBank database and given the name *Buttiauxella agrestis* SW-1 as shown in the phylogenetic tree (Fig. 5). Figure 6 represents a transmission electron micrograph (TEM) of the strain under study. The electron micrograph shows three pili of the following lengths; 123, 125 and 146 nm.

Protein profile of the predominant bacteria membrane

To study the membrane of the predominant bacteria, SDS-PAGE electrophoresis was performed on the bacterial membrane of *B. agrestis*. The results show that there are variations in the protein bands; 15 bands were observed at different sizes (in kDa) as follows: 142.73, 135.88, 126.71, 119.84, 110.10, 103.22, 97.5, 90.5, 84. 32, 73.44, 53.38, 43.65, 28.75, 19.01, the last band was below the range of the protein markers used (Fig. 7).

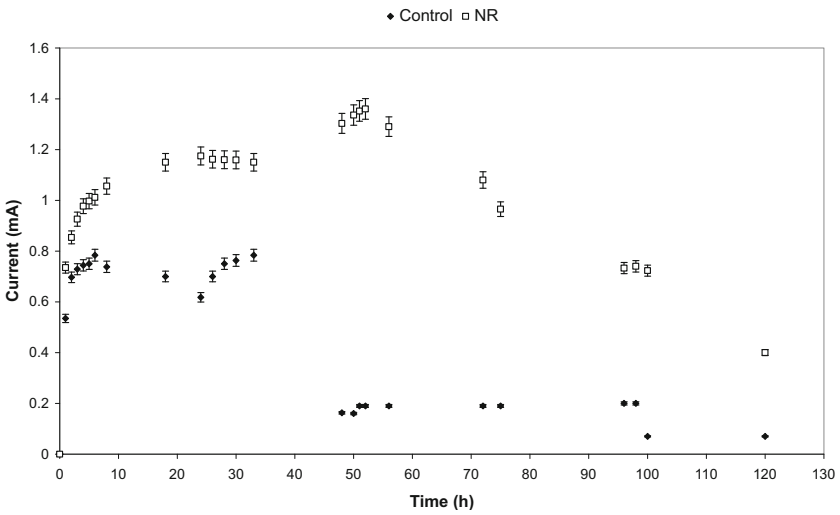

Fig. 2 Current production over time in the presence and absence of NR

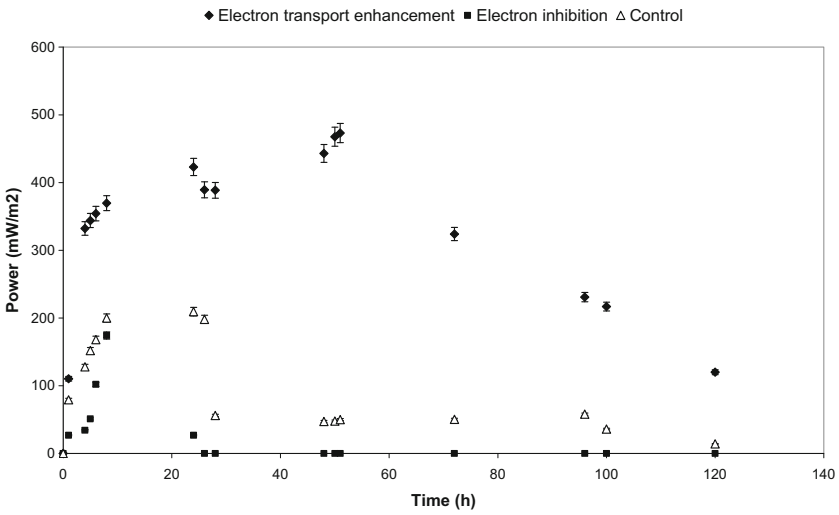

Fig. 3 Changes in power intensity over time in the presence of NR, azole and azide as compared to that of control

Discussion

Microbial fuel cells are considered a very practical approach with which to counteract our diminishing sources of power. The advantages of MFCs are doubled if waste water is used, thus combining waste water treatment with electricity production (Liu et al. 2011). In our previous work, production of electricity was obtained using a mediator-less double chamber MFC. The electricity was sustained for only 24 h after optimization of both the anodic and cathodic chambers. In the current work, the physiology of the microflora was investigated closely with special emphasis on the impact of adding NR to act as a mediator and enhance electron shuttle in the bacterial consortium present in domestic waste water.

How the electron transfer chain of the bacterial strain can be converted to electricity generation or color removal still requires more exploration (Chen et al. 2011).

To test the tolerance of the domestic waste water consortia to NR, the total bacterial count was calculated in the presence of different NR concentrations, the results showed that using 15 mM of the dye was enough to obtain good growth and, at the same time, no decolorization took place, which means that the NR is not consumed by the present consortia. The addition of NR acted to enhance electron transfer and the power increased ten-fold as compared to that obtained in a mediator-less system, NR can pass through or be absorbed by microbial cytoplasmic membranes, specifically through the lipid bilayer (McKinlay and Zeikus 2004), and has the ability to couple with NADH, which facilitates electrical power reduction by the microbe for both growth and production of metabolites (Osman et al. 2012). The redox reaction of NR with NADH is illustrated by the following equations (Lin et al. 2014):

$$NR_{Ox} + NADH \longrightarrow NAD^{+} + NR_{Red}$$
$$NR_{Red} \longrightarrow NR_{Ox} + 2^{e-} + H^{+}().$$

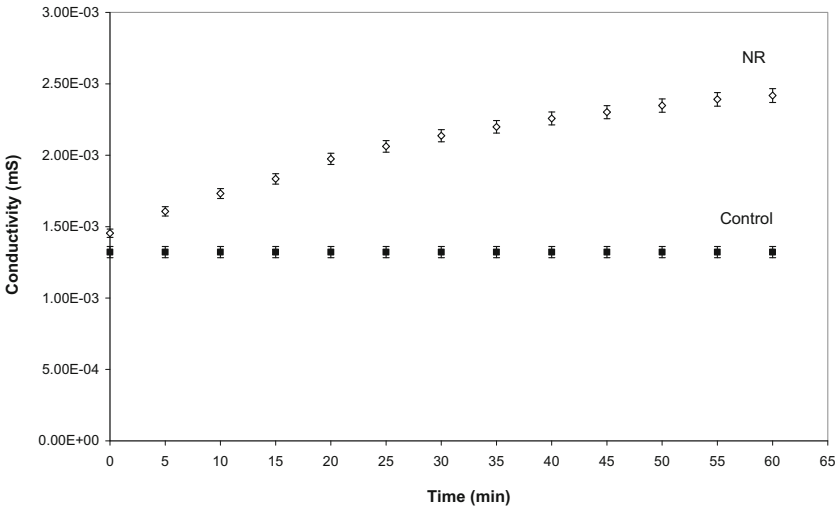

Fig. 4 Conductivity measurements in the presence and absence of NR during the first 60 min; readings were taken every 5 min

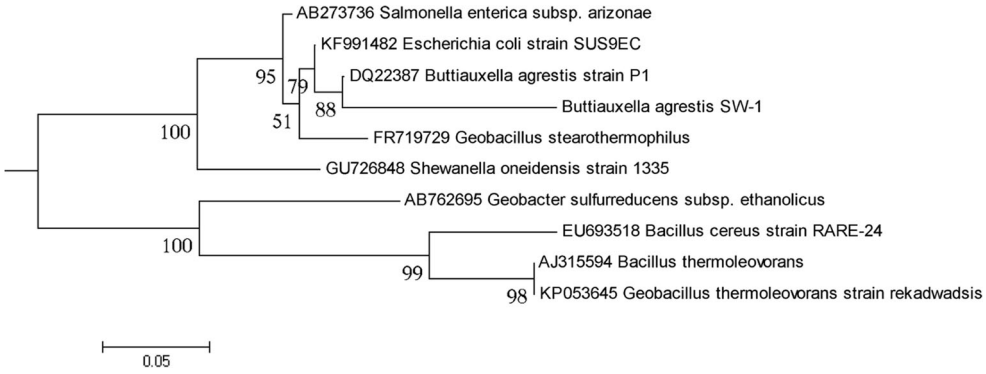

Fig. 5 Phylogenetic tree representing the relatedness of the predominant strain *Buttiauxella agrestis* SW-1 to other relevant strains in GenBank

The addition of azide to the anodic chamber has been reported, with contradictory results. Zhou et al. (2014) reported that the addition of azide to the microbial community resulted in an increase in power density as proven through adding electron mediator inhibitors. On the other hand, Bennett et al. (1996) suggested that the addition of azide inhibits cytochrome oxidase without affecting other respiratory chain enzymes. Barbot et al. (2010) stated that sodium azide causes inhibition of respiration in bacteria. Azole compounds are known to inhibit cytochrome complex I (Fredrich et al. 1994). To study the bioelectrochemical behavior of the predominant bacterium in the presence and absence of NR, conductivity measurements were performed. The results obtained showed a marked difference in both samples, explaining why cultures amended with NR exhibit a sharp increase in electricity production as compared to those lacking NR as a mediator. Different single microorganisms and mixed cultures have been known to be electrochemically active via the electron transfer pathway (Carmona-Martinez et al. 2011).

The microorganisms present in the anodic chamber control the production of bioelectricity. There are two predominant model organisms mostly studied for understanding the bioelectrochemical behavior leading to electron transfer, belonging mainly to the families Geobacteraceae and Shewanellaceae, the former being very well studied from different aspects, the latter still in need of further studies since there is proof of a more complex behavior (Carmona-Martinez et al. 2011). *Proteus hauseri* and *Klebsiella pneumoniae* have also been reported as bioelectricity producers (Chen et al. 2011). It is worth mentioning that microbial structural

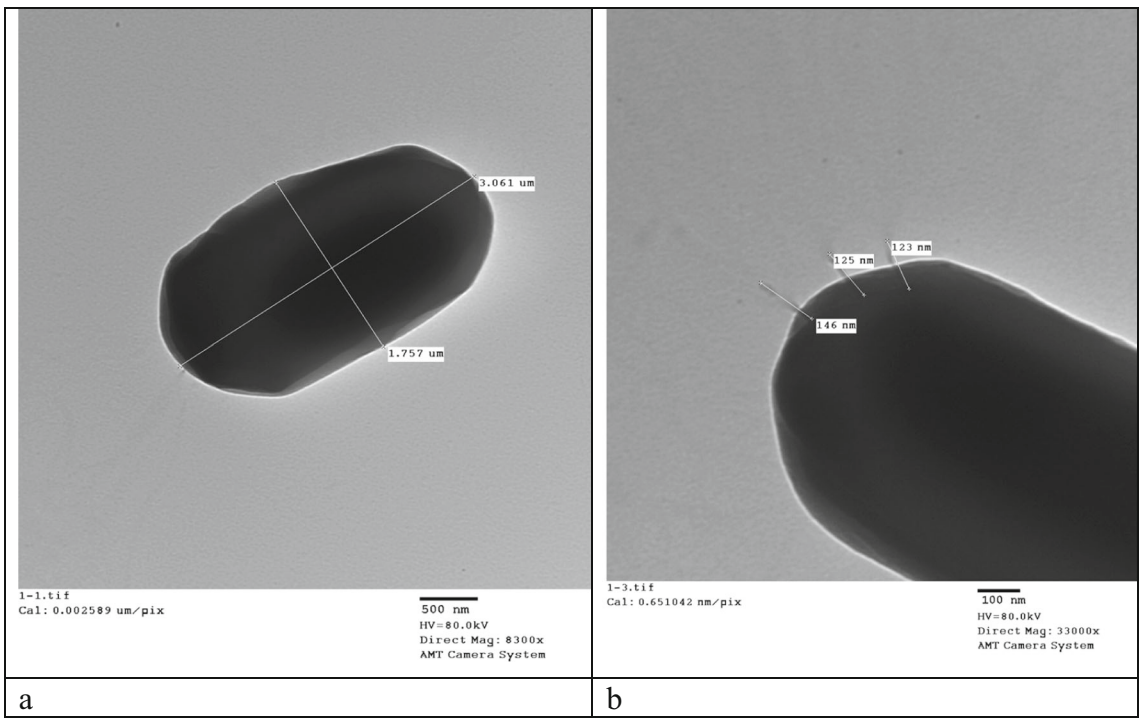

Fig. 6a,b Micrograph of *Buttiauxella agrestis* SW-1 examined using transmission electron microscopy. **a** Whole bacterium at a magnification of 8,300×. **b** Micropilli shown at a magnification of 33,000×

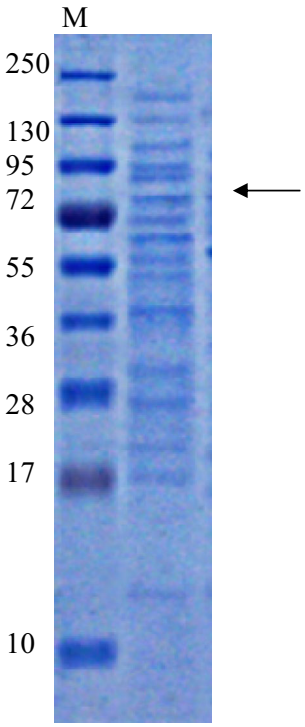

Fig. 7 Protein profile of membrane of the predominant strain *Buttiauxella agrestis* SW-1

communities change under different optimization conditions, this information could be employed in the design of MFCs (Lin et al. 2014). The presence of pollutants in MFCs controls the diversity of the microflora in these environments (Jiang et al. 2015). In the present study, *Buttiauxella agrestis* was the predominant strain and, to our knowledge, this is the first reported research mentioning this strain as a bioelectricity producer. A TEM image of *B. agrestis* showed the presence of nanowire pili, which are suspected to be one of the direct electron transfer mechanisms in bacteria (Carmona-Martinez et al. 2011).

Membrane-bound redox proteins such as cytochromes play a crucial role in influencing the performance of bioelectricity production, and are considered one of the mechanisms of microbial electron transfer (Chen et al. 2011). Multiheme cytochrome is essential for extracellular electron transfer (Costa et al. 2015). The sizes of the proteins observed in the present report are very close to those reported for outer membrane cytochromes. Myers and Myers (1997) reported that the four outer membrane proteins have apparent molecular masses of 150 kDa, 83 kDa, 65 kDa and 53 kDa, and the 83 kDa protein was reported specifically as a *c*-type cytochrome. The relevant band in our study was at 84.32 kDa, which might correspond to cytochrome *c*, but further identification will require a full spectral analysis after purification of the this band.

Understanding the electron transfer process through deciphering the secrets of the structure/function of membrane cytochromes is currently considered one of the most interesting area of research in the field of bioremediation (Edwards et al. 2014); therefore, the aim of our future work is to investigate membrane cytochromes with a view to enhancing electricity production simultaneously with the bioremediation of waste water.

Conclusion

Appropriate optimization of the microbial community plays a crucial role in effective bioelectricity generation in a domestic waste water MFC. The longevity of the MFC can be enhanced through the use of mediators that help to increase electron transport, hence increasing the current.

References

Barbot E, Seyssiecq I, Roche N, Marrot B (2010) Inhibition of activated sludge respiration by sodium azide addition: effect on rheology and oxygen transfer. Chem Eng J 163:230–235

Bennett MC, Maldy GW, Fleshnert M, Rose GM (1996) Synergy between chronic corticosterone and sodium azide treatments in producing spatial learning deficit and inhibiting cytochrome oxidase activity. Proc Natl Acad Sci USA 93:1330–1334

Carmona-Martinez AA, Harnisch F, Fitzgerald LA, Biffinger JC, Ringeisen BR, Schroder U (2011) Cyclic voltametric analysis of the electron transfer of *Shewanella oneidensis* MR-1 and nanofilament and cytochrome knock-out mutants. Bioelectrochemistry 81:74–80

Chen BY, Wang YM, Ng IS (2011) Understanding interactive characteristics of bioelectricity generation and reductive decolorization using *Proteus hauseri*. Bioresour Technol 102:1159–1165

Choi J, Ahn Y (2015) Enhanced bioelectricity harvesting in microbial fuel cells treating food waste leachate produced from biohydrogen fermentation. Bioresour Technol 183:53–60

Costa NL, Carlson HK, Coates JD, Louro RO, Paquete CM (2015) Heterologous expression and purification of a multiheme cytochrome from a Gram-positive bacterium capable of performing extracellular respiration. Protein Expr Purif 111:48–52

Das D, Veziroglu TN (2001) Hydrogen production by biological process: a survey of literature. Int J Hydrog Energy 26:13–28

Du Z, Li H, Gu T (2007) A state of the art review on microbial fuel cell: a promising technology for wastewater treatment and bioenergy. Biotechnol Adv 25:464–482

Edwards U, Rogalt T, Blocker H, Emde M, Bottger EC (1989) Isolation and direct complete nucleotide determination of entire genes, characterization of a gene coding for 16S ribosomal RNA. Nucleic Acid Res 19:7843–7853

Edwards MJ, Baiden NA, Johs A, Tomanicek SJ, Liang L, Shi L, Fredrickson JK, Zachara JM, Gates AJ, Butt JN, Richardson DJ, Clarke TA (2014) The X-ray crystal strucrure of *Shewanella oneidensis* OmcA reveals new insight at the microbe-mineral interface. FEBS Lett 588:1886–1890

El-Hag Ali A, Gomaa OM, Fathey R, Abd El Kareem H, Abou Zaid M (2015) Optimization of double chamber Microbial Fuel Cell for domestic wastewater treatment and electricity production. J Fuel Chem Technol 43:1–8

Fredrich T, Van Heek P, Leif H, Ohnishi T, Forche E, Jansen BR, Trowitzsch-Keinast W, Hofle G, Reichenbach H, Weiss H (1994) Two binding sites of inhibitors in NADH: ubiquinone oxidoreductase (complex I) Relationship of one site with the ubiquinone-binding site of bacterial glucose:ubiquinone oxidoreductase. Eur J Biochem 219:691–698

Iwamoto S, Kumagai H (1998) Analysis of the dielectric relaxation of a gelatin solution. Biosci Biotechnol Biochem 62:1381–1387

Jiang YB, Deng H, Sun DM, Zhong WH (2015) Electrical signals generated by soil microorganisms in microbial fuel cells respond linearly to soil Cd^{2+} pollution. Geoderma 255–256:35–41

Kumar S, Dudley J, Nei M, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299–306

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lin CW, Wu CH, Chiu YH, Tsai SL (2014) Effects of different mediators on electricity generation and microbial structure of a toluene powered microbial fuel cell. Fuel 125:30–35

Liu G, Yates MD, Cheng S, Call DF, Sun D, Logan BE (2011) Examination of microbial fuel cell start-up times with domestic wastewater and additional amendments. Bioresour Technol 102: 7301–7306

Logan BE, Regan JM (2006) Microbial fuel cells-challenges and applications. Environ Sci Technol 40:5172–5180

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:256–275

McKinlay B, Zeikus JG (2004) Extracellular iron reduction is mediated in part by neutral red and hydrogenase in *Escherichia coli*. Appl Environ Microbiol 70:3467–3474

Myers CR, Myers LM (1997) Outer membrane cytochromes of *Shewanella putrefaciens* MR-1 spectral analysis, and purification of the 83-kDa c-type cytochrome. Biochim Biophys Acta 1326: 307–318

Osman MH, Shah AA, Walsh FC (2012) Recent progress and continuing challenges in biofuel cells. Part II Microb Biosensing Bioelectron 26:953–963

Pant D, Bogaert GV, Diels L, Vanbroekhoven K (2010) A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. Bioresour Technol 101:1533–1543

Rahimnejad M, Adhami A, Darvari S, Zirepour A, Oh SE (2015) Microbial fuel cell as new technology for bioelectricity generation: a review. Alexandria Eng J 54:745–756

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY

Wang K, Liu Y, Chen S (2011) Improved microbial electrocatalysis with neutral red immobilized electrode. J Power Sources 196:164–168

Wu S, Xiao Y, Wang L, Zheng Y, Chang K, Zheng Z, Yang Z, Varcoe JR, Zhao F (2014) Extracellular electron transfer mediated by flavins in Gram-positive *Bacillus* sp. WS-XY1 and yeast *Pichia stipitis*. Electrochem Acta 146:564–567

Zhou X, Qu Y, Kim BH, Choo PY, Liu J, Du Y, He W, Chang IS, Ren N, Feng Y (2014) Effects of azide on electron transport of exoelectrogens in air-cathode microbial fuel cell. Bioresour Technol 169:265–270