BAU Journal - Science and Technology

Volume 4 | Issue 1
ISSN: 2706-784X

Article 5

December 2022

ISOLATION AND CHARACTERIZATION OF CELLULOSE DEGRADING MICROORGANISMS GENERATING ELECTRICITY USING MICROBIAL FUEL CELL

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Recommended Citation

Fayad, Lama; Amer, Rania; Abd El Nabi, Ahmad; Hammoud, Ali; and Yusef, Hoda (2022) "ISOLATION AND CHARACTERIZATION OF CELLULOSE DEGRADING MICROORGANISMS GENERATING ELECTRICITY USING MICROBIAL FUEL CELL," *BAU Journal - Science and Technology*: Vol. 4: Iss. 1, Article 5.
DOI: https://www.doi.org/10.54729/ROGR4946
Available at: https://digitalcommons.bau.edu.lb/stjournal/vol4/iss1/5

1. INTRODUCTION

The world is encountering critical ecological and energy issues, essentially attributable to the increased burning of nonrenewable fuel source to insure the basic needs of society (Rojas-Flores *et al*., 2021). Electricity has been developed into fundamental element for living and for achieving daily activities. The need for this power source is consistently rising, which means high fossil fuel production (Rojas Flores *et al*., 2020). However, burning fossil fuels has harmful consequences for the environment as a result of the release of greenhouse gases such as carbon dioxide that cause atmospheric pollution and global warming (Islam *et al*., 2020). As a result of high degree of pollution correlated with this, the academic world is following new strategies to create new ecofriendly sources of energy. One of these sustainable resources is the microbial fuel cells consisted of two chambers anode and cathode, connected by a proton-exchange membrane or a salt bridge (Rojas Flores *et al*., 2020).

A microbial fuel cell is an apparatus which turns chemical energy emitted from the oxidation of organic carbon sources used as substrates by microbes to generate electricity thus confirming to be an effective manner of renewable energy generation. The electrons emitted owing to the bacteriological metabolism are trapped to preserve a stable power density, with the absence of an efficient carbon release in the environment (Tharali *et al*., 2016). At the anodic chamber, the microorganisms oxidize the carbon-based compounds present in a substrate (fuel) and generate electrons and protons. Protons are spreaded from the anodic chamber, through a membrane or a salt bridge, directly to the cathodic chamber. At this place, they react with the incoming electrons that flow through an external circuit. The oxidation reaction in the anodic chamber is stabilized by the oxygen reduction reaction in the cathode, where oxygen play the role of an electron acceptor (Rojas Flores *et al*., 2020).

Anodic oxidation half reaction: $C_6H_{12}O_6 + 6H_2O \longrightarrow 6CO_2 + 24H^+ + 24e^-$

Cathodic reduction half reaction: $6O_2 + 24H^+ + 24e^- \longrightarrow 12H_2O$ (Songera, 2012)

Voltage in MFCs is produced due to the potential difference of the electron acceptor and the oxidative system (Rahmani *et al*., 2022).

The premier fuel cell was constructed in 1839. In 1911, Potter explained the idea of bioconversion to generate electrical current (Li *et al*., 2018). Besides that, in 1999, microbial fuel cell gained a lot of attention because it was confirmed that the mediator was not a mandatory element within MFCs (Parkash, 2016). Moreover, the findings on electricity production from renewable biomass and wastes using microorganisms facilitate the research in microbial fuel cell field. Additionally, MFC innovation was drawn a lot of attention when Time Magazine announced *Geobacter sulfurreducens* KN400 which is able to generate high electric current, as one of the top 50 principal devices for 2009 (Barua *et al*., 2019). A major benefit of MFCs is that they commonly have extended work duration up to five years. Furthermore, they are capable to oxidize organic compounds to carbon dioxide using oxidation-reduction reactions also they can act in moderate conditions. Thus, MFC could perform a significant role in sustainable technology for the generation of electricity and sewage treatment (Islam *et al*., 2020).

Organic compounds like carbohydrates (glucose, sucrose and starch) or low molecular weight organic acids (amino acids, xylose, fumarate, oxalate and acetate) are widely used as electron donors because they are biodegradable. Similarly, sewage and marine sediments have been applied as carbon and energy source in microbial fuel cells (Hassan *et al*., 2012).

Cellulose, the most abundant macromolecule in the world, is of great interest as a renewable energy resource (Rezaei *et al*., 2009a). For that reason, cellulosic products are attractive substrates for biofuels and energy transmitters such as biodiesel, hydrogen, or ethanol (Rezaei *et al*., 2008). In addition, cellulose can be utilized as a fuel in a microbial fuel cell (MFC) for immediate electricity production using exoelectrogenic bacteria (Ren *et al*., 2007; Rezaei *et al*., 2007; Rismani-Yazdi *et al*., 2007; Rezaei *et al*., 2008; Hassan *et al*., 2012). The first research study conducted on the utilization of cellulose as electron donor in microbial fuel cell was done by Niessen *et al*., 2004. It is demonstrated that an insoluble cellulose can be utilized for electricity production (Hassan *et al*., 2012).

In Lebanon, the electricity sector is notably deteriorated, suffering from scant supply for many years. The productive capability is lower than the peak demand that is around 1.5 gigawatts (GW) or 219.78 megawatts (MW) per million inhabitants (Kassem, 2022). Furthermore, waste accumulation in Lebanon has enlarged considerably over the last few decades. Particularly as a result to the increase in society wellbeing, civilization, migration of Syrian emigrants, and high population levels. Examination of waste content shows that the largest waste ratio is organic matter (52%), succeeded by papers and cardboards (16%), plastics (12%), others (11%), metals (6%) and glass (3%). For this reason, new actions are required to treat the Lebanese waste produced nowadays to avoid it from causing problems for future generations (Abbas *et al*., 2017).

The goal of this study was the isolation and identification of exoelectrogens from soil in Lebanon capable of cellulose degradation to generate power using double chambered microbial fuel cell technology.

2. MATERIALS AND METHODS

2.1 Soil Sampling and Maintenance

Soil samples used to isolate electrogenic bacteria were collected from different dumpsite located at Daraya, Iklim El kharoub, Lebanon, by using clean scalpel then transferred to sterile plastic bags. These samples were maintained at 4˚C for further microbiological analysis.

2.2 Soil Property Measurements

Soil physiochemical properties were analyzed using routine methods. Soil electrical conductivity (EC) and soil pH at 1:2 (soil: water) were determined using conductivity meter (Mi 170 Bench Meter) and pH meter (Ohaus starter 3100), respectively (Mylavarapu *et al*., 1993). Moisture content is determined by drying soil sample at 105°C in a drying oven. Soil organic carbon (OC), organic matter and ash were measured by ignition in a muffle furnace at 440℃ using ASTM D 2974 standard test methods (ASTM D2974-13, 2014). In addition, total nitrogen (TN) was measured using Kjeldahl digestion method (Kjeldahl, 1883). Total phosphate and total phosphorus (TP) were also analyzed using colorimetric method that uses a complexation reaction to produce a colored complex of molybdate and phosphorus measured at 650 nm (Method 10209/10210/843/844/845, 2008).

2.3 Isolation and Screening Experiments for Bacterial Production of Cellulase

Soil samples collected from dumpsite were serially diluted. One gram of soil sample was combined with 99 ml of sterile distilled water in a conical flask, and then serial dilution up to 10^{-9} was done. 0.1 ml from each dilution was inoculated on the starch casein agar medium for the isolation of bacteria (Soluble starch, 10.0; K_2HPO_4, 2.0; KNO_3, 2.0; casein, 0.3; $MgSO_4$.7H2O, 0.05; $CaCO_3$, 0.02; $FeSO_4$.7H2O, 0.01; agar, 15.0 g/l). Morphologically different colonies were picked up and purified on carboxymethyl cellulose agar medium (Carboxymethyl cellulose, 2.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; NH_4NO_3, 1.0; $FeCl_3 \cdot 6H_2O$, 0.05; $CaCl_2$, 0.02; yeast extract, 5.0 g/l) and cellulose congo-red agar medium (Cellulose, 2.0; KH_2PO_4, 0.5; $MgSO_4$, 0.25; congo-red, 0.2; gelatin, 2.0; agar, 15.0 g/l).

2.4 Phenotypic and Morphological Characterization of the Bacterial Strain

Cell morphology was inspected microscopically with Gram staining technique. Certain biochemical tests were done such as motility, oxidative fermentative test, catalase, oxidase, citrate utilization, Triple sugar iron test, Methyl Red, Voges-Proskauer, and indole production. Additionally, the production of lipase, amylase, cellulase, pectinase and gelatinase were checked (Shirling & Gottlieb, 1966).

Morphological characters such as colony description, aerial hyphae and substrate mycelium were observed as described by Cross and Goodfellow method (Cross & Goodfellow, 1973; Bhat *et al*., 2014).

The most important physiological criteria used for taxonomical characterizations were: Melanin pigment-production and carbon utilization such as utilization of dextrose, lactose, mannitol and sucrose, were investigated as described in the International Streptomyces Project (Shirling & Gottlieb, 1966; Bhat *et al*., 2014).

DOI: https://www.doi.org/10.54729/ROGR4946

2.5 Molecular Identification of the Bacterial Strain

Molecular characterization was further employed to obtain a full identification of selected microbial isolates. The chosen cellulosic bacterial isolate was detected using 16S ribosomal RNA (rRNA) gene sequencing. Genomic DNA extraction kit was utilized to extract the total genomic DNA from the bacterial cells. The universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG3') and 1492R (5'- TAC CTT GTT ACG ACT T3') were used to perform the amplification reaction in a thermal cycler. The applied polymerase chain reaction programme was started with denaturation at 95° C for 5 minutes succeeded by 30 cycles of 95° C for 1 minute, 55° C for 1 minute and 72 ° C for 2 minutes and a final extension at 72 ° C for 10 minutes. The PCR purification kit (Sigma-Aldrich, USA) was used to purify amplified DNA. The purified PCR product was sequenced by GATC using the ABI 3730xl DNA sequencer and screened for sequence similarity to the known 16S rDNA sequence in the GenBank database by the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was built utilizing the neighbour-joining DNA distance algorithm (Saitou & Nei, 1987) through MEGA 3. The partial sequence of 16S rRNA gene of the selected isolate was presented to NCBI GenBank, and an accession number was determined.

2.6 Construction of Double Chambered Microbial Fuel Cell

A laboratory scale double-chambered microbial fuel cell reactor made of polypropylene plastic was built with an anode and a cathode compartment as shown in Fig.1. The vacant capacity for every compartment is 1.4 L. Each compartment possesses a broad opening closed firmly with a cover containing three bores. The first one in the anodic chamber was to pass a pliable plastic tube to insert Nitrogen gas (N_2). The second one holds a copper wire (3 mm diameter) connected to the carbon anode with total surface area equals 12.5 cm^2. The third bore sealed with a rubber stopper was used to collect sample. While, the first one in the cathodic chamber was also to pass a pliable plastic tube to splash air, the second one holds a copper wire (3 mm diameter) connected to the carbon cathode with total surface area equals 12.5 cm^2.The last one was used to exit the excess air. The two chambers were joined together via an agar salt bridge, a resistant polyethylene tube with an inner diameter of 2 cm and 15 cm length. This tube was placed between anode and cathode chambers and the sealing was established by rubber sheets inserted between each frame. The anolyte was continually stirred by a magnetic stirrer. The catholyte was consistently splashed by air. The experiments have been done at pH 7.0 and at room temperature 25 ° C.

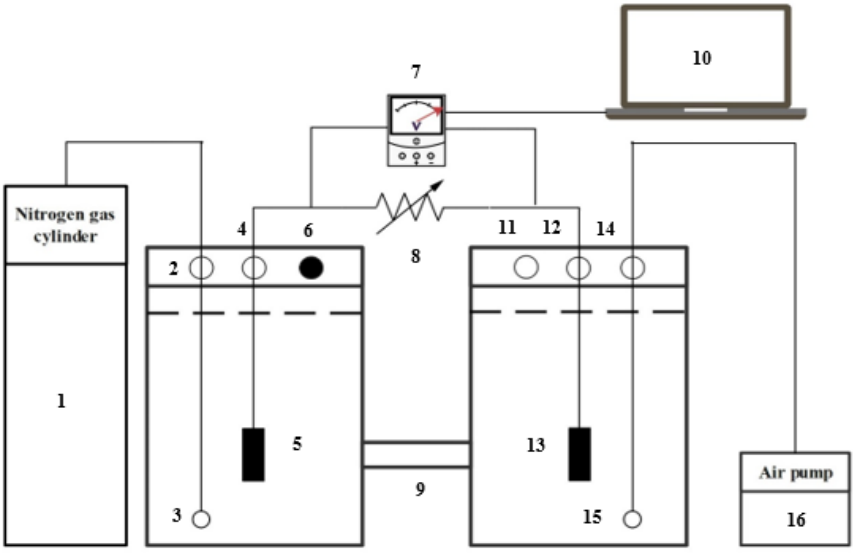


Fig.1: Schematic diagram of double-chambered microbial fuel cell. 1. Nitrogen gas (N_2) cylinder, 2. Bore for N_2 sparging, 3. Plastic tube for N_2 sparging, 4. Bore for passing anode, 5. Anode compartment, 6. Sampling bore, 7. Voltmeter, 8. Variable external resistance, 9. Separator for salt bridge, 10. Computer, 11. Bore for excess air exit, 12. Bore for passing cathode, 13. Cathode compartment, 14. Bore for air sparging, 15. Plastic tube for air sparging, 16. Air pump.

2.7 Preparation of Electrodes

Carbon rods made in Germany were utilized as electrodes to gather the electrons in the two anodic and cathodic compartments by copper wire connections. Both electrodes were soaked in 1M hydrochloric acid succeeded by 1M sodium hydroxide for 1 hour each, to neutralize it and to eliminate impurities. After that, they were kept in distilled water before use.

2.8 Screening Method for Electricity Generation in Double-Chambered Microbial Fuel Cell

2.8.1. Effect of carbon sources used as substrates on electricity production

Two carbon sources were tested for maximum potential difference: glucose and cellulose. The salt bridge was prepared by using 3% agar and 1M KCl, autoclaved at 15 psi for 15 minutes. Sterile 1.2 liter of M9 medium (carbon source, 5; yeast extract, 1.0; NH_4Cl, 1.0; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; NaCl, 5.0; $MgSO_4$, 0.05; $CaCl_2$, 0.005 g/l) was added into anode chamber with different carbon sources. The same medium was added into cathode chamber but without carbon sources and yeast extract. Anaerobic conditions were maintained into anode chamber by Oxygen Free Nitrogen (OFN) gas input. All procedures were done at room temperature (25 ^{o} C) and at pH 7. Results were reported after a specified period of time.

2.8.2. Power calculation

The voltage and the current in the double-chambered microbial fuel cell were measured using Arduino uno instrument connected to PC.

Potential difference measures are transformed to current values through Ohm's law: $V = I \times R$

Where V = voltage, V; I = current, A; R = resistance, Ω

The power output is determined using: $P = I \times V$

Where P = power, W.

3. RESULTS AND DISCUSSION

3.1 Soil Property Measurements

Table 1 represents the tested parameters of the soil sample collected from the dumpsite in Lebanon. pH of the soil sample was 7.49 which indicates that the soil was neutral. The measurement of pH has been described as a straight reflection of the soil chemical quality.

Electrical conductivity is a rapid, easy and reasonable manner to examine the quality of the soil. Electrical conductivity of the soil sample was 1.32 ds/m which is above the normal range between 200 µS/cm and 1200 µS/cm. This value indicates an excess of soil nutrients and a salinity problem as the electrical conductivity of a soil sample rises with high salt concentration. While, soil solution with electrical conductivity less than 200 µS/cm are considered as sterile soil with low microbial activity.

The moisture content of the soil sample was 5.54%, which is less than the standard value ranging from 11 to 17%. The quantity of water present in a substance is termed moisture content. This value describes the soil's absorption of nutrients and their effect on soil texture.

The ash content of the soil sample was 90.76%. The substance remaining after ignition contains the ash which is the inorganic residue that persists after removal of organic matter and water by heating.

The organic matter content of the soil sample was 9.23%. Total organic matter (TOM) or soil organic matter (SOM) composed of animal and plant debris, cells and tissues of soil creatures, and their materials, have several advantages over the physical and chemical properties of soil, as well as its ability to provide controlled benefits to the ecosystem.

The organic carbon content which is the consequence of the rate of carbon input and decayed organic matter of the soil sample was 5.36%. This value is an indication of the activity of microorganisms causing emission of organic carbon and acidic substances.

This high level of organic matter and organic carbon at the dumpsite is the result of the addition of certain garbage like tree branches, sawdust, household waste and the increased microbial activities.

Total nitrogen content of the soil sample was 0.66% which is in the range of the FAO standards value (0.1-2). Nitrogen supply is important in carbohydrate utilization. Severe nitrogen deficiency means deposition of carbohydrate in plant cells causing thickening of the cell wall. While nitrogen abundance increases most of the forage harvests and can postpone grain maturity and leave the harvest vulnerable to fungal infection. Deficiency of nitrogen can be also noticed by a yellow color of the foliage, which is accompanied by a lack of growth.

Total phosphorus content was 22.32 ppm which is above the FAO standards value (10-20). Low values of available phosphorus showed that there is no constituent of domestic wastes such as soaps, and detergents present in the refuse dumpsites.

These outcomes are similar to the findings of several studies investigating soil quality (Agbeshie *et al*., 2020; Akinbile, 2016; Mekonnen *et al*., 2020; Vijayalakshmi *et al*., 2020).

Table 1: Analysis results of parameters of soil sample

Tested parameter	Value
pH	7.49
Electrical conductivity (ds/m)	1.32
Moisture content	5.54%
Ash content	90.76%
Organic matter	9.23%
Organic carbon	5.36%
Total nitrogen	0.66%
Total phosphate	68.432 ppm
Total phosphorus	22.32 ppm

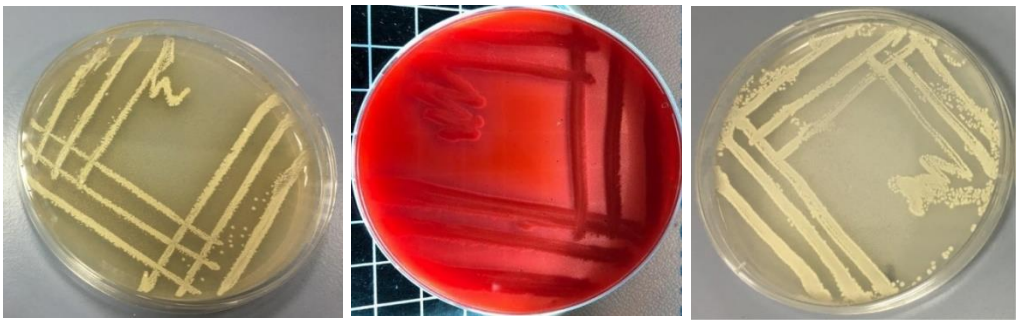
3.2 Isolation and characterization of bacteria capable of producing cellulase

3.2.1 Phenotypic characterization

Six bacterial strains were isolated from soil samples and tested for their capability to generate cellulase on different agar media. As shown in Figure 1, one bacterial strain gave positive results on different screening media (Starch casein agar, Carboxymethyl cellulose agar and Cellulose congo-red agar) yielding clear zone during incubation. Findings of the microscopic observation revealed a Gram positive, rod shaped and non-motile bacterial strain. The biochemical analyses of the bacterial isolate are recapped in Table 2. The oxidative fermentative test showed a facultative anaerobic bacterial strain. The other tests indicated positive triple sugar iron, oxidase, catalase, methyl red, amylase, cellulase, gelatinase, lipase and citrate test. On the other hand, the isolate was found to be negative for pectinase, Voges-Proskauer and indole test.

The morphological characters and melanin production are summarized in Table 3. The colony color was white, the spore chain morphology examined microscopically showed branched filaments with coccoid spores, the color of spore chain was green, the color of substrate mycelium was yellow-brown and no melanin production was observed.

These findings are similar to the results of previously reported studies on biochemical and morphological characters of *Streptomyces* species and *Streptomyces fimicarius* (Sarranyadhevi *et al*., 2015; Talari Vishwanatha *et al*., 2017; Ventorino *et al*., 2016).



(a) (b) (c)

Fig.2: Plates showing growth of the isolated bacterial strain on: (a) Starch casein agar medium, (b) Cellulose congo-red agar medium and (c) Carboxymethyl cellulose agar medium.

Table 2: Biochemical tests of Strain A1

Biochemical tests	Results
Oxidative fermentative test	**Facultative anaerobe**
TSI	+
MR	+
VP	-
Catalase	+
Oxidase	+
Starch hydrolysis	+
Pectin hydrolysis	-
Gelatin hydrolysis	+
Cellulose hydrolysis	+
Lipid hydrolysis	+
Dextrose	+
Lactose	+
Sucrose	+
Mannitol	+
Indole Test	-
Citrate Test	+
Motility Test	Non motile
Gram staining	Positive

Table 3: Morphological characters of Strain A1

Morphological characters	Results
Color of the colony	White
Spore chain morphology	Showing branched filaments with coccoid spores under microscope
Color of spore chain	Green
Color of substrate mycelium	Yellow-brown
Production of melanin	No melanin production

3.2.2 Molecular identification of the strain

The phylogenetic tree revealed that the bacterial strain A1 was a member of the genus *Streptomyces* and showed 99% identity with *Streptomyces fimicarius* strain CSSP537 based on the16S rRNA sequences. Fig. 2 exhibit the phylogenetic tree that demonstrates the highest resemblance of the strain A1 with the other 16S rRNA sequences of pertinent *Streptomyces* species. The GenBank accession number of *Streptomyces fimicarius* A1 was MK463974.

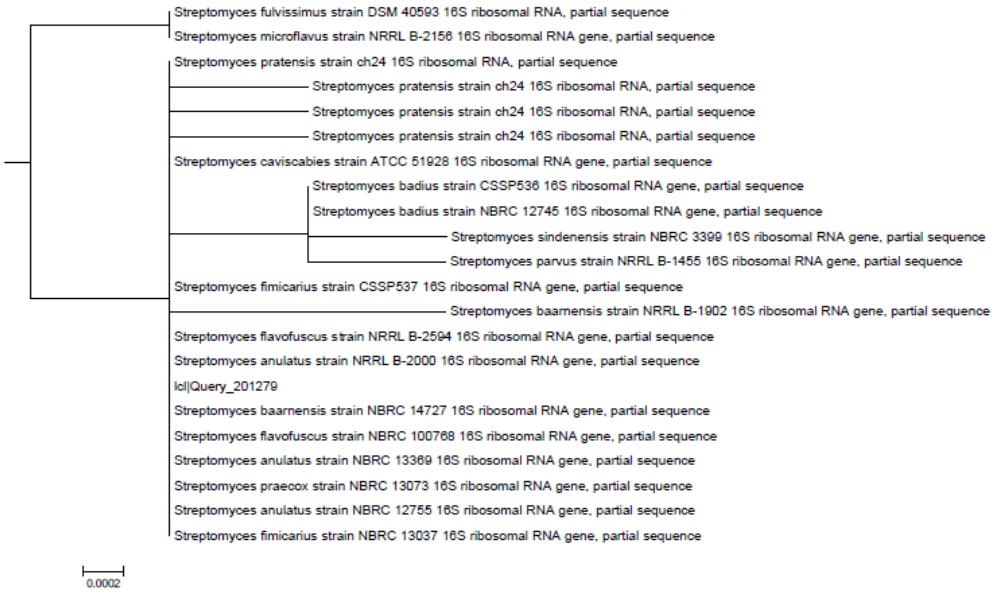


Fig.3: Phylogenetic tree of the bacterial strain *Streptomyces fimicarius* A1 based on 16S rRNA sequence analysis using MEGA 3 (GenBank accession number: MK MK463974).

3.3 Electricity Production Using Different Carbon Sources

Fig. 4 shows that when the microbial fuel cell was inoculated with *Streptomyces fimicarius* and cellulose was utilized as the fuel substrate, there was a significant rise in cell potential with time, till a constant state of 322 mV. While in the presence of glucose as a substrate the voltage was 250 mV. These outcomes are in consonance with the findings of various studies. Rezaei *et al*. (2009) demonstrated that the pure culture of *Enterobacter cloacae* could be used to carry out both cellulose degradation and electricity production. Hassan *et al*. (2012) showed that pure culture of cellulose-degrading bacteria *Streptomyces enissocaesilis* KNU and *Nocardiopsis* sp. KNU can also generate electricity. Bond & Lovley (2003), Hassan *et al*. (2012b) and Huang *et al*. (2008) indicated that the stable duration for electricity production with cellulose was commonly lengthier than that noticed with other pure substrates like acetate, xylose, and glucose.

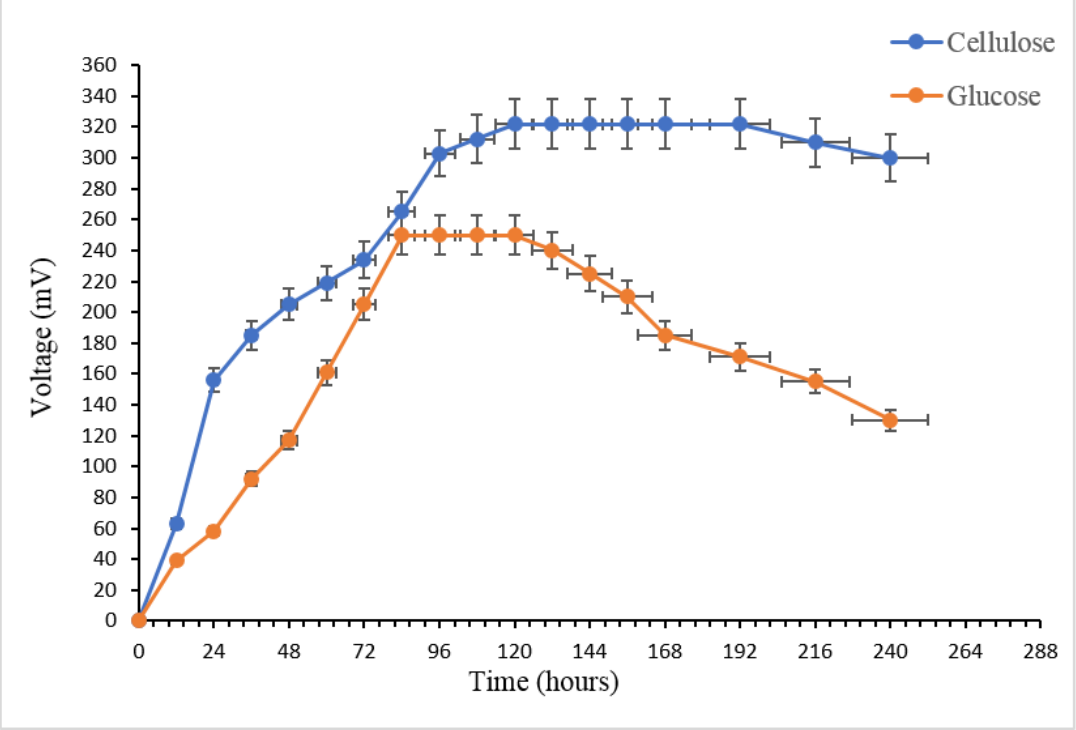


Fig. 4: Generation of electricity by cellulose-degrading bacterium *Streptomyces fimicarius* over time using cellulose and glucose as a substrate and electron donor.

4. CONCLUSION

Fossil fuels are used to produce eighty-six percent of electricity around the world, but they release carbon dioxide, nitrogen and sulfur which are the main cause of climatic problems such as the ozone hole, acid rain, and greenhouse effect. Furthermore, nonrenewable fuel stocks are speedily decreasing. At the same time, energy needs are increasing significantly due to high population density and civilization. This condition draws attention to renewable natural resources like biomass, wind, solar, and others for power production. Microbial fuel cell is a sustainable apparatus that generates electricity. This study investigates a novel cellulose-degrader named *Streptomyces fimicarius* able to generate electricity directly from cellulose, as substrate and electron donor, with a voltage of 322 mV that lasts for 10 days before decreasing. Apart from the generation of electricity, the application of microbial fuel cell can be exploited in several domains, like degradation of wastes, wastewater treatment, CO_2 capture, biosensors, hydrogen production and energy recovery.

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